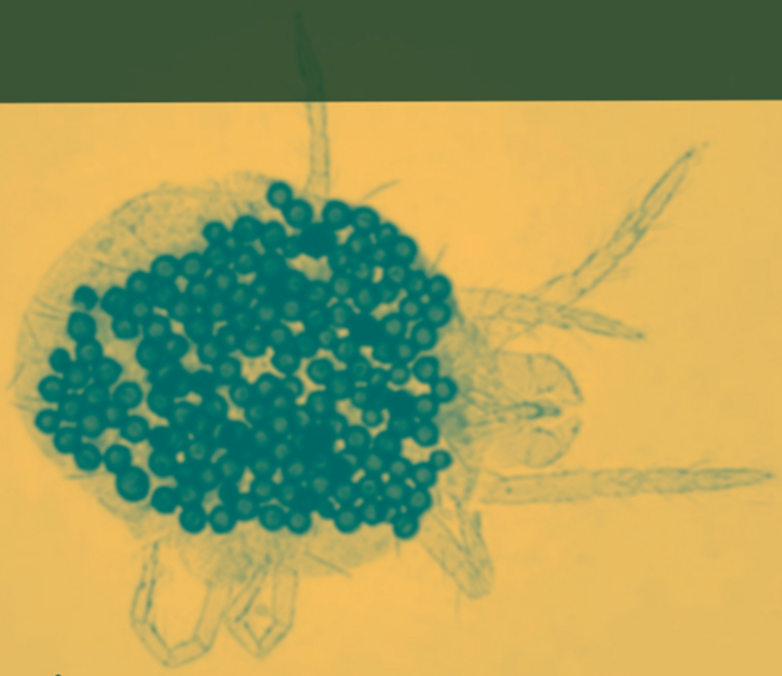
Jan Bruin
Leo P.S. van der Geest
Editors

Diseases of Mites and Ticks



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Jan Bruin · Leo P.S. van der Geest
Editors

Diseases of Mites and Ticks

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Editors

Jan Bruin
University of Amsterdam
IBED
Section Population Biology
Kruislaan 320
1098 SM Amsterdam
The Netherlands
bruinjan@tiscali.nl

Leo P.S. van der Geest
University of Amsterdam
IBED
Section Population Biology
Kruislaan 320
1098 SM Amsterdam
The Netherlands
lpsvdgeest@casema.nl

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Cover illustration: Cassava green mite filled with resting spores of the fungus *Neozygites tanajoae*. photo: George Gorgen and Fabien Hountondji.

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Contents

Preface 1–2

Diseases of mites and ticks: from basic pathology to microbial control—an introduction
L.P.S. van der Geest & J. Bruin 3–6

Using RNA interference to determine the role of varisin in the innate immune system of the hard tick *Dermacentor variabilis* (Acari: Ixodidae)
W.L. Hynes, M.M. Stokes, S.M. Hensley, S.M. Todd & D.E. Sonenshine 7–15

Silencing expression of the defensin, varisin, in male *Dermacentor variabilis* by RNA interference results in reduced *Anaplasma marginale* infections
K.M. Kocan, J. de la Fuente, R. Manzano-Roman, V. Naranjo, W.L. Hynes & D.E. Sonenshine 17–28

Potential effects of mixed infections in ticks on transmission dynamics of pathogens: comparative analysis of published records
H.S. Ginsberg 29–41

An association between the Antarctic mite *Alaskozetes antarcticus* and an entomophthoralean fungus of the genus *Neozygites*
P.D. Bridge & M.R. Worland 43–52

Diversity of acaropathogenic fungi in Poland and other European countries
S. Bałazy, R. Miętkiewski, C. Tkaczuk, R. Wegensteiner & M. Wrzosek 53–70

Entomopathogenic fungi against South American tick species
É.K.K. Fernandes & V.R.E.P. Bittencourt 71–93

Pathogenicity and thermotolerance of entomopathogenic fungi for the control of the scab mite, *Psoroptes ovis*
M. Lekimme, C. Focant, F. Farnir, B. Mignon & B. Losson 95–104

Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on *Varroa* mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health
W.G. Meikle, G. Mercadier, N. Holst & V. Girod 105–117

Topically applied myco-acaricides for the control of cattle ticks: overcoming the challenges
P. Polar, D. Moore, M.T.K. Kairo & A. Ramsubhag 119–148

Protection of *Metarhizium anisopliae* conidia from ultra-violet radiation and their pathogenicity to *Rhipicephalus evertsi evertsi* ticks
M. Hedimbi, G.P. Kaaya, S. Singh, P.M. Chimwamurombe, G. Gindin, I. Glazer & M. Samish 149–156

Evaluation of *Metarhizium anisopliae* (Deuteromycota: Hyphomycetes) for control of broad mite *Polyphagotarsonemus latus* (Acari: Tarsonemidae) in mulberry
M. Maketon, P. Orosz-Coghlan & J. Sinprasert 157–167

Enabling mycelial application of *Hirsutella thompsonii* for managing the coconut mite
P. Sreerama Kumar & L. Singh 169–182

A tale of three acaropathogenic fungi in Israel: *Hirsutella*, *Meira* and *Acaromyces*
U. Gerson, A. Gafni, Z. Paz & A. Sztejnberg 183–194

Lessons from interactions within the cassava green mite fungal pathogen *Neozygites tanajoae* system and prospects for microbial control using Entomophthorales
F.C.C. Hountondji 195–210

Failure of the mite-pathogenic fungus *Neozygites tanajoae* and the predatory mite *Neoseiulus idaeus* to control a population of the cassava green mite, *Mononychellus tanajoa*
S.L. Elliot, G.J. de Moraes & J.D. Mumford 211–222

The effects of *Pseudomonas putida* biotype B on *Tetranychus urticae* (Acari: Tetranychidae)
H.M. Aksoy, S.K. Ozman-Sullivan, H. Ocal, N. Celik & G.T. Sullivan 223–230

Overwintering and prevalence of *Neozygites floridana* (Zygomycetes: Neozygitaceae) in hibernating females of *Tetranychus urticae* (Acari: Tetranychidae) under cold climatic conditions in strawberries
I. Klingen, G. Wærsted & K. Westrum 231–245

Sprays of emulsifiable *Beauveria bassiana* formulation are ovicidal towards *Tetranychus urticae* (Acari: Tetranychidae) at various regimes of temperature and humidity
W.-B. Shi, M.-G. Feng & S.-S. Liu 247–257

Role of entomopathogenic fungi in the control of *Tetranychus evansi* and *Tetranychus urticae* (Acari: Tetranychidae), pests of horticultural crops
N.K. Maniania, D.M. Bugeme, V.W. Wekesa, I. Delalibera Jr. & M. Knapp 259–274

Effect of temperature on virulence of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to *Tetranychus evansi*
D.M. Bugeme, N.K. Maniania, M. Knapp & H.I. Boga 275–285

Side-effects of pesticides on the life cycle of the mite pathogenic fungus *Neozygites floridana*
V.W. Wekesa, M. Knapp & I. Delalibera Jr. 287–297

Natural enemies of mass-reared predatory mites (family Phytoseiidae) used for biological pest control
S. Bjørnson 299–306

Verified and potential pathogens of predatory mites (Acari: Phytoseiidae)
C. Schütte & M. Dicke 307–328

Symbionts, including pathogens, of the predatory mite *Metaseiulus occidentalis*: current and future analysis methods
M.A. Hoy & A. Jeyaprakash 329–347

Diseases of mites and ticks: farewell to Leo van der Geest

Jan Bruin

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After more than 20 years Leo van der Geest has retired as editor of *Experimental and Applied Acarology*. When in March 1985 the first issue of the journal was published, Leo already acted as associate editor, at the side of editor-in-chief prof. Wim Helle. The formal difference between the two was cancelled in 1993, when both were mentioned on the journal's cover as plain 'editor'. In 1994 Helle retired and Frans Jongejan joined Leo as Ticks editor. In 1997 I myself joined as the second Mites editor.

Throughout his research and teaching career Leo has always been involved with pathogens, first of insects, later of mites. Therefore, it should not come as a surprise that I was struck by the idea to honour Leo's editorial retirement with a special journal issue dedicated to the subject that he finds most interesting: acarine pathogens and pathology. I discussed the idea with Leo, and was very pleased to learn that he not only appreciated the token, but also that he was willing to lend his expertise to the enterprise. Next, we asked about all relevant researchers we could think of to participate, and much to my surprise almost all accepted the invitation. This can only mean that pathologists throughout the world value Leo's work.

J. Bruin (✉)
IBED, Section Population Biology, University of Amsterdam, Kruislaan 320,
1098 SM Amsterdam, The Netherlands
e-mail: bruinjan@tiscali.nl

So many authors joined that we can now proudly present a quadruple issue on Diseases of Mites and Ticks. The 24 contributions provide a wide variety of aspects of acaropathogens, just as we had hoped for. There are numerous highlights, but one particularly worthy of mention—because it is the first time in the journal's history, and because it is yet another token of appreciation—is the formal description of an acaropathogenic fungus, new to science: *Hirsutella vandergeesti*. Cheers, Leo!

Diseases of mites and ticks: from basic pathology to microbial control—an introduction

Leo P. S. van der Geest · Jan Bruin

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One of the earliest observations of a pathogen infecting mites goes back to 1924 when Speare and Yothers (1924) noted a vast decimation of large populations of the citrus rust mite, *Phyllocoptruta oleivora* (Ashmead) on grapefruit in Florida, USA. This effect was ascribed to the presence of an acaropathogenic fungus that was later described by Fisher (1950) as *Hirsutella thompsonii*. It would still take several decades before any comprehensive research would be conducted on pathogens of Acari. Nowadays, pathological investigations on mites and ticks form an important discipline within the field of acarology, as is apparent from several recent reviews: McCoy (1996), Poinar and Poinar (1998), Samish and Řeháček (1999), Chandler et al. (2000) and Van der Geest et al. (2000).

The current collection of 24 papers is a mixture of primary research articles and literature reviews, presenting a broad overview of the developments in about all possible aspects of acarine diseases, stretching from basic pathology to microbial pest control. The pathogens include fungi, bacteria, and protozoa (as well as an occasional virus and unidentified organism), the hosts are mites and ticks from a variety of taxa (e.g., Eriophyidae, Ixodidae, Oribatida, Phytoseiidae, Psoroptida, Tarsonemidae, Tetranychidae, Varroidae), and the authors come from all over the world (e.g., Belgium, Benin, Brazil, Canada, China, France, India, Israel, Kenya, Namibia, Norway, Poland, Thailand, The Netherlands, Trinidad and Tobago, Turkey, UK, USA). With such variety the contributions can be ordered in a near infinite number of coherent ways, and we had to pick just one.

The issue kicks off with two interrelated papers, applying a molecular–biological technique (RNA interference) to investigate a mechanistic detail of the immune system of a hard tick, *Dermacentor variabilis* (Say), involved in its reaction to infections by the rickettsial bacterium *Anaplasma marginale* (Hynes et al., Kocan et al.). Individual ticks are frequently infected by more than one (type of) pathogen, which may interact in various possible ways. Ginsberg presents a literature review of the possible effects of coinfection of ticks, and a simple model linking the implications of coinfection with pathogen transmission. Very different, yet equally basic, is the intriguing case study of the association

L. P. S. van der Geest (✉) · J. Bruin
IBED, Section Population Biology, University of Amsterdam, Kruislaan 320,
1098 SM Amsterdam, The Netherlands
e-mail: lpsvdgeest@casema.nl

between the Antarctic oribatid mite *Alaskozetes antarcticus* (Michael) and a member of the fungus genus *Neozygites* (Bridge and Worland)—this seems to be only the fourth reported case of an arthropod infected by a fungus under these harsh climatic conditions. The genus *Neozygites* has a worldwide distribution and it is well known for its entomo-/acaropathogenicity. Inventories in Poland and several other Central-European countries show the presence of *Neozygites* spp., as well as the other genera *Hirsutella* spp. and *Conidiobolus* spp., on various species of mites (Bałazy et al.). The authors even describe two species new to science. Still mainly descriptive, but increasingly applied, is the extensive review of fungi found in association with a variety of tick species in South America (Fernandes and Bittencourt). The wealth of information in this paper will help develop IPM (integrated pest management) or biocontrol strategies and may help persuade tick controllers to deviate from traditional chemical methods.

Lekimme et al. screened isolates of fungi from four genera, for their pathogenicity and temperature sensitivity, aiming to find suitable control agents of *Psoroptes ovis* (Hering), the notorious pest mite of cattle and sheep. An isolate of *Beauveria bassiana* has previously been found to induce the falling of *Varroa destructor* Anderson and Trueman from bees. Following up on this, Meikle et al. compared several isolates and application methods to improve this type of biological control of the bee parasite. Commercial products based on entomopathogenic fungi are being applied in a growing number of control programmes against plant pests, but their application against animal ectoparasites lacks behind, as Polar et al. point out. Still, laboratory studies as well as pasture applications of several fungal products have shown great promise. Instead of treating large areas of field, it could be much more cost effective if cattle were treated topically, but so far topical application has shown variable results. Polar et al. thoroughly review the literature on the use of myco-acaricides against ticks in general, but emphasis is on the topical application, the host skin environment, and how to improve the pathogens' performance in this environment. One relevant feature of topical application of a myco-acaricide—exposure to sunlight—is explored in more detail in an experimental study by Hedimbi et al. These authors tested the sensitivity to UV-radiation of different formulations of conidia of *Metarhizium anisopliae*, in combination with their pathogenicity against *Rhipicephalus* ticks—commercial sunscreens yielded some encouraging results!

The next block of papers deals with (the control of) pest mites on plants. Conidial suspensions of a variety of fungus species and strains were tested against the broad mite, *Polyphagotarsonemus latus* (Banks) on mulberry (Maketon et al.). *Metarhizium anisopliae* came out on top and showed great potential. Acaropathogenic fungi may also help in the control of another mite—also tiny, yet devastating, with a hidden lifestyle—the coconut mite *Aceria guerreronis* Keifer. Kumar and Singh report the effect of a mycelial suspension of *Hirsutella thompsonii* against this eriophyid mite. *Hirsutella thompsonii* has been developed to a biological miticide by McCoy and co-workers in the 1970s for the control of the citrus rust mite in citrus orchards (cf. McCoy 1996). The fungus is in particular infectious for eriophyids in a variety of crops. However, commercial development of mycelial preparations against the citrus rust mite failed because of lysis of hyphal material during storage and for that reason, the work was discontinued. Recently, the fungus has received renewed attention for the control of other eriophyids, e.g., in coconut in Asia (India) and in Latin America. Successful attempts have been made in India to develop a biological acaricide with the fungus as active ingredient (Kumar and Singh). *Hirsutella thompsonii* and several other fungi are also studied in Israel by Gerson and co-workers, especially for their effect on citrus rust mite, but also some others. They report some very promising results of a couple of recently described fungi (two *Meira* species and

an *Acaromyces*), including their probable compatibility with currently used chemical pesticides.

Entomophthoralean infections are well-known in insect and mites, particularly in spider mites. The best-studied species is probably *Neozygites tanajoae*, a fungus that is specific for the cassava green mite, *Mononychellus tanajoa* (Bondar). In the 1980s, the fungus was considered to be a possible classical biological control agent that could be the solution for the enormous infestations in cassava by *M. tanajoa* in the rural areas in the African continent, after its unfortunate introduction in eastern Africa. An international collaboration between the International Institute for Tropical Agriculture (IITA, Cotonou, Benin), the International Center for Tropical Agriculture (CIAT, Cali, Colombia), Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Brazil), and the University of Amsterdam (The Netherlands), was set up in order to guide the introduction of the fungus into Africa. In this issue, Hountondji reviews the current status of microbial control of the cassava green mite in Africa. A second paper on much the same players, but now on the Brazilian site of the system, explores the possible causes of failing control of *M. tanajoa* with *N. tanajoae* (Elliot et al.). The importance of timing is nicely motivated.

A series of contributions deals with the control of *Tetranychus* species, most noticeably the two-spotted spider mite, *T. urticae* Koch, known from literally hundreds of host plants (e.g., Bolland et al. 1998). *Pseudomonas putida*, a saprotrophic bacterium isolated from the soil in a Turkish greenhouse, appears to have *T. urticae* control potential in a carefully arranged laboratory set-up (Aksoy et al.). Next, this potential should be corroborated in greenhouse and field trials. Klingen and colleagues investigated the overwintering capability of *Neozygites floridana* in hibernating *T. urticae* females in strawberry in the Norwegian field (minimum ambient temperature ca. −15°C). It turns out that the insulation experienced within the mites' bodies allows the fungus to survive the winter and to sporulate—and infect new spider mites—in early spring. The influence of temperature and humidity regimes on the efficacy of conidial suspensions of *Beauveria bassiana* against especially the egg stage of *T. urticae* was investigated in China (Shi et al.). The ovicidal activity also at rather low air humidity (ca. 50%) indicates its potential for spider mite control under practical field conditions.

Like the cassava green mite, also the tomato red spider mite, *Tetranychus evansi* Baker and Pritchard, is suspected to originate from South America. And like the CGM, *T. evansi* has caused huge problems after entering Africa, in the late 1970s. *Tetranychus evansi* specializes on solanaceous horticultural crops, especially tomato. Under the hot and dry conditions of eastern and southern Africa it can wipe out whole tomato plants within a month. The size of the problems caused by *T. evansi* has stimulated much research effort, aiming for control of this mite—the application of acaropathogens is the subject of three contributions to this volume. First, Maniania et al. review the relative successes of a variety of fungi in the control of both *T. evansi* and *T. urticae*, in the context of various biological control strategies. Then Bugeme et al. focus on the effect of temperature on the efficacy of isolates of *B. bassiana* and *M. anisopliae* against *T. evansi*. Thirdly, Wekesa et al. report on a detailed study on the effects of various pesticides on *N. floridana* as control agent of *T. evansi*. The compatibility between fungal strains and chemicals used in commercial tomato production is a prerequisite for successful IPM programmes.

Biological pest (mite) control by means of beneficial insects and/or mites has seen a rapid development since the 1960s. A well-known example is the phytoseiid mite *Phytoseiulus persimilis* Athias-Henriot. This predator is very successful in controlling spider mites, in particular *T. urticae*, in a variety of greenhouse and outdoor crops. However, in a number of instances, control was unsatisfactory due to suboptimal performance of the

predator. It turned out that pathogens could negatively affect predator populations, thus hampering the success of pest control. This aspect of acaropathology is dealt with in the last three contributions. Bjørnson gives a crisp and concise overview of the various types of pathogens (viruses, bacteria, microsporidia) that can pop up in mass-rearings of commercial phytoseiid biocontrol agents. Schütte and Dicke treat partly the same subject, but it a much wider context—they systematically, consistently and carefully review all (possible) microorganisms known to have some negative effect on phytoseiid mites. In passing, they provide much inside information on their own 10-year quest for the agent, causing the strange behavior and poor performance of *P. persimilis* from their cultures in Wageningen, starting in the early 1990s. Finally, Hoy and Jeyaprakash extend the subject of pathogens from predatory mites to endosymbionts, focussing on their pet phytoseiid biocontrol agent, *Metaseiulus occidentalis*. Although the study of these endosymbiotic bacteria, such as *Wolbachia* and *Cardinium*, is still relatively new, they have already been found to be virtually omnipresent—they have been found in many groups of invertebrates and they may have a great impact on the population dynamics of a species.

Although we feel this collection of papers offers a rich variety of sides to the subject of diseases in mites and ticks, complete coverage of mite pathology is not possible in a single volume of *Experimental and Applied Acarology*. We cordially thank all contributing authors, as well as the more than 50 peer reviewers, who helped us to prepare this end result. We sincerely hope this issue will stimulate further research in this most fascinating field.

Acknowledgments A personal note by LvdG: *Experimental and Applied Acarology* was launched in March 1985. Dr. Wim Helle was the first editor-in-chief and I acted as associate-editor, until Wim decided to step down because of his retirement. Now it is my turn. I have served the journal for a great many years as editor, and I found it a stimulating and inspiring task to promote acarology. As many of you may know, my main field of interest lies in invertebrate pathology and I am glad that we have had the opportunity to prepare an issue on pathogens of the Acari. I also would like to thank all authors who have contributed to the journal and the reviewers for their critical remarks when reviewing manuscripts, not just for this present issue but for all the years that I was editor.

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Using RNA interference to determine the role of varisin in the innate immune system of the hard tick *Dermacentor variabilis* (Acari: Ixodidae)

Wayne L. Hynes · Martha M. Stokes · Shannon M. Hensley · S. Michelle Todd · Daniel E. Sonenshine

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Abstract Defensins are an important component of the innate immune system of ticks. These small peptides are produced by various genera of ticks, and expressed in various tissues. In this study we used RNA interference to silence the expression of the defensin varisin produced by the hemocytes of the American dog tick, *Dermacentor variabilis*. Ticks were injected with double stranded varisin RNA prior to being placed on a rabbit. After feeding, the ticks were removed, bled, and the hemolymph plasma and hemocytes separated. Hemocytes were screened for the presence (or absence) of both varisin transcript and peptide. Varisin peptide was below detectable levels and the transcript showed a greater than 99% knockdown. The antimicrobial activity of the hemolymph plasma was reduced 2–4 fold compared to that of control injected ticks indicating varisin accounts for a large portion of the antimicrobial activity of the hemolymph.

Keywords Innate immunity · Defensin · Varisin · RNAi · Antimicrobial activity

Introduction

Ticks are obligate blood-feeding ectoparasites that have the ability to transmit a wide variety of disease causing microbes. In fact, they have the ability to transmit more disease causing microbes than any other blood feeding arthropod, including mosquitoes, although more human illness is caused by mosquito-borne agents than by tick-borne ones. The hard tick *Dermacentor variabilis* (American dog tick) can be found throughout the southeastern USA, and up the east coast to Nova Scotia, Canada (Brown et al. 2005). This hard tick is a vector of the pathogens responsible for Rocky Mountain Spotted Fever (RMSF), human monocytotrophic ehrlichiosis (HME), tularemia, and is capable of causing tick paralysis. However, ticks are more than 'syringes-on-legs'. Although lacking the highly developed, adaptive immune response found in vertebrates, ticks have an efficient innate immune response. The

W. L. Hynes (✉) · M. M. Stokes · S. M. Hensley · S. M. Todd · D. E. Sonenshine
Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529-0266, USA
e-mail: whynes@odu.edu

innate immune system consists of both cellular and soluble components (Gillespie et al. 1997; Schmid-Hempel 2005; Taylor 2006) that are effective in eliminating many microbes. The cellular responses include phagocytosis, nodulation, and encapsulation (Eggenberger et al. 1990; Inoue et al. 2001; Ceraul et al. 2002; Taylor 2006; Sonenshine and Hynes 2008). The soluble aspects of the innate immune response include production of antimicrobial peptides, including defensins (Bulet et al. 2003; Taylor 2006; Sonenshine and Hynes 2008).

Invertebrates produce many different types of antimicrobial molecules when challenged by microbes or parasites (Cociancich et al. 1994; Bulet et al. 2003; Taylor 2006; Tsuji et al. 2007; Sonenshine and Hynes 2008). Among the most conserved of these molecules are the defensins. Defensins are small cationic peptides, generally comprising 34–61 amino acids for the mature peptide (3–6 kDa), produced by organisms from plants to invertebrates to the most complex mammals (Bulet et al. 2003; Sonenshine and Hynes 2008). Insect defensins generally have six cysteine residues that form three disulfide bridges with linkages between Cys1-Cys4, Cys2-Cys5, and Cys3-Cys6 (Bulet et al. 1999, 2003; Taylor 2006); the exception being drosomycin, produced by *Drosophila melanogaster* which has four disulfide bonds and antifungal activity (Michaut et al. 1996; Bulet et al. 2003). Most insect defensins are active against gram positive bacteria, with some showing activity against other microbial challengers such as gram negative bacteria, parasites and fungi (Cociancich et al. 1994). To date, more than 20 defensins have been identified from 11 species of tick (Taylor 2006; Sonenshine and Hynes 2008). Defensins are produced as a prepro form, which is cleaved, at a highly conserved RVRR site, to release the mature peptide (Sonenshine and Hynes 2008). Some species of tick have multiple isoforms of defensin, with *Ornithodoros moubata* having four isoforms (Nakajima et al. 2001, 2002) and *I. ricinus* having two isoforms (Rudenko et al. 2007), while others appear to have only one form (Hynes et al. 2005; Todd et al. 2007). The first defensin recognized from a hard tick was varisin, isolated from the hemolymph of American dog tick, *Dermacentor variabilis* (Johns et al. 2001a, b). The transcript sequence of varisin was determined following RT-PCR from RNA isolated from hemocytes (Ceraul et al. 2003). It appears that varisin is produced in the hemocytes and released into the hemolymph following microbial challenge. Although transcript has been detected in various tissues (Ceraul 2005; Sonenshine et al. 2005; Ceraul et al. 2007) the peptide has only been detected in the hemolymph/hemocytes. Recently a second defensin has been reported from *D. variabilis* which had less than 50% similarity to varisin (Ceraul et al. 2007). In order to understand the role of varisin in the innate immune system of *D. variabilis*, the expression of the varisin gene was silenced using RNA interference (RNAi). RNAi has been used to study gene function in ticks due to the lack of other means of genetic manipulation (de la Fuente et al. 2005, 2007). In this study we show that RNAi can be used to silence the gene for varisin and that silencing results in a decreased level of antimicrobial activity of tick hemolymph.

Materials and methods

Ticks, injection, and bleeding

Dermacentor variabilis were obtained from a colony maintained at Old Dominion University as previously described (Johns et al. 1998, 2001a, b). All use of animals in this research was done in accordance with protocols approved by the Old Dominion University Institutional Animal Use and Care Committee. Unfed virgin female ticks were injected with 1–5 μl of

double stranded RNA (dsRNA), or Shen's solution (Oliver et al. 1974) for the controls, using a 30 gauge × ½" hypodermic needle attached to a 10 μl Hamilton syringe. Injection was via the foramen between the capitulum and anterior end of the scutum. After injection, the needle was held in the tick's body for 30 min to prevent leakage of the injected material. Subsequently, the ticks were confined within plastic capsules attached to New Zealand white rabbits (*Oryctolagus cuniculus*). After feeding for 5 days the ticks were forcibly removed from the rabbit and hemolymph was collected by severing the forelegs at the coxal–trochanteral joint and applying gentle pressure to the body. The clear, amber-colored liquid expressed was collected in a glass micropipette and put into either 20 μl Shen's saline solution or 100 μl RNA later (Applied Biosystems, Foster City, CA) depending on the assay; Shen's for protein and antimicrobial assays, RNA later for RT-PCR reactions. Approximately the same number of ticks were used for both control and test injections.

Previous results have suggested storage of varisin in the hemocytes which is released on challenge (Ceraul et al. 2003). Stored varisin should be released on injection of the dsRNA. To show that no new varisin was made and stored after injection of the dsRNA, some ticks were pin-pricked (wounded) and allowed to incubate for 1 h prior to bleeding.

Double stranded RNA production

Double-stranded RNA was prepared from a PCR product, containing the entire 624 bp cDNA fragment of varisin derived from *D. variabilis* hemocytes (Ceraul et al. 2003), using the MEGAscript RNAi kit (Applied Biosystems). The gene was amplified from a plasmid using the primers DEFT75: 5′-*TAATACGACTCACTATAGGGTACT*ATGCGCGGACTTTGCATCTGC and DEFT733: 5′-*TAATACGACTCACTATAGGGTACT*TACGTCGACAAAGCGCTTCGG, which contain the T7 promoter for in vitro transcription (shown in italics). Amplification was carried out using the following cycle parameters: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s and 68°C for 1 min; 68°C for 7 min completed the run. The PCR fragment was then used in the transcription reaction as described by the manufacturer. After transcription, the reaction was treated to remove DNA and single stranded RNA, then purified, and ethanol precipitated. The dsRNA was then resuspended in Shen's solution for injection into ticks. Ticks were injected with 10^{11}–10^{12} molecules of dsRNA as described above. Control ticks were injected with the same volume of Shen's solution.

Quantitative RT-PCR

Using the "illustra QuickPrep Micro mRNA purification kit" (GE Healthcare, NJ) the mRNA was isolated from hemolymph collected in RNA-later. The isolated mRNA was then treated with Turbo DNA-free (Applied Biosystems) to remove any residual DNA; this step was repeated if necessary. Reverse transcription reactions were carried out using the ImProm-II Reverse Transcription System in the presence of RNasin according to the manufacturer's instructions (Promega Corp, Madison, WI) using either Oligo(dT) or random hexamer primers. The synthesized cDNA was then used in real-time PCR reactions. Controls, in which reverse transcriptase was not added, were set up for each reaction to ensure no DNA contamination.

Real-time PCR

Real-time PCR was carried out using the RT^2 SYBR Green qPCR Master Mix (SuperArray Bioscience Corporation, Frederick, MD) on a Cepheid Smartcycler (Cepheid, Sunnyvale,

CA). Control real-time reactions were set up monitoring actin expression using 0.2 μM final concentration of actF (5′-GTACGCCAACACCGTTCTC-3′) and actR (5′-ATCTT GATCTTCATGGTGGAA-3′) primers. Reactions for the detection of varisin were 0.2 μM vsnF (5′-ATGCGCGGACTTTGCATCT-3′) and vsnR (5′-TTAATTCCTGTAGCAGGTG CA-3′). The cDNA template (1 μl) was added last to the 25 μl reaction. All reactions were run on the same program: 95°C for 60 s then 40 cycles of 95°C for 15 s, 60°C for 60 s, and followed by a melt curve. C_T values were determined and used either in the comparative C_T method ($\Delta\Delta C_T$) for relative quantification, or to determine the actual number of molecules based on a standard curve derived from adding known amounts of amplified DNA to a set of real-time reactions. Controls without reverse transcriptase and without template were set up with each set of samples.

Protein gels

Hemolymph collected in Shen's solution was used to check for the presence or absence of the varisin band in both hemolymph plasma and hemocyte lysate. Hemocytes were collected from the hemolymph by centrifugation at 1,000 × *g* for 20 min at 4°C. The pellet was resuspended in 10 μl water and frozen until needed. Cells were thawed and 4 μl of the hemocytes were mixed with loading buffer and reducing agent, then heated at 70°C for 10 min before loading on a 4–12% NuPAGE Tris-Bis SDS gel (Invitrogen) with See Blue molecular weight marker (Invitrogen). After electrophoresis at 200 V for 35 min, the gel was silver stained using the Silver Express staining kit (Invitrogen). A western blot analysis of a similarly run gel was carried out as previously described (Ceraul et al. 2003) except that the primary antibody (anti-varisin) was diluted 1:50.

Antimicrobial assay

Antimicrobial activity of hemolymph plasma was assessed using a well diffusion assay. Samples (10 μl) to be tested were pipetted into 4 mm diameter wells cut into tryptic soy agar plates, then allowed to dry, exposed to chloroform vapors for 20 min, and aired. An overnight culture of the gram positive bacterium *Micrococcus luteus* was seeded onto the surface of the plate using a sterile swab, and the plate incubated at 37°C overnight. Zones of inhibition were seen as areas of no growth around the wells. Two fold dilution series were carried out using 0.9% saline as the diluent. The titer (arbitrary units, AU) was determined as the last dilution to show a zone of growth inhibition.

Results

Antimicrobial assay

Screening of undiluted hemolymph plasma for antimicrobial activity against the sensitive gram positive bacteria *M. luteus* indicated less activity in the dsRNA treated tick hemolymph, than in the ticks injected with Shen's solution (Fig. 1). When the hemolymph was two-fold serially diluted, titers for the control (Shen's injected) were 2–4 times higher than the test (dsRNA injected) sample. Control titers were 8–16 AU whereas the dsRNA injected tick hemolymph was 4 AU.

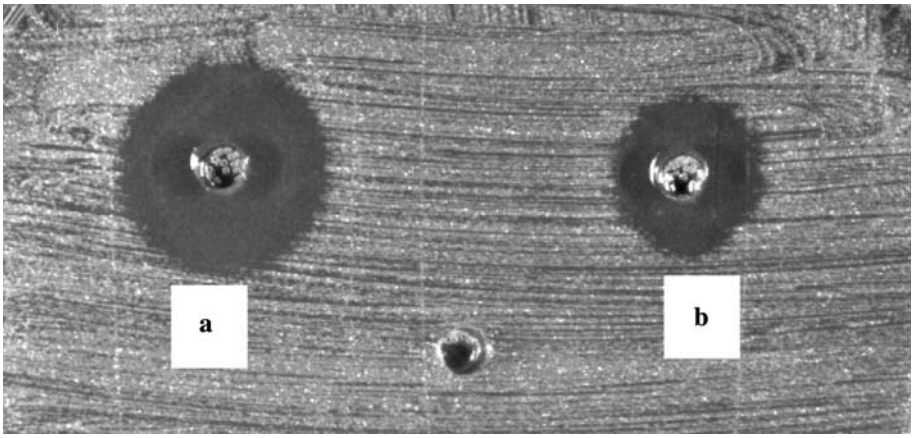

Fig. 1 Antimicrobial activity of hemolymph against *Micrococcus luteus* from control (**a**) and treated (**b**) ticks. The same volume of hemolymph was added to each well. The lower well was buffer control. Hemolymph used in this assay was from 2 separate injections of varisin dsRNA

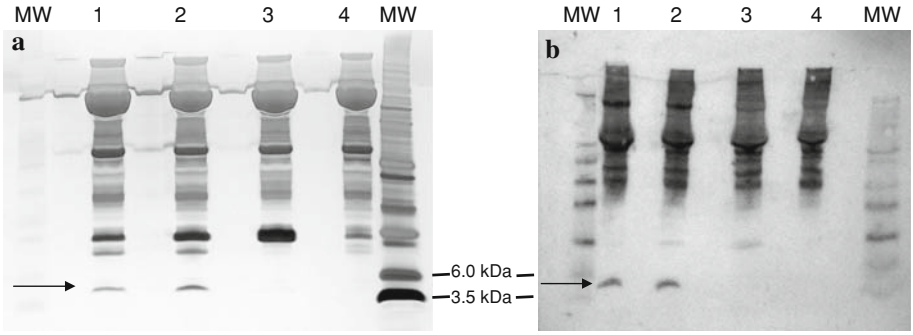

Fig. 2 PAGE (**a**) and Western blot (**b**) showing inhibition of defensin production by vsn dsRNA in tick hemolymph plasma. MW is molecular weight markers. Lanes 1 and 2 are hemolymph from ticks injected with Shen's, lanes 3 and 4 are hemolymph from ticks injected with dsRNA. Lanes 1 and 3 are hemolymph samples in which the tick was bled after removal from the rabbit, 2 and 4 are hemolymph samples obtained 1 h after the ticks were wounded. The arrow indicates the varisin band

Loss of varisin peptide in hemolymph plasma and cells

Cell lysate and hemolymph plasma were visualized on a polyacrylamide gel, stained for protein. A representative gel of tick hemolymph plasma is shown in Fig. 2a. The varisin peptide band is missing, or very faint, in the hemolymph plasma of those ticks treated with dsRNA, while those treated with buffer still show the presence of a varisin-sized band. The same effect was seen with hemocyte extracts, in that the varisin band is present in the hemocytes of control ticks but not those injected with the dsRNA construct (not shown). Western blot analysis using the anti-varisin antibody confirmed the loss of the defensin in both hemolymph plasma (Fig. 2b) and hemocytes (not shown). The presence of multiple bands in the western blot is most likely due to the non-specific binding of antibodies to other tick proteins as previously described (Ceraul et al. 2003).

To detect release of the varisin peptide following a wounding response, ticks were also wounded 1 h prior to bleeding and collecting the hemolymph. Figure 2 shows that the

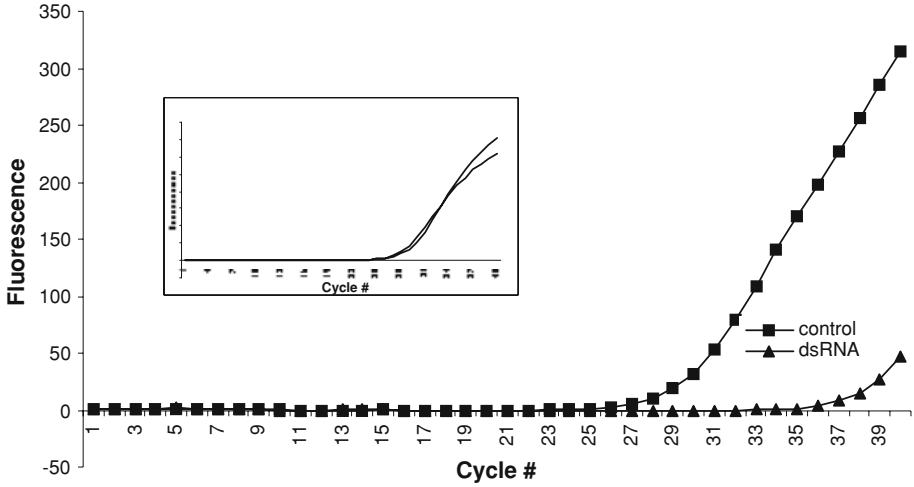

Fig. 3 Real time PCR results following amplification of cDNA derived from control and treated (dsRNA) tick hemocytes. The insert shows the same graph with amplifcation for actin from the same samples as used for varisin amplification

wounding does not result in release of varisin from dsRNA treated ticks. No difference was seen in the presence or absence of the varisin band between non-wounded and wounded ticks.

Real-time PCR assays

Real-time PCR was used to determine the degree to which varisin transcription was silenced in treated ticks. Representative results are shown in Fig. 3. Treatment with dsRNA results in a decrease in the amount of varisin transcript as seen by the number of cycles required for the transcript to be detected. Figure 3 shows control and treated samples amplified using the varisin primers. The insert shows the graphs of control and treated reactions with the actin amplification; this ensures equal amounts of template were added to the reaction.

The effect of treatment can easily be seen in this graph but to gain insight into how much the gene is silenced in our samples we determined the amount of transcript remaining using both the relative quantification and standard curve methodologies. A standard curve was prepared in which the C_T was plotted against the number of molecules present; we used ten-fold dilutions from 10^9 through 10^3 copies of the varisin gene. Using the standard curve to determine the number of molecules of varisin, we found that injection of dsRNA for varisin resulted in a 99–99.5% knockdown. Using the $\Delta\Delta C_T$ method for relative quantification treatment with dsRNA the knockdown was as high as 99.9%. These results show that injection of varisin dsRNA effectively silences the expression of the varisin gene.

Discussion

RNA interference is an effective way of examining gene function in ticks, and has been used to target a number of different genes (de la Fuente et al. 2007; Karim et al. 2008). We can now add to this list varisin, which we believe to be the major defensin in tick hemolymph. Inactivation of

varisin results in a 2–4 fold reduction in the antimicrobial activity of tick hemolymph as determined by our plate assays. However, it does not account for all the inhibitory activity since hemolymph plasma from the treated ticks was still able to inhibit the growth of *M. luteus*. *Micrococcus luteus* was chosen as an indicator of antimicrobial activity because it is sensitive to and used for detecting activity of numerous antimicrobial agents. We currently do not know the antibacterial spectrum of activity (Gram positive, gram negative or fungi) of the varisin peptide but *M. luteus* is very sensitive to the action of the peptide. What accounts for the other antimicrobial activity seen in the hemolymph? Perhaps the most likely candidate is lysozyme, which is known to be expressed by *D. variabilis* hemocytes (Simser et al. 2004; Ceraul et al. 2007) and possibly released into the hemolymph plasma fraction. We have previously shown that a lysozyme is able to enhance the antimicrobial activity of varisin (Johns et al. 2001a, b). Whether authentic tick lysozyme functions with varisin in the same manner as the egg white lysozyme remains to be determined. What would happen to the antimicrobial titer of tick hemolymph if we silenced lysozyme expression as well as varisin? RNAi studies into this aspect are currently underway in our laboratory.

There are a number of other antimicrobial molecules present in tick hemolymph (Sonenshine and Hynes 2008; Taylor 2006) that could also result inhibition of microbial growth. Activity of these other molecules would be indicated by a smaller zone of inhibition as shown in Fig. 1. One possible peptide that could be responsible for some activity would be the second defensin reported from *D. variabilis*, defensin-2 (Ceraul et al. 2007). However, this is an unlikely player in the antimicrobial activity of hemolymph since it is expressed by the midgut and not expressed by hemocytes.

Defensins have been implicated as major players in the innate immune response of ticks. We have shown that RNAi can be used to target and silence varisin expression in hemocytes and therefore in hemolymph. We have previously reported that varisin was most likely produced and stored in the hemocytes (Ceraul et al. 2003) then released into the hemolymph upon wounding or following microbial challenge. New defensin would then be made at a later time (Ceraul 2005). In this study, the initial challenge would be the injection of dsRNA into the hemocoel, resulting in release of the stored defensin. Varisin released at the time of dsRNA injection would be expected to have been lost from the tick within 24 h (Johns 2003). Since we do not detect varisin in the hemocytes or released into the hemolymph, even after another wounding (Fig. 2), it appears that RNAi effectively prevents synthesis of any new varisin. Whether inhibition of varisin production has an effect on the tick, beyond that of its role in the innate immune response is unknown. What, if any, additional or alternative roles varisin has in tick immunity requires further investigation, since defensin has been suggested to have an alternative function in mosquito immunity (Bartholomay et al. 2004). Similarly, we do not know what is the actual in vivo effect of varisin silencing. In a separate study reported elsewhere in this issue (Kocan et al. 2008), RNAi silencing of varisin in male *D. variabilis* resulted in a reduction of *Anaplasma marginale* infections as well as morphological changes in the colonies of these rickettsiae in the tick midgut.

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Silencing expression of the defensin, varisin, in male *Dermacentor variabilis* by RNA interference results in reduced *Anaplasma marginale* infections

Katherine M. Kocan · José de la Fuente · Raúl Manzano-Roman · Victoria Naranjo · Wayne L. Hynes · Daniel E. Sonenshine

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Abstract Antimicrobial peptides, including defensins, are components of the innate immune system in ticks that have been shown to provide protection against both gram-negative and gram-positive bacteria. Varisin, one of the defensins identified in *Dermacentor variabilis,* was shown to be produced primarily in hemocytes but transcript levels were also expressed in midguts and other tick cells. In this research, we studied the role of varisin in the immunity of ticks to the gram-negative cattle pathogen, *Anaplasma marginale*. Expression of the varisin gene was silenced by RNA interference (RNAi) in which male ticks were injected with varisin dsRNA and then allowed to feed and acquire *A. marginale* infection on an experimentally-infected calf. Silencing expression of varisin in hemocytes, midguts and salivary glands was confirmed by real time RT-PCR. We expected that silencing of varisin would increase *A. marginale* infections in ticks, but the results demonstrated that bacterial numbers, as determined by an *A. marginale msp4* quantitative PCR, were significantly reduced in the varisin-silenced ticks. Furthermore, colonies of *A. marginale* in ticks used for RNAi were morphologically abnormal from those seen in elution buffer injected control ticks. The colony shape was irregular and in some cases the *A. marginale* appeared to be free in the cytoplasm of midgut cells. Some ticks were found to be systemically infected with a microbe that may have been related to the silencing of varisin. This appears to be the first report of the silencing of expression of a defensin in ticks by RNAi that resulted in reduced *A. marginale* infections.

Keywords Defensin · Varisin · RNA interference · *Dermacentor variabilis* · *Anaplasma marginale*

K. M. Kocan · J. de la Fuente · R. Manzano-Roman
Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK, USA

J. de la Fuente · V. Naranjo
Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain

W. L. Hynes · D. E. Sonenshine (✉)
Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529-0266, USA
e-mail: dsonensh@odu.edu

Introduction

Ticks transmit a greater variety of pathogens than any other group of hemotophagous arthropods (Sonenshine 1993). In ticks, the midgut is the first site of exposure to a wide variety of hemoparasites that may be ingested with the bloodmeal. Some of these hemoparasites are either not infective for ticks and rapidly digested or cleared by the innate tick immune system. Others infect midgut epithelial cells where they multiply and subsequently infect other tissues including the salivary glands. Transmission may occur when the tick is ingested by the vertebrate host or from salivary glands via the saliva to vertebrate hosts when the tick feeds again. Tick-borne pathogens have apparently co-evolved with ticks for their mutual survival because, while pathogens undergo considerable multiplication in ticks, these infections do not appear to be detrimental to tick feeding or their biology (Kocan et al. 1992a, 2005; Sonenshine et al. 2005).

Among the various tick-borne pathogens, those belonging to the genus *Anaplasma* (Rickettsiales: Anaplasmataceae) are obligate intracellular organisms found exclusively within parasitophorous vacuoles in the cytoplasm of both vertebrate and tick host cells (Kocan 1986; Dumler et al. 2001). The type species, *A. marginale*, causes the economically important cattle disease, anaplasmosis, with *Dermacentor variabilis* comprising one of the main tick vectors of this pathogen in the USA (Kocan et al. 2004).

While the molecular relationship between ticks and pathogens is not well understood, these molecular interactions may enhance or be necessary for tick and pathogen biology (de la Fuente et al. 2007a). In this emerging area of research, initial studies of tick host cell response to *Anaplasma* infection revealed genes that are differentially expressed in response to pathogen infection. These genes, therefore may be necessary for and facilitate pathogen infection, multiplication and transmission (i.e. receptors) or limit infections that favor tick survival (de la Fuente et al. 2001, 2005, 2007a, b; Manzano-Roman et al. 2007).

One component of innate immune systems of eukaryotic organisms are the small cationic peptides known as defensins, which have been identified in a wide range of species ranging from the simplest invertebrates to mammals, as well as plants (Gillespie et al. 1997). Among invertebrates, the most completely characterized defensins contain six cysteines and provide immunity against gram-positive bacteria (Ganz and Lherer 1994; Fogaca et al. 2004). In insects, these defensins were found to be expressed primarily in fat body and midgut epithelial cells (Hoffmann and Hetru 1992; Boulanger et al. 2002).

Defensins have been identified in a variety of ixodid ticks, including *D. variabilis* (Johns et al. 2001a; Ceraul et al. 2003), *Ixodes scapularis* (Hynes et al. 2005), *Amblyomma americanum* (Todd et al. 2007), *A. hebraeum* (Lai et al. 2004) and *R. microplus* (Fogaca et al. 2004; Tsuji et al. 2007). While defensins have clearly been shown to be expressed in tick hemocytes (Johns et al. 2000, 2001a), they were also found to be expressed or at least transcribed in midguts and other tick tissues in the soft tick *Ornithodoros moubata* (Nakajima et al. 2002) and the hard ticks *Amblyomma americanum* and *Ixodes scapularis* (Hynes et al. 2005; Todd et al. 2007). Tick defensins were shown to be involved in protection against a wide range of organisms such as *Micrococcus luteus* in *Dermacentor variabilis* (Johns et al. 2001a) or *Escherichia coli* and *Staphylococcus aureus* as demonstrated in *A. hebraeum* (Lai et al. 2004). Upregulation of a defensin occurred in response to challenge-exposure of *D. variabilis* with the gram-negative rickettsia, *Rickettsia montanensis,* fed to ticks via capillary tubes (Ceraul et al. 2007). In addition, defensins were also found to provide immunity against the protozoan parasites, *Babesia equi*, *B. gibsoni* and *B. microti* (Tsuji et al. 2007). When varisin, the defensin found in *D. variabilis*, was silenced, antimicrobial activity of hemolymph was reduced 2–4 fold

compared to controls, indicating that this peptide is essential for the tick's innate immune response (Hynes et al. 2008). This collective research suggests that defensins contribute to the elimination or modulation of microbes to which ticks are exposed.

In this study we hypothesized that expression of varisin would provide protection in *D. variabilis* against infection by the gram-negative *A. marginale.* RNA interference (RNAi) was used to silence the varisin gene in male *D. variabilis*, after which the ticks were allowed to feed on an *A. marginale*-infected calf to acquire bacteria. Varisin gene silencing was confirmed by real time RT-PCR and *A. marginale* abundance was determined by use of a quantitative PCR assay for *A. marginale msp4* gene. Surprisingly, the results derived from this research were contrary to our hypothesis and demonstrated that silencing of varisin resulted in significantly reduced *A. marginale* numbers. Further studies are needed to determine whether defensin may be necessary for the development of *A. marginale* in ticks.

Materials and methods

Ticks

Dermacentor variabilis males were purchased from a laboratory colony maintained at the Oklahoma State University (OSU), Tick Rearing Facility, Stillwater, OK, USA. Larvae and nymphs were fed on rabbits and male ticks derived from the engorged nymphs were used for these studies. Male ticks were used for these studies because they become persistently infected with *A. marginale* and the pathogen's developmental cycle has been well described in the intrastadial cycle. In addition intrastadial studies avoid the possible influence of molting. Off-host ticks were maintained in a L12:D12 photoperiod at 22–25°C and 95% relative humidity.

Infection of ticks with *A. marginale*

For infection of ticks with *A. marginale*, male *D. variabilis* ticks injected with either varisin dsRNA or elution buffer alone were allowed to acquire bacteria during feeding (acquisition feeding, AF). Acquisition was done by feeding the ticks for 7 days on a splenectomized calf that was experimentally-infected with the Virginia isolate of *A. marginale* which was shown previously to be infective and transmissible by ticks (Kocan et al. 1992a, b) when the ascending percent parasitized erythrocytes (PPE) was 3–4%. The ticks were then removed and maintained off-host for 4 days, after which they were allowed to feed for 7 days on a sheep to allow for development of *A. marginale* in tick salivary glands and transmission (transmission feeding, TF). Two days after infestation of the sheep all unattached ticks were removed and discarded. All ticks were removed after 7 days of feeding and held in the humidity chamber for 4 days. The calf and sheep were housed at the OSU Center for Veterinary Health Sciences, Laboratory Animal Resources with a protocol approved by OSU Institutional Animal Care and Use Committee.

RNA interference in ticks

Oligonucleotide primers homologous to *D. variabilis* defensin and containing T7 promoters for in vitro transcription and synthesis of dsRNA (DEFT75: 5′-*TAATACGACTCA CTATAGGGTACT*ATGCGCGGACTTTGCATCTGC and DEFT733: 5′-*TAATACGACTC ACTATAGGGTACT*TACGTCGACAAAGCGCTTCGG) were synthesized to amplify tick

defensin. RT-PCR and dsRNA synthesis reactions were performed as described previously (de la Fuente et al. 2006a, b), using the Access RT-PCR system (Promega) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The purified dsRNA was quantified by spectrometry (BioRad SMART SPEC 3000).

In order to test the effect of injection with varisin dsRNA on development of *A. marginale* in male *D. variabilis,* 20 ticks per group were injected in the lower right quadrant of the ventral surface of the exoskeleton with approximately 0.4 µl of varisin dsRNA (5×10^{10}–5×10^{11} molecules per µl) (de la Fuente et al. 2006a, b). The exoskeleton was first pierced with the tip of a 30 g needle to create an opening and then the dsRNA was injected through this opening into the hemocoel using a Hamilton® syringe fitted with a 33 g needle. Twenty ticks were injected with *D. variabilis* subolesin dsRNA to serve as positive controls (de la Fuente et al. 2006a, b) or elution buffer used in the final step of purification of dsRNA (10 mM Tris–HCl, pH 7, 1 mM EDTA) alone to serve as negative controls. The ticks were held in a humidity chamber for 24 h after which they were allowed to feed on an experimentally infected calf.

Analysis of tick attachment and feeding

Tick attachment was evaluated during AF and TF as the ratio of attached ticks 48 h after infestation on the calf to the total number of ticks. Tick mortality was evaluated as the ratio of dead ticks after feeding on the calf (AF) or the sheep (TF) to the total number of fed ticks. Tick attachment and mortality were compared between dsRNA and elution buffer-injected ticks by χ^2-test as implemented in Mstat 4.01 ($\alpha = 0.01$).

Dissection of tick tissues and hemolymph collection for determination of mRNA levels and *A. marginale* infections

Midguts were dissected from five ticks after AF and stored in RNAlater (Ambion) for extraction of DNA and RNA using Tri-Reagent (Sigma) according to manufacturer's instructions to determine the *A. marginale* levels by *msp4* quantitative PCR (de la Fuente et al. 2001) and to confirm gene expression silencing by real-time RT-PCR as described below. After TF, salivary glands and guts were dissected from five ticks from each group and processed for RNA and DNA studies as described. Tick tissues were processed and analyzed individually. Midguts and salivary glands were also collected from another five ticks and fixed for microscopy studies (see following section).

To assess the effect of defensin RNAi on the expression of defensin in tick hemocytes, 50 male *D. variabilis* ticks were injected with defensin dsRNA or elution buffer alone as described above. Injected ticks were allowed to feed on a calf for 3 days after which they were removed with forceps. Hemolymph was collected from the severed legs of two groups of 25 ticks each from both the RNAi and control groups using finely drawn 100 µl glass collecting micropipets (VWR International, Suwanee, GA, USA), and dispensed into 30 µl of sterile phosphate-buffered saline (PBS). Total RNA was extracted and the expression of defensin was quantified by real time RT-PCR as described below.

Real-time reverse transcription (RT)-PCR analysis

Total RNA was extracted from five individual uninfected and *A. marginale*-infected male *D. variabilis* guts and salivary glands and from two hemolymph pools from 25 ticks each using TriReagent (Sigma) according to manufacturer's instructions. Two primers were

synthesized based on the sequences of *D. variabilis* defensin (Genbank accession number AY181027; Ceraul et al. 2003) (DvDEFEN-5: TCTGGCATCATCAAGCAGAC and DvDEFEN-3: CTGCAAGTATTCCGGGGTTA) and used for real-time RT-PCR analysis of mRNA levels in uninfected and *A. marginale*-infected ticks. Subolesin mRNA levels were determined as described previously (de la Fuente et al. 2006b). Real-time RT-PCR was done using the QuantiTec SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) and a Bio-Rad iQ5 thermal cycler (Hercules, CA, USA) following manufacturer's recommendations. Amplification efficiencies were normalized against tick β-actin (forward primer: 5′-GAGAAGATGACCCAGATCA; reverse primer: 5′-GTTGCCGATGGTGATCACC) using the comparative Ct method (de la Fuente et al. 2007a, b). mRNA levels were compared between infected and uninfected ticks by Student's *t*-test ($P = 0.05$) and average mRNA levels were used to calculate percent silencing in dsRNA-injected ticks with respect to elution buffer-injected controls.

Quantification of *A. marginale* infections in ticks by PCR

Anaplasma marginale infections in dsRNA injected and control ticks were determined by a major surface protein 4 (*msp4*) quantitative PCR as reported previously (de la Fuente et al. 2001). Total DNA was extracted from five individual *A. marginale*-infected and uninfected male *D. variabilis* collected after TF using TriReagent (Sigma) according to manufacturer's instructions. *Anaplasma marginale* infection levels in tick midguts and salivary glands were compared between dsRNA and saline injected ticks by Student's *t*-test ($P = 0.05$).

Light microscopy studies of *D. variabilis* gut and salivary glands

Ticks were cut in half, separating the right and left halves, and fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). Tick halves were then post-fixed in 0.2 M sodium cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol and embedded in epoxy resin (Kocan et al. 1980). Thick sections (1.0 μm) were cut with an ultramicrotome and stained with Mallory's stain (Richardson et al. 1960). Photomicrographs were recorded using a light microscope equipped with a 3-chip digital camera.

Results

Tick attachment, feeding and *A. marginale* calf infection levels during tick feeding

Tick attachment and survival after AF (95% attachment and 85% survival) and TF (95% attachment and 89% survival) did not appear to be affected by injection of ticks with varisin dsRNA when compared to the elution buffer (100 and 97% attachment and 88 and 91% survival after AF and TF, respectively; $\alpha > 0.01$) and subolesin-injected controls (95 and 100% attachment and 88 and 90% survival after AF and TF, respectively; $\alpha > 0.01$). The PPE during tick feeding on the calf experimentally infected with the Virginia isolate of *A. marginale* ranged from 4.8 to 35.9%.

Silencing of expression of varisin in tick tissues

RNAi resulted in 99.4% silencing of varisin expression in tick hemolymph as determined by real-time RT-PCR (Table 1). Silencing of the varisin gene by RNAi was also confirmed

Table 1 Confirmation of gene silencing in midguts, salivary glands and hemolymph from male *D. variabilis* that were injected with varisin and subolesin dsRNA

Tick tissue/collection time	Expression silencing ± *SD* (%)[a]	
	Varisin	Subolesin
Midguts after AF	89.9 ± 0.1*	90.0 ± 21.5*
Midguts after TF	97.4 ± 0.1*	99.7 ± 0.7*
Salivary glands after TF	57.9 ± 0.2*	99.4 ± 0.9*
Hemolymph[b]	99.4 ± 0.5*	ND

[a] Total RNA was extracted from five individual ticks from each group and varisin and subolesin expression silencing was determined with respect to control ticks after RNAi. mRNA levels were determined by real-time RT-PCR and compared between dsRNA-treated and control ticks by Student's *t*-test (*$P < 0.05$). Amplification efficiencies were normalized against β-actin using the comparative Ct method and average mRNA levels were used to calculate percent silencing in dsRNA-injected ticks with respect to elution buffer-injected controls

[b] Ticks were allowed to feed for 3 days after treatment on an uninfected calf and hemolymph was collected from two groups of 25 ticks each. Varisin mRNA levels were determined with respect to control ticks after RNAi by real-time RT-PCR and compared between dsRNA-treated and control ticks by Student's *t*-test (*$P < 0.05$) as described above for tick guts and salivary glands. ND, not determined

by real time RT-PCR in tick midguts after AF (89%) and in the midguts (97%) and salivary glands (57.9%) after TF as compared with the elution buffer-injected controls (Table 1). For the positive control ticks injected with subolesin dsRNA, silencing in midguts after AF was 90.0%; after TF, it was 99.7% in midguts and 99.4% in salivary glands (Table 1).

The effect of varisin RNAi on *A. marginale* infections in male *D. variabilis*

Levels of *A. marginale* tick infections, as determined by a *msp4* quantitative PCR and analyzed by Student's *t*-test, were significantly reduced in tick midguts after AF and in salivary glands after TF as compared with the elution buffer-injected controls ($P < 0.05$) (Table 2). Although not statistically significant, *A. marginale* infection levels were also lower in tick midguts after TF as compared with the elution buffer-injected controls (Table 2). Reduction of *A. marginale* levels after RNAi of the subolesin gene (positive control) was statistically significant only in salivary glands collected from transmission fed ticks (Table 2).

Expression levels of varisin in *A. marginale*-infected and uninfected *D. variabilis*

Varisin mRNA levels were higher after TF in the midguts of uninfected ticks as compared to infected ticks ($P = 0.02$). In contrast, varisin levels were significantly higher in the salivary glands from *A. marginale* infected ticks ($P = 0.05$) as compared to the salivary glands from uninfected ticks (Table 3).

Light microscopic changes in ticks injected with varisin dsRNA

Morphologic changes were observed in the colonies of *A. marginale* in tick midguts after injection of ticks with varisin dsRNA as compared with the elution buffer-injected control ticks. While typical large, round colonies of *A. marginale*, were observed in the control ticks, colonies in the varisin dsRNA injected ticks were irregular in shape (Fig. 1a, b).

Table 2 *Anaplasma marginale* infection levels in *D. variabilis* males that were injected with varisin and subolesin dsRNAs and then allowed to acquire *A. marginale* infection by feeding on an experimentally infected calf

Tick tissue	Average *A. marginale*/tick ± *SD*[a]		
	Varisin RNAi	Subolesin RNAi	Control
Midguts after AF	340 ± 535*	814 ± 122	40579 ± 6993
Midguts after TF	1006 ± 470	1517 ± 1025	28252 ± 27788
Salivary glands after TF	2 ± 0*	2 ± 0*	287 ± 144

[a] *A. marginale* infection levels in midguts or salivary glands from five ticks per group were determined by *msp4* PCR and compared between dsRNA-treated and control ticks by Student's *t*-test (*$P < 0.05$)

Table 3 Varisin expression levels in *A. marginale*-infected and uninfected *D. variabilis*[a]

Tick tissue	Average mRNA levels ± *SD* (arbitrary units)		I/U	*P*
	Uninfected	Infected		
Gut	5.5 ± 0.6	1.8 ± 1.5	0.3	0.02
Salivary gland	12.5 ± 5.2	29.2 ± 11.1	2.3	0.05

[a] Varisin mRNA levels were determined by real-time RT-PCR and compared between dsRNA-treated and control ticks by Student's *t*-test ($N = 5$). Amplification efficiencies were normalized against β-actin using the comparative Ct method. The infected to uninfected mRNA ratio (I/U) was calculated and showed that defensin mRNA levels significantly decreased in tick guts but increased in tick salivary glands after infection with *A. marginale*

Some tick midgut cells appeared to contain *A. marginale* free in the cytoplasm rather than within the parasitophorous vacuole (Fig. 1b, arrowheads). Hemocytes in the varisin dsRNA injected ticks were degranulated as compared with those from the controls (Fig. 1c, d). Two of these ticks appeared to be systemically infected with microbes of unknown identity. Large numbers of these organisms were observed in most tissues, including midguts (Fig. 1e) and spermatogonia (Fig 1f). Similar systemic microbial infections were not observed in the elution buffer or subolesin dsRNA-injected controls (data not shown).

Discussion

Ticks are exposed to a wide variety of organisms from mammalian hosts during their extended feeding periods. While some of these organisms are not infective for ticks, others infect tick midguts, where they undergo development and are subsequently transmitted to other hosts during feeding or when the ticks are ingested by the host. During attachment and blood feeding, tick genes express a variety of proteins and peptides involved in the innate immune response that function to inhibit microbial infection, as well as mitigating the oxidative stress and the toxic byproducts (e.g., heme) of hemoglobin digestion. These proteins may include several stress reducing proteins such as glutathione-*S*-transferases (Dreher-Lesnick et al. 2006), protease inhibitors, lectins and others (Lehane et al. 1997; Rudenko et al. 2005; Zhou et al. 2006). In addition, anti-microbial peptides in ticks have been reported to be upregulated in response to microbial challenge. For example, lysozyme was found to be upregulated in tick hemolymph after challenge-exposure with *E. coli* (Simser et al. 2004).

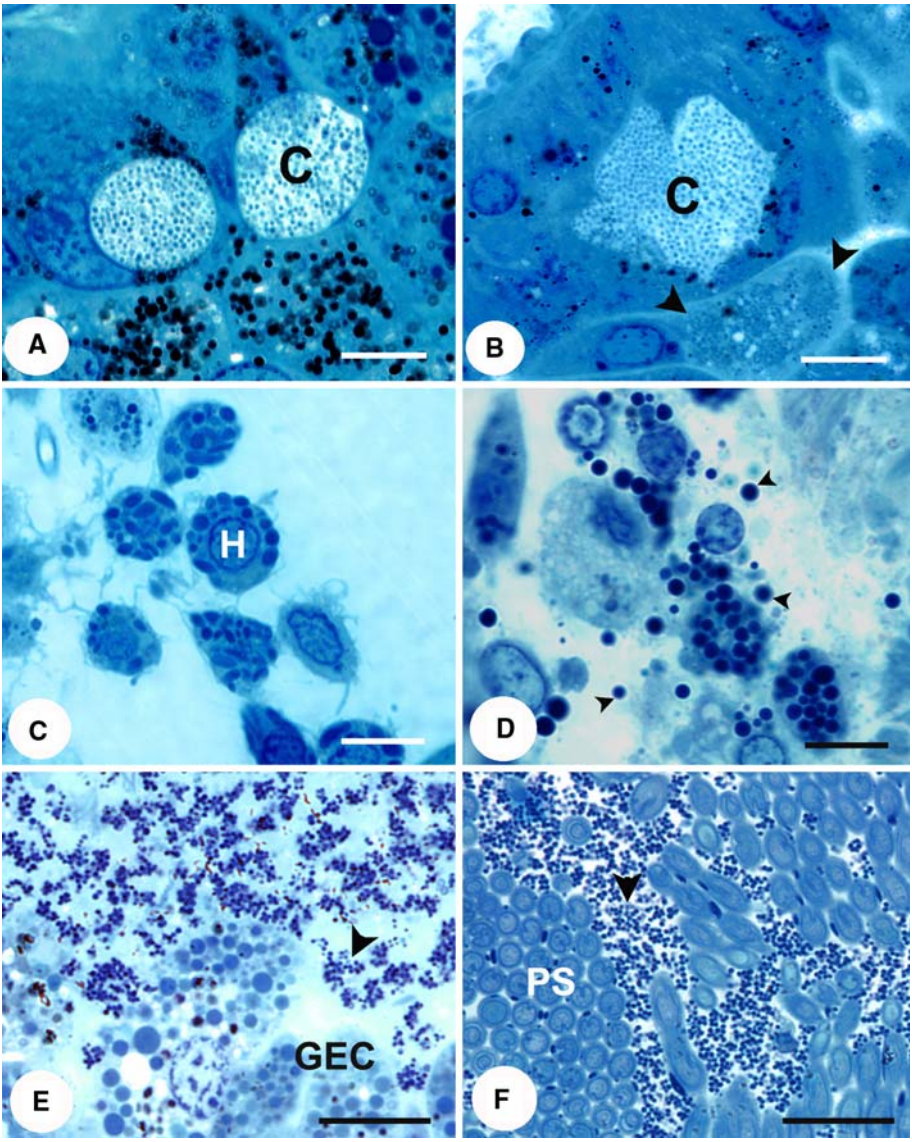

Fig. 1 Light micrographs of tissues in cross sections of ticks that were either injected with varisin dsRNA or elution buffer to serve as controls. (**a**) Typical large round colonies (C) of *A. marginale,* as described previously by Kocan et al. (1992a, b), were observed in the midguts of the elution buffer injected control ticks, (**b**) *A. marginale* colonies (C) observed in the varisin dsRNA males were irregular in shape or appeared to be disrupted in the cytoplasm of gut cells (arrows), (**c**) granulated hemocytes (H) were observed in the hemocoel of elution buffer injected control ticks, (**d**) in contrast to the control ticks, many hemocytes in the varisin dsRNA injected ticks had degranulated (small arrows), (**e**) some ticks appeared to be systemically infected with microbes (arrow) which were seen in the midguts lumen (arrow) near gut epithelial cells (GEC), and (**f**) in spermatogonia (small arrow) among prospermatids (PS). **a** and **b**, bars = 10 μm; **c** and **d**, bars = 5 μm; **e** and **f**, bars = 10 μm

An example of the ability of ticks to rapidly eliminate noninfective organisms was demonstrated by de la Fuente et al. (2001) in which *D. variabilis* males that fed for 7 days on calves with >70% erythrocytes infected with a non-tick transmissible isolate (Florida isolate) of *A. marginale* were found to be clear of *A. marginale* DNA 4 days after being removed from the infected calf.

The small cationic peptides, defensins, are a notable part of the innate response in ticks. Defensins were found to be upregulated in response to challenge with *B. burgdorferi* or gram positive bacteria (Johns et al. 2001b; Nakajima et al. 2001, 2002; Ceraul et al. 2003). Upregulation of tick defensins has also been reported in response to gram negative bacteria such as the intracellular rickettsia, *R. montanensis* (Ceraul et al. 2007) and to protozoan pathogens such as *Babesia* species (Tsuji et al. 2007). The reports cited above suggest that ticks are able to eliminate or at least curtail most microbial infections to which they are exposed.

In this research we tested the hypothesis that one of the defensins identified in *D. variabilis*, varisin, was involved in the tick innate immune response in response to infection with the gram negative cattle pathogen, *A. marginale.* If the results supported our hypothesis, silencing the expression of the varisin gene by RNAi would have resulted in greater numbers of *A. marginale* in the ticks. While expression of varisin was confirmed to be silenced in the midguts and hemocytes of the male *D. variabilis* after AF and in the midguts and salivary glands after TF, both sites of varisin expression (Johns et al. 2001a; Ceraul et al. 2003), the results of these studies were opposite to those expected. Silencing of varisin resulted in significantly lower numbers of *A. marginale* organisms in these male ticks. These results suggested that defensin may play a role in *A. marginale* infection and multiplication in *D. variabilis* in a manner different than we had expected. Interestingly, varisin appeared down-regulated in the gut of infected ticks but it was up-regulated in the salivary glands after TF. These results suggest a mechanism by which *A. marginale* may down-regulate varisin expression to establish infection in the guts while in the salivary glands varisin may plays a role in pathogen infection and multiplication.

Although these studies were not designed to quantify morphologic changes, the appearance and integrity of the *A. marginale* colonies in midgut epithelial cells suggested an impact of varisin RNAi on parasite development. Within host cells, *A. marginale* develop within a parasitophorous vacuole (called colonies) which is uniformly round. However, in ticks in which varisin was silenced by RNAi, *A. marginale* colonies were highly irregular and some organisms appeared to be free within the cell cytoplasm.

Another explanation for the reduction in the numbers of *A. marginale* organisms is that it may have resulted from divergent changes in the levels of expression of off-target genes (Scacheri et al. 2004; Ma et al. 2006). At least in mammalian systems, RNAi is known to induce unexpected and divergent changes in the levels of expression of off-target genes (Scacheri et al. 2004). Specifically, in some mammalian systems, RNAi resulted in global upregulation of the interferon system with unexpected consequences (Siedz et al. 2003). Similarly, as reported for *salps* 16 and other tick genes (Sukumaran et al. 2006; de la Fuente et al. 2007c), defensin expression may be manipulated by the pathogen to aid in its multiplication by an as yet undefined mechanism. Alternatively, RNAi treatment may have affected other physiological processes that modified tick susceptibility to infection by other pathogens. Finally, due to the redundant gene function of other defensin genes (Ceraul et al. 2007), the possibility that silencing of the varisin gene targeted in these studies may not be sufficient to suppress all defensin response in ticks should be considered.

Interestingly, other effects were noted in ticks after varisin RNAi. We observed that two of five ticks appeared to have a systemic infection with an unknown microbe. Although the

microbes were seen in most tissues, infections were most notable in the midgut and testis. However, similar systemic infections were not seen in sections of five control ticks (elution buffer- or subolesin dsRNA-injected ticks). While the microscopy studies herein were not designed to be quantitative, this observation provided evidence that the silencing of varisin by RNAi may have been related to extensive multiplication of a microbe other than *A. marginale*. Further studies are needed to define the relationship between other microbes and *A. marginale*. We also noted degranulation of hemocytes in the ticks injected with varisin dsRNA. However, whether either of these observations were directly related to varisin knockdown is not known.

The results reported here illustrate the utility of RNAi as a powerful tool for studying the effect of gene silencing in ticks as reported previously (de la Fuente et al. 2007c). However, the effect of gene silencing may be indirect rather than direct due to off-target RNAi effects and may be limited by our understanding of the molecular biology of tick-pathogen interactions. Since ticks and the pathogens they transmit have co-evolved molecular interactions to assure their survival, these interactions are likely to involve loci in both the pathogen and the tick. Further studies are needed to fully explore the impact of defensins on the infection and development of *A. marginale* in ticks.

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Potential effects of mixed infections in ticks on transmission dynamics of pathogens: comparative analysis of published records

Howard S. Ginsberg

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Abstract Ticks are often infected with more than one pathogen, and several field surveys have documented nonrandom levels of coinfection. Levels of coinfection by pathogens in four tick species were analyzed using published infection data. Coinfection patterns of pathogens in field-collected ticks include numerous cases of higher or lower levels of coinfection than would be expected due to chance alone, but the vast majority of these cases can be explained on the basis of vertebrate host associations of the pathogens, without invoking interactions between pathogens within ticks. Nevertheless, some studies have demonstrated antagonistic interactions, and some have suggested potential mutualisms, between pathogens in ticks. Negative or positive interactions between pathogens within ticks can affect pathogen prevalence, and thus transmission patterns. Probabilistic projections suggest that the effect on transmission depends on initial conditions. When the number of tick bites is relatively low (e.g., for ticks biting humans) changes in prevalence in ticks are predicted to have a commensurate effects on pathogen transmission. In contrast, when the number of tick bites is high (e.g., for wild animal hosts) changes in pathogen prevalence in ticks have relatively little effect on levels of transmission to reservoir hosts, and thus on natural transmission cycles.

Keywords Mixed infections · Ticks · Coinfection · Transmission dynamics

Introduction

Ticks serve as vectors for numerous pathogens, and individual ticks are often infected with more than one pathogenic organism. Multiple infections can have medical significance, because coinfection can increase severity of symptoms in humans and animals (Belongia 2002; Thomas et al. 2001). Mixed infections in ticks can also potentially influence transmission dynamics, because of either interactions between the pathogens within the

H. S. Ginsberg (✉)
USGS Patuxent Wildlife Research Center, Coastal Field Station, Woodward Hall-PLS,
University of Rhode Island, Kingston, RI 02881, USA
e-mail: hginsberg@usgs.gov

ticks, or pathogenic effects on tick behavior or survival. This applies both to vertebrate pathogens in ticks and to entompathogenic organisms. For example, Ross and Levin (2004) found that some strains of *Anaplasma phagocytophilum*, the etiologic agent of granulocytic anaplasmosis in humans, affect molting of *Ixodes scapularis* ticks. Hornbostel et al. (2004) found sublethal effects of the entomopathogenic fungus *Metarhizium anisopliae* on fecundity and body mass of *I. scapularis*. In cases of mixed infections, these pathogens could potentially influence transmission of other pathogens in the tick by virtue of their effects on tick behavior and survival.

Investigators have used two major approaches to studying the ecological features of mixed infections in ticks. One is to infect ticks with single or multiple pathogens in the laboratory, and to quantify differences in pathogen persistence and transmission to lab animals. This approach gives direct information about pathogen interactions, but these interactions might differ in the laboratory than under field conditions. The second approach is to quantify infection with each pathogen in field collected ticks, and to test whether the prevalence of mixed infections is higher or lower than would be expected on the basis of chance alone. This approach provides field tests of the interactions of pathogens, but interpretation can be difficult because the number of mixed infections results from factors other than just interactions of pathogens. For example, if two pathogens occur in different vertebrate host species, then these pathogens will generally not be found together in nymphal ticks, even if they have no interaction within the tick. Even for ticks with broad host ranges, nymphs have generally fed only once (as larvae) and have therefore acquired pathogens from only one vertebrate host species. In this case, proportions of mixed infections in nymphs would be expected to be lower than in adult ticks, because the nymphs have only fed once while adults have fed twice, and the adults might have picked up infections from different host species.

To fully assess the causes of observed levels of coinfection at a given site, it is necessary to conduct laboratory studies and in-depth local field studies. However, it is also worthwhile to ask whether broad patterns of coinfection from numerous field sites fit the hypotheses of antagonistic or of mutualistic interactions within ticks, or whether tick host associations are adequate to explain the observed patterns. I briefly review some relevant research below. A comparative analysis of published data from numerous field sites follows.

Evidence of interactions between pathogens within tick hosts

Negative relationships between pathogens

A well-known example of negative interactions of rickettsiae within ticks is the transovarial transmission interference of *Rickettsia rickettsii* (agent of Rocky Mountain Spotted Fever) in *Dermacentor andersoni* ticks coinfected with the nonpathogenic Spotted Fever Group rickettsia *R. peacockii* (Burgdorfer et al. 1981; Macaluso and Azad 2005). Ticks coinfected with both rickettsiae vertically transmit only the nonpathogenic species, which influences the distribution of *R. rickettsii*. Similar negative interactions apparently occur among other arthropod-transmitted rickettsiae (Macaluso et al. 2002; Rudakov et al. 2003).

De la Fuente et al. (2002) inoculated *I. scapularis* cells with different strains of *Anaplasma marginale*, and found that only one strain persisted. Furthermore, *A. ovis* infection excluded infection by *A. marginale* in *I. scapularis* cells. When *Dermacentor variabilis*

males fed sequentially on calves infected with different strains of *A. marginale*, only one strain persisted in the ticks (De la Fuente et al. 2003).

Negative interactions in ticks have also been reported for pathogens other than rickettsia. For example, Alekseev et al. (1996) presented evidence that *Borrelia* infection suppressed replication of tick-borne encephalitis virus in *Ixodes persulcatus*. Mather et al. (1987) found that *I. scapularis* that were parasitized by the encyrtid wasp *Ixodiphagus hookeri* were not infected with *Borrelia burgdorferi* and were rarely infected with *Babesia microti*, two pathogens that were common in ticks not parasitized by wasp larvae. However, this phenomenon could have resulted from the host-finding behavior of the wasp (rather than from pathogen interactions within ticks), because *I. hookeri* might have preferentially parasitized ticks attached to white-tailed deer, which is a poor reservoir for both pathogens (Samish et al. 2004).

Positive relationships between pathogens

Sutáková and Rehácek (1990) found increased spread of *Coxiella burnetii* into tissues of *Dermacentor reticulatus* in the presence of *Rickettsia phytoseiuli*. In a survey of 738 *Ixodes persulcatus* ticks in Russia for infection with *Babesia microti* (Alekseev et al. 2003), all 7 infected ticks were also infected with other pathogens (including *Borrelia* spp. and tick-borne encephalitis virus (TBEV)). Postic et al. (1997) found high prevalence of mixed infections of *Borrelia* genospecies in ticks and hosts, also in Russia. In the United States, Mixson et al. (2006) found higher than random levels of coinfection of *Ehrlichia chafeensis* and *E. ewingi* in *Amblyomma americanum* ticks. *Rickettsia amblyommii* and *Borrelia lonestari* also showed higher than random levels of association in this tick. Of course, these high levels of coinfection in field-collected ticks might have resulted from ecological factors relating to pathogen infections in reservoir hosts and tick feeding preferences, rather than from mutualistic interactions of pathogens within the ticks.

No interactions between pathogens

Levin and Fish (2000) studied transmission of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* by *Ixodes scapularis* to white-footed mice in the laboratory. They found no differences in transmission rates between singly infected and coinfected ticks. In field studies, Korenberg et al. (1999) found no evidence of interference between TBEV and *Borrelia burgdorferi* s.l. in *Ixodes persulcatus* ticks in Russia, and Morozova et al. (2002) found these pathogens to be distributed independently in *I. persulcatus* in western Siberia. Hornbostel et al. (2005) found no differences in prevalence of *B. burgdorferi* s.s. in *Ixodes scapularis* ticks regardless of whether or not they were infected with the entomopathogenic fungus *Metarhizium anisopliae*. Swanson and Norris (2007) found that *Borrelia burgdorferi* s.s. and *Rickettsia* spp. were distributed independently in *I. scapularis* ticks. They found that some other pathogens co-occurred with *B. burgdorferi* more frequently than expected due to chance alone, but they attributed these cases to shared enzootic cycles rather than to interactions within the ticks.

These examples provide evidence that some pathogens display antagonistic interactions in ticks, others display mutualisms, and many apparently do not interact within the ticks. However, they do not provide insight into the frequency of each type of interaction among pathogens within ticks. In the following sections I assess the frequencies of various types of interactions between pathogens within ticks by compiling data from several field studies

that measured infections of various pathogens within ticks at various sites, and testing whether the proportion of coinfections was significantly higher or lower than would be expected due to chance alone. I then consider the implications of coinfections for transmission dynamics of these pathogens.

Methods

The scientific literature was surveyed to find studies that reported raw data on infection rates of pathogens in ticks, including mixed infections, with sample sizes large enough for statistical analysis. This survey was restricted to papers where the pathogen strains were identified (thus mostly to recent literature) and where the numbers of individuals infected singly with each pathogen, the number coinfected with both, and the number not infected with either, could be ascertained. Much of this work has been restricted to pathogens of public health importance, so mostly pathogens that have been at least tentatively implicated in human illness were included. The numbers of ticks positive and negative for each pathogen at each site were compiled in 2 × 2 contingency tables, and simple chi-square tests (SAS, version 9.2) was used to assess significance. To quantify the degree of departure of the number of mixed infections from independence, I developed an index of coinfection (I_c), defined as the difference of the number of coinfections from the number expected due to chance alone, as a percentage of the total number of infected ticks in the sample. If a = number of ticks infected with both pathogens, b = number infected only with pathogen 1, c = number infected only with pathogen 2, and d = number not infected with either pathogen, then the number of observed coinfections (O) equals a, the expected number of coinfected ticks due to chance alone (E) is given by: $E = ((a + b)(a + c))/(a + b + c + d)$, and the total number of ticks infected by either or both pathogens (N) is: $N = a + b + c$. The index of coinfection (I_c) is:

$$I_c = ((O - E)/N) \times 100$$

Note that I_c is positive when the number of coinfections is greater than expected, and negative when there are fewer coinfections than would be expected due to chance alone.

To assess the potential implications of coinfection for pathogen transmission dynamics, I used a simple binomial model of the probability of exposure to a pathogen (P_e) when an animal is bitten by n ticks, and with a prevalence of infection in ticks of k_v (Ginsberg 1993, 2001). P_e is the probability that at least one of the n ticks is infected:

$$P_e = 1 - (1 - k_v)^n$$

Results

Levels of coinfection of various pathogens in *Ixodes ricinus* and *I. persulcatus* are shown in Table 1. The number of mixed infections differed signficantly from the expectation due to chance alone in about half the cases. I_c was positive (when significant) for mixed infections of *B. burgdorferi s.s.* and *B. afzelii*, of *B. garinii* and *B. valaisiana*, of *B. burgdorferi s.s.* and *B. garinii*, and of *B. burgdorferi* s.l. and *Anaplasma phagocytophilum*. I_c was negative (when significant) for interactions between *B. afzelii* and *B. valaisiana*. I_c was sometimes positive and sometimes negative for coinfections between *B. afzelii* and *B. garinii*.

Table 1 Patterns of coinfection between pathogen species in *Ixodes ricinus* and in *I. persulcatus* ticks. P = probability from chi-square test, I_c = index of coinfection

Tick species	Pathogen 1	Pathogen 2	Stage(s)	Number sampled	Number coinfected	Total number infected	P	I_c	Reference
Ixodes ricinus	*Borrelia burgdorferi* s.s.	*B. afzelii*	nymphs	411	3	25	<0.0001	+10.25	Kirstein et al. (1997)
			adults	90	1	26	0.478	+1.71	Golubić et al. (1998)
	B. afzelii	*B. garinii*	nymphs	411	0	30	0.516	−1.31	Kirstein et al. (1997)
			nymphs	198	1	30	0.878	−0.51	Hanincová et al. (2003b)
			adults	90	0	28	0.275	−2.98	Golubić et al. (1998)
			adults	239	0	65	0.018	−6.44	Hanincová et al. (2003b)
			adults	147	1	46	0.135	−4.92	Hanincová et al. (2003b)
			adults	154	0	35	0.229	−3.23	Hanincová et al. (2003b)
			nymphs + adults	200	10	53	0.007	+9.53	Rauter et al. (2002)
			nymphs + adults	256	26	126	0.022	+5.52	Rauter et al. (2002)
			nymphs + adults	196	8	33	<0.0001	+18.21	Rauter et al. (2002)
			nymphs + adults	200	9	74	0.507	+2.00	Rauter et al. (2002)
			nymphs + adults	203	7	45	0.001	+10.01	Rauter et al. (2002)
	B. afzelii	*B. valaisiana*	nymphs	411	2	41	0.062	+3.38	Kirstein et al. (1997)
			nymphs	130	0	28	0.393	−2.06	Hanincová et al. (2003b)
			nymphs	198	2	29	0.391	+2.93	Hanincová et al. (2003b)

Table 1 continued

Tick species	Pathogen 1	Pathogen 2	Stage(s)	Number sampled	Number coinfected	Total number infected	P	I_c	Reference
			nymphs	174	0	31	0.197	−4.45	Hanincová et al. (2003b)
			adults	239	0	52	0.111	−3.86	Hanincová et al. (2003b)
			adults	147	0	46	0.038	−6.63	Hanincová et al. (2003b)
			adults	154	0	67	0.0006	−10.68	Hanincová et al. (2003b)
			adults	90	4	30	0.239	+5.00	Golubić et al. (1998)
	B. garinii	*B. valaisiana*	nymphs	411	8	51	<0.0001	+11.74	Kirstein et al. (1997)
			nymphs	198	3	21	0.005	+10.82	Hanincová et al. (2003b)
			adults	90	0	12	0.557	−2.50	Golubić et al. (1998)
			adults	239	4	33	0.008	+8.32	Hanincová et al. (2003b)
			adults	147	2	27	0.596	+2.12	Hanincová et al. (2003b)
			adults	154	0	44	0.153	−3.36	Hanincová et al. (2003b)
	Borrelia burgdorferi s.l.	*Anaplasma phagocytophilum*	nymphs	114	1	16	0.260	+3.95	Hildebrandt et al. (2003)
			adults	112	15	59	0.234	+4.72	Christova et al. (2001)
			adults	424	21	112	<0.0001	+10.08	Stańczak et al. (2002)

Table 1 continued

Tick species	Pathogen 1	Pathogen 2	Stage(s)	Number sampled	Number coinfected	Total number infected	P	I_c	Reference
			adults	303	25	117	0.004	+7.62	Stańczak et al. (2004)
Ixodes persulcatus	*Borrelia burgdorferi* s.s.	*B. afzelii*	adults	1280	10	185	<0.0001	+4.62	Alekseev et al. (2003)
	B. burgdorferi s.s.	*B. garinii*	adults	1280	28	85	<0.0001	+30.75	Alekseev et al. (2003)
	B. afzelii	*B. garinii*	adults	1280	68	300	<0.0001	+14.76	Alekseev et al. (2003)

Mixed infections of diverse pathogens in *I. scapularis* and *A. americanum* are shown in Table 2. I_c was positive for coinfections of *Ehrlichia chafeensis* and *E. ewingi* in *A. americanum* ticks. No other significant levels of coinfection were observed.

One major effect of positive or negative interactions among pathogens within a tick would be to raise or lower infection prevalence of the affected pathogen. The potential effects on transmission dynamics are shown in Fig. 1. The effects differ when tick populations are low (e.g., one tick bite in Fig. 1) compared to when tick populations are high (e.g., 50 tick bites) because the probability of exposure to the pathogen rapidly reaches 1.0 when hosts are exposed to numerous tick bites. When tick numbers are low, the effect on P_e of changes in the proportion of ticks infected is more or less linear. When the number of tick bites is high, on the other hand, P_e is near 1.0 unless the proportion of ticks infected is near zero (Fig. 1).

Discussion

Nymphal ticks have generally fed only once, as larvae. Therefore, mixed infections in nymphs (for pathogens such as *Borrelia* spp. that are not generally passed vertically) presumably result from feeding on a host infected with both pathogens. Of course, some ticks might carry mixed infections because, for example, *Borrelia* infections are occasionally passed vertically, and larval feeding can be interrupted, which can result in a second larval feeding, but these are probably relatively rare phenomena (Nefedova et al. 2004). Only two significant positive associations of *Borrelia* spp. were found in nymphal ticks. One was for *B. burgdorferi* s.s. and *B. afzelii* in *I. ricinus*. Both of these *Borrelia* species commonly infect rodents, suggesting that these ticks fed as larvae on rodents with mixed infections. The other was for *B. valaisiana* and *B. garinii* in *I. ricinus*. These *Borrelia* species commonly infect songbirds (Hanincová et al. 2003b), and these ticks might have attached to coinfected birds. In both cases, the positive associations suggest that there are no negative interactions between these *Borrelia* species within *I. ricinus*. In fact, I found no significant negative associations between *B. burgdorferi* s.s and *B. afzelii* or between *B. valaisiana* and *B. garinii* in any stage of any tick species.

Adult ticks have fed twice, once as larvae and once as nymphs. Higher than expected occurrence of pathogens in adult ticks could result from immature ticks feeding on frequently coinfected hosts or from positive interactions among the pathogen species (e.g., higher transmission efficiency of one pathogen when the tick is already infected with the other, or higher pathogen survival in ticks in mixed infections than in single infections). Negative associations presumably result from negative interactions between the pathogens. Some *Borrelia* species primarily infect mammals while others primarily infect birds, but this would only lead to a negative association within a tick species if individual ticks tended to feed on mammals as both larvae and nymphs, while other individuals of the same species tended to feed on birds as both larvae and nymphs. This seems unlikely, although it is plausible that when engorged larvae drop from their hosts, they might be left in microhabitats that would favor them biting the same type of host after molting to the nymphal stage. This possibility is worth additional study.

Most pairs of *Borrelia* species showed at least some examples of higher than expected coinfections. Reservoirs of *B. burgdorferi* s.s. include both mammals (Levine et al. 1985; Kurtenbach et al. 2002) and birds (Richter et al. 2000; Ginsberg et al. 2005). Thus positive associations could result from ticks feeding on coinfected hosts of both mammal-associated species such as *B. afzelii* (Hu et al. 1997; Hanincová et al. 2003a), and with other *Borrelia*

Table 2 Patterns of coinfection between pathogen species in *Ixodes scapularis* and in *Amblyomma americanum* ticks. P = probability from chi-square test, I_c = index of coinfection

Tick species	Pathogen 1	Pathogen 2	Stage	Number sampled	Number coinfected	Total number infected	P	I_c	Reference
Ixodes scapularis	*Borrelia burgdorferi*	*Anaplasma phagocytophilum*	adults	147	4	79	0.715	−0.67	Schulze et al. (2005)
	Anaplasma phagocytophilum	*Babesia odocoilei*	adults	68	0	15	0.308	−5.49	Steiner et al. (2006)
Amblyomma americanum	*Ehrlichia chaffeensis*	*E. ewingi*	adults	121	5	20	0.0002	+18.80	Schulze et al. (2005)
	Borrelia lonestari	*E. chaffeensis*	adults	121	1	25	0.721	−0.67	Schulze et al. (2005)
	E. ewingi	*B. lonestari*	adults	121	0	21	0.297	−4.33	Schulze et al. (2005)

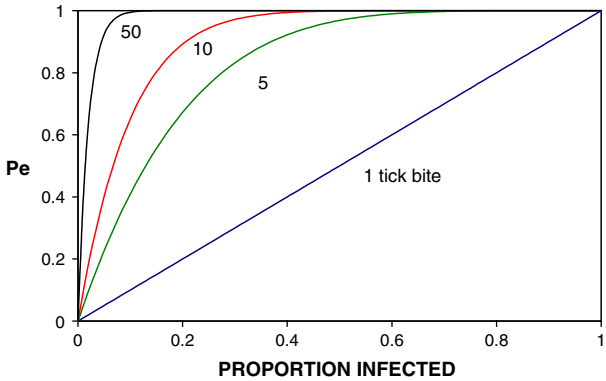

Fig. 1 Probability of host exposure to a pathogen (P_e) at different levels of tick abundance and pathogen prevalence in ticks

species associated with both mammals and birds such as *B. garinii* (Kurtenbach et al. 2002). The one example of a lower than expected level of coinfection of *B. afzelii* and *B. garinii* in *I. ricinus* (Table 1) suggests that there is no positive interaction of these pathogens within the tick.

The one pair of *Borrelia* genospecies that generally showed lower than expected levels of coinfection was *B. afzelii* with *B. valaisiana* in *I. ricinus* (Table 1). As already mentioned, *B. afzelii* primarily infects mammals while *B. valaisiana* primarily infects birds, so individual ticks feeding on coinfected hosts is unlikely. Nevertheless, nymphal ticks can attach to different hosts than larvae, so this negative association might suggest a negative interaction between these pathogens within *I. ricinus*. However, the one case in Table 1 of a marginally positive association in nymphs (data from Kirstein et al. 1997) suggests that there is no negative interaction between these pathogens within *I. ricinus* ticks. Infection and transmission experiments with these *Borrelia* species in *I. ricinus* in the lab could help clarify the mechanism responsible for these results.

The positive associations between *B. burgdorferi* s.l. and *A. phagocytophilum* (Table 1) could have resulted largely from ticks feeding on coinfected hosts because mice and other small mammals serve as reservoirs for both of these pathogens (Kurtenbach et al. 2002; Telford et al. 1996). Similarly, the positive association of *Ehrlichia chaffeensis* with *E. ewingi* in *Amblyomma americanum* ticks (Table 2) could result from the likely role of white-tailed deer, *Odocoileus virginianus*, as the primary reservoir of both rickettsial species (Dawson et al. 2005; Paddock et al. 2005).

The abundant examples of higher than expected levels of coinfection in ticks suggest that hosts are frequently infected with more than one pathogen species. This could result from positive interactions of pathogens within the vertebrate hosts, or it could simply result from large tick populations. When tick populations are large enough that individual host animals are bitten by numerous ticks, then the probability that individual host animals are exposed to more than one pathogen is high.

Implications for pathogen transmission patterns

In general, these results provide little evidence of negative interactions among pathogens within ticks (with the possible exception of *B. afzelii* and *B. valaisiana*). Nevertheless, there are a few examples in which negative interactions between pathogens have been documented, such as the interaction of *Rickettsia peacockii* with *R. rickettsii* (Macaluso

and Azad 2005), and the interactions among selected strains of *Anaplasma marginale* (de la Fuente et al. 2003). There have also been some reports of positive interactions among pathogens, such as those of *Babesia microti* with other pathogens in *I. persulcatus* (Alekseev et al. 2003). These interactions could potentially influence transmission dynamics by lowering or raising infection prevalence in ticks, and thus affecting the probability that an individual vertebrate will be exposed to the bite of an infected tick. The potential implications of negative or positive interactions among pathogens in mixed infections apparently differ for humans than for reservoir hosts involved in natural transmission cycles. Most humans are bitten by relatively few ticks per year, even in high-incidence sites for Lyme borreliosis (Ginsberg 1993). For a person who is bitten by one tick in a given year, a negative interaction among pathogens within the tick would lower the probability of exposure to the pathogen linearly with the lowering of infection prevalence in ticks (Fig. 1). If infection prevalence is lowered from 0.4 to 0.2, for example, then the probability of exposure is also lowered from 0.4 to 0.2. In contrast, for a wild reservoir host that is constantly exposed to ticks, and is bitten by 50 or more ticks per year, the probability of exposure remains 1.0, even when the prevalence of infection in ticks has been lowered from 0.4 to 0.2 (Fig. 1).

This result applies to positive interactions between pathogens within ticks as well. If prevalence of a pathogen in ticks increases from 0.4 to 0.6, the risk of human disease would increase to the same extent (for humans bitten by one tick per year), but the natural transmission cycle would not be affected. Therefore, interactions among pathogens in ticks that influence pathogen prevalence will tend to have greater direct effects on human disease incidence than on the dynamics of natural transmission cycles. This result does not apply to pathogens with low prevalence in ticks (prevalences below 0.2 in Fig. 1) where changes in prevalence have substantial effects on the probability of exposure. The prevalence level at which changes in prevalence affect transmission depends on tick abundance. For example, at sites where individual hosts are bitten by 1,000 ticks or more (e.g., deer in some locales), P_e is nearly 1.0 even at low pathogen prevalence levels in ticks.

This analysis pertains primarily to cases where transmission is primarily horizontal, such as for *Borrelia burgdorferi* s.l. In contrast, when vertical transmission contributes strongly to pathogen maintenance, as in *R. rickettsii* in *D. andersoni* (Schriefer and Azad 1994), transmission interference by other rickettsia can apparently have strong effects on prevalence.

Hornbostel et al. (2005) found no effect of infection of *I. scapularis* ticks with the entomopathogenic fungus, *Metarhizium anisopliae*, on the prevalence of *B. burgdorferi* in these ticks. Beyond this observation, however, interactions between entomopathogens and zoonotic pathogens in ticks have received little attention. Such interactions warrant further study because they could potentially influence the effectiveness of entomopathogens as biocontrol agents for vector-borne diseases.

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An association between the Antarctic mite *Alaskozetes antarcticus* and an entomophthoralean fungus of the genus *Neozygites*

P. D. Bridge · M. R. Worland

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Abstract A fungal pathogen provisionally identified as *Neozygites* cf. *acaridis* has recently been isolated from the Antarctic oribatid mite *Alaskozetes antarcticus*. The identification of the fungus is discussed with reference to recent changes in the taxonomy of *Neozygites*. The potential role of the fungus in the Antarctic mite populations is considered in relation to the known mite life cycles, and the particular environmental conditions in the Antarctic.

Keywords Oribatida · Ameronothridae · Entomopathogen · Entomophthorales · Environment · Zygomycete

The Antarctic mite *Alaskozetes antarcticus*

Alaskozetes antarcticus (Michael) (Oribatida: Ameronothridae) is a free-living, terrestrial cryptostigmatid mite that is widely distributed throughout the maritime Antarctic. The species has a range that extends from the cool temperate Falkland Islands (c. 51° S) to South Eastern Alexander Island on the Antarctic Peninsula (c. 67° S) (Convey 1998). The adult mite is heavily sclerotized, grows to about 1 mm in length and has a live mass of 150–300 μg. Populations of the mite are typically found in dense aggregations in a variety of nutrient enriched ornithogenic habitats, e.g. under rocks and in crevices close to penguin colonies.

In common with all Antarctic arthropods so far studied, *A. antarcticus* is freeze susceptible but avoids freezing by depressing the freezing point of its body fluids and surviving in a supercooled state. This is achieved by the accumulation of low molecular weight cryoprotective compounds such as glycerol (Montiel 1998), together with the removal or masking of ice-nucleating material from its body. The mite is a detritivore and feeds largely on algal thalli, crustose lichens, fungi and bacteria. These are all potential ice nucleators when

P. D. Bridge (✉) · M. R. Worland
British Antarctic Survey, NERC, High Cross, Madingley Rd, Cambridge CB3 0ET, UK
e-mail: pdbr@bas.ac.uk

contained in the mite gut and so must be removed from its body for the mite to achieve and maintain freezing points below −30°C.

Antarctic environment habitats

The maritime Antarctic typically experiences long periods of severe, variable weather (low temperature with frequent freeze thaw cycles and desiccating conditions) with only short summers when conditions are suitable for growth and reproduction. Air temperatures are typically only above zero for some 1–4 months in the summer each year (Walton 1982). In contrast, large temperature variations can occur on a daily basis with soil temperatures occasionally reaching as high as 22°C at sites where *A. antarcticus* is typically found (Davey et al. 1992).

Survival of arthropods under such harsh conditions demands an extended life cycle, typically lasting more than 5 years from egg to egg (Convey 1994). In order to achieve this all life stages of the population (eggs, nymphs and adults) can survive over winter. Fecundity is low with individuals surviving up to 7 years (Convey 1998; Mitchell 1977) due to low competition for food and virtually no predators.

Antarctic biodiversity

The maritime Antarctic supports only an impoverished terrestrial animal fauna dominated by micro-arthropods (Acari, Collembola) and other micro-invertebrates (nematodes and tardigrades) with just two species of Diptera (Convey 2001). Oribatid mites have a functionally important role in the maritime Antarctic where they are primary decomposers of lower plant material. Microbial autotrophs form the basis of polar terrestrial ecosystem processes (Wynn-Williams 1996) and play a fundamental role in primary colonization and stabilization of mineral soils. Terrestrial plant biodiversity is also very limited and dominated by bryophytes and lichens with only two flowering plants.

Despite the relatively limited terrestrial biota in the Antarctic, a wide range of fungal species have been described from the region. Around 1,000 species names having been recorded for Antarctic fungi from the late 1800s to date, and allowing for synonyms and changed species concepts this reduces to around 700 species names commonly in use (Bridge et al. 2008c). This figure is much larger than the equivalents for other taxa and so the fungi may be the most diverse and numerous terrestrial group (in terms of species numbers). Representatives of all of the major fungal phyla have been reported, and although isolation and observation studies suggest some groups, such as filamentous basidiomycetes, may be very rare (Onofri et al. 2007), recent molecular diversity studies have suggested that the overall distribution of taxa may be quite different to that seen from observation and culture (Lawley et al. 2004). Most of the fungal isolations have been from soils, and although many fungi have very close associations with plants and animals, soil is frequently an important component of their life-cycle (Bridge and Spooner 2001). One particularly interesting observation is that some fungi that are well known from particular niches and environments elsewhere in the world can occur in an alternative niche in the Antarctic. Examples of this include the normally ericoid mycorrhizal species *Rhizoscyphus ericae* that is found on liverwort roots in the Antarctic (Upson et al. 2007), and the normally coprophilous species *Pirella circinans* that has been reported as the almost exclusive coloniser of beetle carcasses on the sub-Antarctic Bird Island (Bridge et al. 2008a).

Entomogenous fungi

An entomogenous habit is relatively common among fungi, and some 750 species in 56 genera are known to be pathogens or parasites of arthropod pests alone (Hawksworth et al. 1995). In comparison the number of species of entomogenous fungi reported from Acari is relatively small at some 40+ named and unnamed species from 18 genera (see Waterhouse and Brady 1982; Keller and Petrini 2005; Van der Geest et al. 2000; Humber and Hansen 2005). Among these the most common pathogens of mites in the natural environment are species of the ascomycete genus *Hirsutella* and the entomophthoralean genus *Neozygites*. Some species of these genera such as *H. thompsonii* have a wide host range and can infect a broad range of invertebrates, whereas others such as *N. floridana* and *N. tetranychi* occur exclusively on mites. Entomogenous fungi have been reported in mite populations world wide, and although much research has been focused on their role in agricultural systems in temperate and tropical regions, they are also known to infect mites in cooler environments including north central Europe, Iceland and the Canadian North-west territories (e.g., Van der Geest et al. 2000; Hallas and Gudleifsson 2004; Humber and Hansen 2005).

Entomogenous fungi on mites grow largely within the host body and then produce either conidia or other resting spores for further dissemination. The spread of infection is not therefore strictly directly from individual to individual and often relies on the new fungal propagules being transferred in soil or vegetation. The entire process may take only a few days or several weeks, but it can occur repeatedly during a season or year, so that the number of infected individuals, and the number of infective particles, usually increases with the invertebrate population. Entomogenous fungi are important regulators of invertebrate populations, and the increasing infection during their life cycle can result in the formation of epizootics. In some cases these can reduce the invertebrate population by 90% by late-summer or autumn (e.g., Klubertanz et al. 1991; Steenberg et al. 1996; Van der Geest et al. 2000). The primary over-wintering mechanism in entomophthorales is large thick walled resting spores that develop either in or on the dead insect and that subsequently germinate to produce new infective conidia (Van der Geest et al. 2000).

Antarctic entomogenous fungi

Although fungal pathogens have been reported from Antarctic nematodes, plants and mosses (see Gray and Lewis-Smith 1984; Bridge et al. 2008b; Pegler et al. 1980), there have only been three reports of fungi appearing as potential pathogens occurring on Antarctic arthropods. The first of these was *Arthrobotrys ferox*, a springtail-capturing fungus that was described from moss in Victoria Land (Onofri and Tosi 1992). Subsequently, an entomophthoralean fungus resembling *N. acaridis* was reported on *A. antarcticus* (Bridge and Worland 2004), and a novel ascomycete *Cordyceps* anamorph on *Cryptopygus* was described as *Paecilomyces antarcticus* (Bridge et al. 2005).

A number of fungal species that have been reported as entomogenous over a wide host range in other environments have been identified in the broad Antarctic area, including among others *Beauveria bassiana*, *Lecanicillium lecanii*, *Metarhizium anisopliae* and *Tolypocladium* species (Lopez Lastra et al. 1991; Hughes and Lawley 2003; Möller and Dreyfuss 1996; Roddam and Rath 1997). These have exclusively been isolated from plants, soils and other environmental features. Fungal species that can infect mites elsewhere in the world have been reported from other environmental sources in the region (see Table 1).

Table 1 Fungal species reported as pathogens on Acari and known to be present in the Antarctic

	Mite host (from Van der Geest et al. 2000 and Humber and Hansen 2005)	Antarctic substrate/environment (from Bridge et al. 2008c)
Acremonium implicatum (syn. *Paecilomyces terricola*)	*Tetranychus urticae*	Soil and litter
Aspergillus flavus	*Dinothrombium giganteum, Thrombidium gigas*	Lake water and air samples
Beauveria bassiana	*Polyphagotarsonemus latus*	Lake sediment and soil
Cladosporium sp.	*T. urticae*	Soil and lichen
Cladosporium cladosporoides	*Eotranchyus* sp.	Tussock grass, mosses & bryophytes, soil, introduced wood
Lecanicillium lecanii (syn. *Verticillium lecanii*)	*Abacarus hystrix*, *T. urticae*, Oribatid species	Gypsum crusts, bryophytes and soil
Simplicillium lamellicola (syn. *Verticillium lamellicola*)	Oribatid species	Moss

Mites collected in the Antarctic frequently 'become mouldy' when, or as, they die in soil-free cultures. Anecdotal evidence suggests that in most cases there is considerable hyphal growth of largely zygomycete fungi. These are often species of *Mucor* and *Mortierella* that are common soil fungi in the Antarctic. There are a number of potential associations that could occur between Antarctic fungi and mites. In other environments viable fungi are routinely isolated from invertebrate frass, and in the Antarctic viable fungi have been reported from the surface of beetle carapaces, and from the gut of soil-based herbivorous larvae (Bridge and Denton 2007: Bridge et al. 2008a).

Infection of *Alskozetes antarcticus*

A soil-free colony of *A. antarcticus* collected at Rip Point, Nelson Island (62°14.93′ S 058°58.73′ W) off the West coast of the Antarctic Peninsula early in 2003, was examined at the British Antarctic Survey in Cambridge in the summer of that year. Typical soil zygomycete growth was present in some collections but a few dead individuals showed a different fungal growth form, with short conidiophores being formed from within the body to produce conidia. This gave individuals a 'dusty' appearance, typical of entomophthoralean infection. Dissection of individuals, together with light microscopy revealed ovoid hyphal bodies, and dark resting spores within the mite bodies, together with curved unbranched conidiophores and greyish light-brown primary conidia. No rhizoids were produced and the only external hyphal growth was from soil zygomycetes (Bridge and Worland 2004). The above characters are typical of the entomophthoralean genus *Neozygites*, and closely match those shown by the known mite pathogenic species in the genus (see Table 2). The fungal collection was originally described as *Neozygites* sp., and most of the features relating to conidiogenesis matched fairly well to those for *N. floridana* and *N. acaridis*. Secondary conidia were not observed, and the relatively scarce hyphal bodies in the infected hosts were distinctly oval, and did not match either the rod shaped or spherical hyphal bodies reported for *N. floridana* and *N. acaridis*, respectively (see Bridge and Worland 2004). *Neozygites floridana* has been reported on a number of different tetranychid mite taxa world wide (Keller 1997; Van der Geest et al. 2000; Delalibera et al. 2004). The isolation of *N. floridana* from an oribatid mite would represent a major extension to its known host range, as all previous reports have been from prostigmatid taxa, and it has been

Table 2 Key morphological features of main named Entomophthoralean mite pathogens[a]

Characteristic	*Neozygites tanajoae*	*Pandora phalangicida*[b] (syn. *Erynia phalangicida*)	*Neozygites* cf. *acaridis*	*Apterivorax. acaricida*[c] (syn. *Neozygites acaricida*)	*Neozygites adjarica*[d]	*Neozygites tetranychi*	*Neozygites floridana*
Rhizoids	Absent	? Present	Absent	Absent	Absent	Absent	Absent
Hyphal bodies	Rod shaped	n/a	Spherical	Spherical	Short, tubular	Tubular or clavate	Rod shaped
Conidiophore	Unbranched	Wide, branched, coalescing	Short	Short, stout	Single	Digitate	Slightly broadened, curved
Primary conidia shape	Globose or ovoid	Hyaline, ovoid	Ovoid	Ovoid	Colourless, Pyriform	Hyaline, broadly pyriform	Pyriform
Primary conidia size	13.7–16.4 × 11.6 × 14.9	19–22 × 10	n/a	9–12 × 5–7	12–17 × 10–14	15–17 × 12–15	13–18 × 11–13
Secondary conidia	As primary, capilliconidia almond shaped, pale brown	As primary	As primary	As primary	Clavate, brownish warty	As primary, capilliconidia almond shaped with brown verruclose wall	As primary, capilliconidia claviform with striate brownish wall
Resting spore shape	Sub-globose, dark brown, roughened surface	n/a	Dark brown	n/a	Spherical or subspherical, black	Pyriform, brown to black, ridged with a hilum	Spherical to ovoid, dark brown, smooth
Resting spore size	17.8–23.1 diam.	n/a	n/a	n/a	14–25 × 14–22	21.7 × 17.6	22–26 × 20–23
Most frequent host(s)	*Mononychellus tanajoa*	*Pergamusus* sp.	*Penthaleus major*, *Halodyteus destructor*, *Euseius citrifolius*	*H. destructor*, *E. citrifolius*	*Tetranychus urticae*, *Oligonychus pratensis*	*Tetranychus* sp.	*Bryobia* sp., *Eotetranychus banksi*, *Oligonychus* spp., *Panonychus citri*, *Tetranychus* spp.

Data partly aggregated from Waterhouse and Brady (1982), Keller (1997) and Delalibera et al. (2004)

[a] A number of *Tarichium* species names have been proposed for some Entomophthoralean mite pathogens that were recorded only as resting spores (Bałazy and Wisniewski 1982, 1984). These are not included here due to the lack of further morphological information

[b] Humber (1989)

[c] Keller and Petrini (2005); Keller (2006)

[d] Included in *N. floridana* by Keller (1991) and Bałazy (1993)

suggested that it is restricted to this family (Pell et al. 2001). On this basis the *A. antarcticus* associated fungus was provisionally identified as *Neozygites* cf. *acaridis* (Bridge and Worland 2004).

Neozygites pathogens of mites

The morphological characteristics of the mite-associated *Neozygites* species are very similar. It is very difficult to grow isolates in artificial culture (Waterhouse and Brady 1982; Leite et al. 2000), and so the identification of isolates is often limited to the morphological features produced on the host at the time of observation. *Neozygites adjarica* has been considered a synonym of *N. floridana* (Keller 1991) and *N. tetranychi* is known only from a single collection (Delalibera et al. 2004). Most isolations from mites have been identified as either *N. floridana* or *Neozygites* sp. Recently, isolates from cassava green mite that had been assigned to *N. floridana* were re-examined and were found to have a reduced host range and reduced cold tolerance in comparison to other *N. floridana* collections. 18S ribosomal DNA analysis recovered the cassava green mite isolates as a separate group and this has been named as *N. tanajoae* (Delalibera et al. 2004). It is therefore possible that isolates labelled as *N. floridana* may represent a complex of morphologically similar species and some of these may correlate with different host ranges or environments (Delalibera et al. 2004). It was not possible to isolate the *Alaskozetes* pathogen in pure culture and so comparable DNA, host range and temperature tolerance data are not available. Given that the maximum summer air temperature at Nelson Island does not exceed 6°C, it would seem likely that the Antarctic isolate would have increased cold tolerance in comparison to typical temperate and tropical isolates of all species. A definite identification of the Antarctic isolate is therefore not yet possible, and will require further collections and some in vitro culture.

Neozygites in sub-polar regions

The genus *Neozygites* is cosmopolitan in distribution, and has been widely reported from arthropod hosts in tropical and temperate regions. The genus is considered by some authors to function best in hot weather, but individual species have also been reported in alpine and sub-polar areas of the northern hemisphere as pathogens of mites and aphids (Pell et al. 2001; Keller 1991; Nielsen et al. 2001). In general conidial viability appears to be better at reduced temperatures (Oduor et al. 1995), and their presence in cool temperate, alpine and sub-polar regions would suggest that resting spores in the soil can survive at very low temperatures.

Neozygites cf. *acaridis* has been isolated from the mite *Penthaleus major* in northern Iceland, and *N. fresenii* has also been reported from aphids in that country (Nielsen et al. 2001; Hallas and Gudleifsson 2004). These reports demonstrate that some strains of *Neozygites* can function in relatively cold environments. The population dynamics for the strain of *N.* cf. *acaridis* in the Icelandic mite infection appear to be rather different to those reported elsewhere. Peak infection was recorded in June from adults of the previous winter generation, with infection in the summer adults slowly rising to only low levels in the latter part of the summer (Hallas and Gudleifsson 2004). This may suggest that in colder climates the fungal life cycle is slowed or interrupted during the winter, with infected adults overwintering and the infection continuing in spring. Given the extended life cycle of

A. antarcticus with all life stages overwintering it would seem likely that a similar process may occur in the Antarctic. In contrast, a recent study of the *Cordyceps* anamorph from Antarctic springtails found that the fungus was three times more common in autumn than in summer (Bridge et al. 2005), suggesting a more typical season-based life cycle for the fungus. This contrast may be related to the differing methods of pathogenicity between ascomycetes and entomophthoraleans but no comparable time-based information is available for the mite pathogen.

There are no reports of significant disease constraints to Antarctic arthropod communities and populations are generally considered to be limited by environmental factors. In the case of *A. antarcticus* the extended life cycle results in all stages of the invertebrate being present in a population (Convey 1994, 1998). Final stages of *Neozygites* infections are normally seen with infected adults and so in a heterogeneous population the epizootics typical of temperate and tropical regions may not become established. There is some anecdotal support for this from the Icelandic observations that the prevalence of the fungal disease was related to the high density of adult females in June (Hallas and Gudleifsson 2004). While *Neozygites* appears to have a functional role within the mite population in the environment, it may be that there is a balance between infection and recruitment but considerable further sampling will be required, particularly in relation to life cycles and population numbers and make-up before this can be considered.

Colonisiation and endemism

There are high levels of endemism among the Antarctic plants and animals, and recent biogeography suggests that most taxa have either evolved in isolation in the Antarctic or are relicts from pre-glacial times (Convey and Stevens 2007). Aerobiological sampling carried out in the Antarctic has shown that invertebrate colonization from airborne propagules is limited to micro-invertebrates which have a desiccation-resistant (anhydrobiotic) stage and which can rehydrate and resume activity if they reach a suitable habitat. Although propagule densities in the maritime Antarctic are much lower than found in temperate regions, they have been shown to include algae, bacteria, fungi and bryophytes (Kinlan and Gaines 2003). Many fungi isolated from the region have airborne propagules or yeast phases. These species have been isolated from air currents in the region and so there is the potential for their continual introduction (Marshall 1996).

There is very little evidence for endemism among Antarctic fungi, and although some 22 fungal species have been reported as endemic (Onofri et al. 2007), a number of these have since been isolated from other regions, including both Arctic and alpine environments (Wuczkowski and Prillinger 2004). Airborne dispersal may explain the low level of endemism seen for some fungi, but the situation for those that do not have a significant airborne stage is less clear. There is very little evidence for co-evolution between fungi and their hosts in the Antarctic, and all of the fungi so far recovered from endemic Antarctic nematodes have been identified as cosmopolitan species (Duddington et al. 1973; Gray and Lewis-Smith 1984). This may be the case with the Antarctic *Neozygites*, as although *Alaskozetes* is restricted to Southern latitudes (Convey 1998), the apparent host shift for *Neozygites* from prostigmatid mites to the oribatid species may be the result of a cosmopolitan fungus being able to adapt in the absence of its usual host and under a different competition regime. This possibility could also explain other apparent host or environmental shifts seen with Antarctic fungi such as *Lecanicillium lecanii* and *Rhizoscyphus ericae* (Hughes and Lawley 2003; Upson et al. 2007).

Future possibilities

Long-term monitoring records have shown that the environmental temperature on the Antarctic Peninsula is rising faster than any other area of the world (King et al. 2003), and this is resulting in shorter winter periods, an increase in available water and more ice-free areas. In particular the availability of liquid water may be more important to biological activity than increased temperature in Antarctic habitats (Kennedy 1993). These environmental changes may affect the current interactions between the Antarctic mites and fungi, and a number of possible scenarios might occur.

Increased temperatures and moisture with a longer summer season could allow a greater proportion of the mite life cycle to be undertaken in a year. This would then alter the composition of the current heterogeneous populations. It is however, unclear as to what level of change in population structure and environmental conditions would be necessary to change the existing interaction and lead to epizootics. Major changes in the population structure in the Antarctic are unlikely, but population structures on some sub-Antarctic islands and those further north could be affected. However, there have been no targeted surveys for entomopathogens in these areas, and so the current range of occurrence and frequency of *Neozygites* sp. in the wider mite population is unknown.

At least seven of the 40+ fungal species that include pathogens of Acari have been reported from the Antarctic environment (Table 1), where they have largely been isolated as viable cultures from soil or vegetation. These fungi may have the potential to infect the existing mite populations under less extreme environmental conditions, and they could provide future sources of infections in Antarctic arthropods as conditions change.

Aerial studies have shown opportunities for colonisation by new fungal species, but it is likely that new colonisation is currently limited by low winter temperatures and a lack of available water together with limited opportunities for dispersal (Convey 2001). A third scenario is that climate change and increasing human activity (such as tourism) could increase the likelihood of colonisation and this could result in the introduction and establishment of more aggressive mite pathogens.

Changes in the radiation climate have already occurred with the regular formation of the 'Ozone hole' over large areas of the Antarctic. Although there are no recorded effects of increased ultraviolet radiation on terrestrial arthropods, the growth of fungi, cyanobacteria, algae and cryptograms is known to be affected (Wynn-Williams 1994; Hughes et al. 2003). In *Neozygites* the infective conidia are typically only lightly melanized and so may be more sensitive to UV than the dark resting spores, although this has not been tested in an Antarctic context. Conversely the conidia are relatively short-lived and so would have short exposure times. The fungal growth is almost entirely within the mite body and so any UV effect would probably be largely limited to the initial infection period. This adds a further uncertainty to any predictions regarding future Antarctic fungal-invertebrate interactions.

The presence of the *Neozygites* and other potential mite pathogens provides a starting point for future changes, but the large number of environmental and functional uncertainties prevent any clear models being developed until more baseline data, particularly on the range and occurrence of the fungus in the population is available.

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Diversity of acaropathogenic fungi in Poland and other European countries

Stanisław Bałazy · Ryszard Miętkiewski · Cezary Tkaczuk · Rudolf Wegensteiner · Marta Wrzosek

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Abstract The occurrence, species diversity and some aspects of taxonomical affinity and host selectivity of acaropathogenic fungi associated with phytophagous, saprotrophic and predacious mites in Poland and other European countries were investigated on wild and cultivated plants, in insect feeding sites under the bark and in decayed wood. From among 33 species of fungi affecting mites only five species of Entomophthorales were separated and the most numerous were *Neozygites floridana* mostly on *Tetranychus urticae*, *N. abacaridis* on a few eriophyid species, and *Conidiobolus coronatus* attacking gamasid mites most frequently of the genus *Dendrolaelaps*. The most frequent mite pathogens occurring in mite communities on plants and in wood infested by insects were of the genus *Hirsutella*. Until now 13 of their form-species have been recognized in these habitats, but only *H. kirchneri*, *H. necatrix* and *H. thompsonii* (including its variety *synnematosa*) can be treated as exclusive oligophagous pathogens of phytophagous mites, though their potential host range seems to embrace only selected eriophyid or tarsonemid mites. Taxonomical differentiation of fungal strains was based on close morphological observations and molecular analysis of ITS region sequences. Two new species of acaropathogenic fungi were described in these studies. *Hirsutella danubiensis* sp. nov. was found in the tetranychid *T. urticae*, whereas *H. vandergeesti* sp. nov. affected phytoseiid mites of the genera *Amblyseius*, *Neoseiulus*, *Seiulus* and *Typhlodromus*, and the tarsonemid *Tarsonemus lacustris.*

S. Bałazy
Research Centre for Agricultural and Forest Environment PAS, Bukowska 19, 60-809 Poznan, Poland

R. Miętkiewski · C. Tkaczuk (✉)
University of Podlasie, Prusa 14, 08-110 Siedlce, Poland
e-mail: tkaczuk@ap.siedlce.pl

R. Wegensteiner
Department of Forest and Soil Sciences, BOKU University, Hasenauerstrasse 38, 1190 Vienna, Austria

M. Wrzosek
Department of Plant Systematics and Phytogeography, Warsaw University, Al. Ujazdowskie 4, 00-478 Warsaw, Poland

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Introduction

Among the relatively small number of microbiological agents that cause infective diseases of mites the entomopathogenic fungi constitute the most numerous group (Lipa 1971; Van der Geest 1985; McCoy 1996; Chandler et al. 2000; Van der Geest et al. 2000). Although fungi affecting mites and insects generally belong to the same taxonomical entities, only little more than 50 species show acaropathogenic capabilities, compared to >1,000 actually known insect pathogenic species excluding Laboulbeniales (Bałazy 2000; Van der Geest et al. 2000). The first cases of mite mycoses were described about 70 years after the recognition of the infective character of insect mycoses and this delay remained until the 1970s, except for a few subtropical and tropical institutes, where mycoses of mites had been included into some research, but usually on a very limited scale (e.g., Fisher 1950, 1951). In Poland the first records on mite mycoses appeared in the 1970s, but during the last three decades they have been developed and widened continuously (Miętkiewski et al. 2000). The political and economical changes of the turn of the 1980s made it possible not only to accelerate these studies in Poland but also to enter into international cooperation and to stimulate acaropathogenic research in other European countries (a.o., Austria, France, Germany, Great Britain, Greece, The Netherlands, Spain). Until now the mycoses of two groups of mites have been partly recognized: (1) phytophagous spider and gall mites, in gardens, orchards and grasslands, and (2) saprotrophic and predacious mites, common in subcortical insects' feeding grounds in forests and mid-field afforestations on trees and decayed wood.

Data on mite pathogens are scarce, hence recent investigations on mycoses of the two groups of host mites widens our knowledge about the diversity of fungal pathogens as well as their taxonomical affinity and host selectivity. Most material included here has been collected or identified in 2003–2007, and this paper is supplementary to three previous publications (Miętkiewski and Bałazy 2003; Miętkiewski et al. 2003; Bałazy et al. 2008).

Materials and methods

Bark and wood samples were preliminarily investigated and selected in local laboratories. Further rearing and periodical checking for the appearance of mycosed arthropod individuals, and subsequent analytical processing (isolation and identification of disease agents, their frequency, duration and succession) were performed in the Research Centre for Agricultural and Forest Environment of the Polish Academy of Sciences in Poznań.

Materials were kept in rearing containers for several months and periodically checked for the presence of fungal insect and mite pathogens at 2–4-week intervals. Fungi were isolated by common insect pathology techniques (Bałazy et al. 2008). The standard potato-dextrose agar (PDA), Sabouraud-dextrose agar (SDA) and SDA enriched with egg-yolk (SDEYA) media were used, the latter sometimes supplemented with powdered milk and/or antibiotics. Quantitative estimations of the fungal pathogens' share in mite mortality were done only in high prevalence cases. Fungi were preserved alive, but in cases of epizootics only representative strains were retained. For details on sampling methods and further

treatment of collected material we refer to previous publications (Miętkiewski et al. 2000, 2003; Bałazy et al. 2008). In recent years attempts have been made to verify the pathogenicity of particular strains by artificial infection of experimental host mites or other invertebrates (Bałazy et al. 2008). On a small scale this research has also concerned mites from forest litter, moss and lichens covering tree logs and trunks, decayed tree hollows and occasionally abandoned bird nests.

The acaropathogenic fungal species considered in this paper have been mostly collected in orchards, meadows and settlements in the vicinity of Siedlce, Białowieża Forest, Wielkopolski National Park and the coniferous or mixed forests of the Wielkopolska region, spruce forests and shelterbelts of southern Bavaria (Germany), similar ecosystems in central and eastern Austria, Parisian Basin (Picardie, Gâtinais, in north-eastern France) and single localities in other countries. In the Siedlce area systematic collections were continued in 2003–2007 on different plants, mostly grasses, weeds and fruit trees. Most samples were taken during the vegetation seasons, but hibernating forms were also studied in dead mites on fruit trees (Tkaczuk et al. 2003). Sometimes so little was left of the host mites, that species identification was impossible.

For taxonomical differentiation of fungal strains, the macro- and micromorphological features of the mycelium and sporulation on the host mites and in cultures were compared, including molecular markers of the whole ITS region (ITS 1, 5.8S and ITS 2). Twenty of them were obtained by ourselves, others were received from GenBank. The total alignment used in this study has 636 bp. Table 1 contains information on the origin of four sequences of recently isolated *Hirsutella* strains and one strain of *Paecilomyces farinosus*, newly introduced as an outgroup. The sequences obtained from GenBank are characterized by accession numbers following on the species names. Accession numbers of 15 strains earlier characterized by the authors can be found in Bałazy et al. (2008). *Cordyceps* strains considered in this analysis were included as the closest teleomorphic relatives based on BLAST search.

Total genomic DNA was extracted from fresh mycelium using the Plant DNeasy Extraction Kit (Qiagen, Valencia, CA, USA). The whole ITS region was amplified using universal primers ITS 4 and ITS 5 proposed by White et al. (1990). The sequences with some ambiguous positions were amplified with the primers ITS 2 and ITS 3 as well. PCR reactions were performed using a Mastercycler personal (Eppendorf), according to Stensrud et al. (2005). Amplified fragments were visualized on 1% agarose gels. The DNA band was subsequently excised and eluted using the QIAEX II Gel Extraction Kit (Qiagen). Cycle sequencing reactions were performed using the purified PCR product, AmpliTaq DNA polymerase and fluorescent dye-labeled terminators BigDye v.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacture's instructions. The ITS was sequenced bidirectionally using the same primers as before. The products were resolved by electrophoresis using Applied Biosystems' 310 DNA sequencing system.

Table 1 New *Hirsutella* and *Paecilomyces* strains used in this study

Species	Strain number	Host	Geographic origin
H. kirchneri	3661/1a	*Abacarus hystrix*	Fontainebleau, France
H. gregis	0207	*Abacarus hystrix*	Siedlce, Poland
H. tydeicola	1107	*Tydeus* sp.	Siedlce, Poland
H. cf. *brownorum*	3787m	Tarsonemid larva	Strass, Bavaria, Germany
P. farinosus	P5	Unidentified caterpillar	Białowieża Forest, Poland

Sequences (about 510 bp long) were aligned using the program CLUSTAL X (Thompson et al. 1994) and thereafter manually adjusted by the eye. The ITS region made as contig from two or more singular sequences from both strands of DNA were compared with those from GenBank NCBI using BLASTN 2.2.5 (Altschul et al. 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al. 2004) and MrBayes (Ronquist and Huelsenbeck 2003).

Results and discussion

Diversity of mite pathogenic fungi and phylogenetic relationships of *Hirsutella* species

From among several dozen species of fungi affecting mites (Table 2) only five species of Entomophthorales were separated, i.e., two *Conidiobolus* and three *Neozygites* species. *Conidiobolus coronatus* is characterized by low host selectivity and strong post-infection aggressiveness caused by highly toxic metabolites (Boguś et al. 1998; Boguś and Szczepanik 2000). Apart from frequent infections of gamasids, mostly *Dendrolaelaps* spp., it was isolated from pupae and adults of predacious beetles including *Crypturgus* spp., all instars of sciarid flies, a few juvenile springtails and one larva of the longicorn beetle *Pogonochaerus hispidus*. Although *C. coronatus* is a constant component of soil fungi especially in dump habitats (Domsch et al. 1980), it has only exceptionally been isolated from mites on plants. In turn, *Neozygites* species show very limited spectra of infected hosts, often restricted to one or few closely related species. For instance, *N. floridana* hosts are about 10 closely related spider mites (Tetranychidae), and often the fungus causes severe epizootics in their populations. However, Delalibera et al. (2004) found that the pathogenic specificity is (or can be) reflected in molecular structure (18S rDNA). On this basis these authors described the new species *N. tanajoae*, strictly adapted to infect the spider mite *Mononychellus tanajoae*, though the micromorphology of this pathogen did not differ from *N. floridana*. Another species, *N. abacaridis*, infects only a few eriophyids of the genera *Abacarus*, *Aculodes* (Miętkiewski and Bałazy 2003) and *Aculus fockeui*—the latter host is newly reported here. Its incidence in populations of the latter host often exceeded 50% in autumn 2007. Only a few species of the genus *Neozygites* are known as mite pathogens (Keller and Petrini 2005).

The most frequent pathogens occurring in mite communities on plants and in wood infested by insects are *Hirsutella* species. Until now 13 of their form species have been recognized in the habitats under study, among them three undescribed species. On the basis of accessible data and the authors' own observations only the species *H. kirchneri*, *H. necatrix* and *H. thompsonii* (including its variety *synnematosa*) can be treated as effective oligophagous pathogens of phytophagous mites, though their potential host ranges seem to embrace only selected eriophyid and/or tarsonemid mites, and sometimes tetranychids. In the Central and Western European climate conditions, only *H. thompsonii* and *H. kirchneri* seem to significantly reduce some eriophyid populations (Minter et al. 1983; Miętkiewski et al. 2003). *Hirsutella thompsonii* was discovered in the Tropics and principally considered a tropical species (Fisher 1950, 1951; McCoy 1996). Recent studies showed that this fungus is the common pathogen of phytophagous mites, mainly eriophyids, in central Europe on grasses and fruit trees (Minter and Brady 1980; Minter et al. 1983; Miętkiewski et al. 2003). In Poland both its synnematous and mononematous forms commonly occur and some isolates also produce stromatic outgrowths, though perithecia were not obtained.

Table 2 Acaropathogenic fungi collected in the years 2003–2007 on phytophagous, predacious and saproxylic mites in European countries, and unpublished data of some earlier collections

Fungus species	Host mite	Host plant or substrate, country, date, other remarks
Entomophthorales		
Conidiobolus coronatus (Costantin) Batko	*Dendrolaelaps* spp.	*Picea abies*, *Pinus sylvestris*, *Quercus robur*; subcortical detritus; single individuals found in several months rearings; GE 2003–2006, PO 2003–2007
Conidiobolus sp.	*Bryobia* sp.	*Potentilla anserina*; leaf underside; PO, Jun. 2004
Neozygites abacaridis Miętkiewski et Bałazy	*Abacarus hystrix*, *Aculodes* spp., *Aculus fockeui*[a]	Grasses, young cereals, fruit trees; leaves, exrescences, PO, Aug.–Nov., 2006–2007
Neozygites floridana (Weiser et Muma) Remaudière et Keller	*Tetranychus urticae*, *Bryobia rubrioculus*	Many cultivated species, some weeds; AU, August 2006, DK, Sep. 2001; PO, Jun.–Oct. (max. in Sep.) 2003–2007, SP, Apr. 2005
Neozygites sp.	Two infected gamasids	*Lolium perenne*; PO, Wielkopolski Nat. Park, Jul. 2002; scarce material
Hypocreales—*Clavicipitaceous anamorphs*		
Haptocillium sp.	*Abacarus hystrix*	*L. perenne*; PO, Aug. 2003, AU, Aug. 2006
Hirsutella cf. *brownorum* Minter et Brady	*Tarsonemus potentillae*, *Tarsonemus* sp., *Dendrolaelaps* sp. and juvenile acaridids	*P. anserina*, leaves; PO, Aug. 2004. Subcortical insect feeding galleries on *P. abies* and *P. sylvestris* (laboratory rearings); GE and PO, 2003–2007; sometimes epizootic in deteriorated cambium; *Q. robur*; FR, Jul. 1997
H. danubiensis sp. nov.	*Tetranychus urticae*[a]	*P. anserina*; Danube Valley, AU, Aug. 2007
H. gregis Minter, Brady et Hall	*Abacarus hystrix*	On grasses; GB, FR, GE, PO, Jul.–Oct. 2003–2007
H. haptospora Bałazy et Wiśniewski	*Dendrolaelaps nostricornutus*, *Proctolaelaps* sp.	*P. sylvestris*; galleries of bark beetles; PO, 1997–2007
H. kirchneri (Rostrup) Minter Brady et Hall	*Abacarus hystrix*, *Aculodes dubius*, *A. mckenziei*, *Aceria tussilagofoliae*,[a] *Aceria* sp., [a] *Phyllocoptes* sp.,[a] Oribatid mite[a]	Common on grasses in Central and West European countries. On *Tussilago farfara*, *Arctium lappa* and *Viola sylvestris;* PO, Sep.–Nov. 2006–2007. On a branch of *Corylus avellana*; FR, Dec. 2004
H. minnesotensis Chen, Liu et Chen	*Tarsonemus* sp.	*P. abies*; subcortical detritus; GE, PO, 2003–2006; usually in strongly deteriorated material; rare
H. necatrix Miner, Brady et Hall	*Abacarus hystrix*[a]	*L. perenne*; FR, Sep. 2005–2007; PO, 2003–2007

Table 2 continued

Fungus species	Host mite	Host plant or substrate, country, date, other remarks
H. nodulosa Petch	*Phytonemus pallidus* ssp. *Fragariae*, *Tarsonemus potentillae*	On leaf underside of *Fragaria grandiflora*, PO. In *Abies alba*, *Larix dahurica*, *P. abies*, *Pinus halepensis*, *P. maritima*, *P. sylvestris*, *Q. robur*; on leaves of *P. anserina*; in subcortical detritus of AU, Aug. 2006–2007, GE, GR, Apr. 2004, FI, Sep. 2005, FR, PO, 2003–2007, RU, Aug. 1990
H. rostrata Bałazy et Wiśniewski	*Dendrolaelaps* spp., some uropodids and parasitids	Subcortical detritus *P. sylvestris*, *P. abies*, *Q. robur*, of common species, PO, GE, 2003–2007
H. thompsonii Samson, McCoy et O'Donnell	*Abacarus hystrix*, *Aculodes* spp., *Aceria* sp., *A. tussilagofoliae*[a]	Central-, West- and South-European countries, on grasses, coltsfoot and rosaceous shrub leaves, Jul.–Dec. 2003–2007; GR, Apr. 2004; SP, Apr. 2007
H. thompsonii var. *synnematosa* Fisher: Samson, McCoy et O'Donnell	*Eriophyes pyri*	*Pyrus communis*, *Malus domestica*, on leaves Jun.–Oct. 2003 in flower and leaf buds, PO, Nov.–Mar. 2003; DK, Sep. 2001; GB, Sep. 2004
H. tydeicola Samson et McCoy	*Aceria phloeocoptes*[a], *Phyllocoptes abaenus*[a], *Tydeus* sp. (Eriophyidae, Tydeidae)	*Prunus domestica*; PO, in bruises, Jan. 2007; on leaves, Sep.–Oct. 2007
H. vandergeesti sp. nov.	*Amblyseius* sp.[a], *Neoseiulus* sp., *Seiulus* sp., *Typhlodromus* sp., *Tarsonemus lucustris*	*Stachys sylvatica*; on leaf underside; FR, Sep.–Oct. 2007
Paecilomyces farinosus (Holm ex S. F. Gray) Fr.	*Phylocoptes gracilis* (Eriophyidae)	*Rubus idaeus*; in buds; PO, Oct. 2007
P. fumosoroseus (Wize)	Remaining of an oribatid carapace	Forest floor vegetation and litter; AU, Aug. 2007
Lecanicillium dimorphum (J. D. Chen) Zare et W. Gams	*Dendrolaelaps* spp. and some other gamasids	*P. sylvestris*, *P. abies*, *Ulmus* spp.; in galleries of subcortical insects; AU, GR, GE, FR, PO, Apr.–Nov. 2003–2007
Lecanicillium cf. *lecanii* (Zimmermann) Zare et W. Gams	*Aceria phyllocoptes Dendrolaelaps* sp., some gamasids and oribatids	Forest and meadow litter, plant leaves and subcortical detritus; GE, FR, PO; few singular isolates, rare; Apr.–Nov. 2004–2007
Lecanicillium cf. *longisporum* (Petch) Zare et W. Gams	Gamasids and tetranychids	In subcortical galleries and on leaves; GE, FR, PO; few isolates; Jun.–Dec. 2003–2006
Lecanicillium muscarium (Petch) Zare et W. Gams	Acaridids, eriophyids, gamasids, uropodids, tetranychids	On plants and in subcortical galleries, common in moist habitats or during wet periods on insects; AU, FR, GE, PO, Apr.–Nov. 2003–2007; RU, Aug. 1990; mycoses of mites singular.

Table 2 continued

Fungus species	Host mite	Host plant or substrate, country, date, other remarks
Lecanicillium psalliotae (Treschow) Zare et W. Gams	*Dendrolaelaps* spp.	In subcortical material; AU, GE, FR, PO, 2004–2007; occasionally isolated even in dry conditions
Lecanicillium spp. (various strains)	*Abacarus hystrix*, *Aculodes* sp., *Euseius finlandicus*,[a] eriophyids, gamasids, and other oribatids	On plants, in subcortical detritus and in organic soil or litter; not rare; AU, FR, GE, PO, Jul.–Nov. 2004–2007; RU, Aug. 1990, singular isolates
Simplicillium lanosoniveum (van Beyma) Zare et W. Gams	*Proctolaelaps* sp., uropodids	In subcortical detritus and litter from a meadow; GE, PO, Jun.–Nov. 2003–2006; singular isolates
Simplicillium spp. (various strains)	Gamasid mites	In subcortical materials; GE, PO, Jun.–Nov. 2003–2007; singular isolates
Other hyphomycetales		
Acrodontium crateriforme (van Beyma) de Hoog	*Neotetranychus rubi*, *Dendrolaelaps* sp., uropodids	On plant stems and leaves and under bark; often numerous, suggesting pathogenicity to arthropods; GE, FR, PO, Jul.–Sep. 2004–2005, 2007
Cladosporium cladosporoides (Fres.) de Vries	*Abacarus hystrix*, *Aculus schlechtendali*,[a] *Eriophyes pyri*[a]	*Agropyron repens*, *P. communis*, *P. domestica*; most common on plants and tree branchlets; all countries and sites; Jul.–Nov. 2003–2007
Ramularia ludoviciana Minter, Brady et Hall	*Abacarus hystrix*, *Aculodes mckenziei*[a]	On *L. perenne*, *Festuca rubra*, *Agrostis stolonifera*; AU, GE, FR, PO, Aug.–Dec. 2003–2007; in late summer and autumn
Sporothrix schenki Hektoen ex Parkins	Eriophyids, gamasids, acaridids and others	On grasses, in subcortical detritus and feeding galleries in wood; GE, FR, PO, Jul.–Nov., every year; pathogenic to eriophyids; not rare; particularly on juvenile instars

AU Austria, *DK* Denmark, *FI* Finland, *FR* France, *GE* Germany, *GB* Great Britain, *GR* Greece, *PO* Poland, *RU* Russia, *SP* Spain

[a] Newly recorded host mites

ITS sequences indicated practically no differences between *H. kirchneri* and *H. necatrix* on the genetic level; these species form together with *H. gregis* a well separated clade (Figs. 1, 2). The strains of *H. thompsonii* display much greater heterogeneity. A tree made with Neighbor Joining only with *H. thompsonii* strains and *H. necatrix* as an outgroup reveals close affinity of all three specimens (not shown). The strain Dq345579 (Xiang et al. 2007) was isolated in the USA by C. W. McCoy in 1981 from citrus rust mite *Phyllocoptruta oleivora*. The strain 3699 came from *Eriophes pyri* (Łosice, Poland) and was described as *H. thompsonii* cf. var. *synnematosa*. Data on the origin or morphology of strain Af293899 are missing in the GenBank Database, as in publications. Hodge (1998) obtained two sequences of *H. thompsonii* var. *synnematosa*. Both are similar to the 3699 strain sequence and form one clade also with Dq345579, Af293844. A similar result for other DNA regions could be a reason for separation of a new taxon from the large and divergent *H. thompsonii*. The closest to this group appeared the rather homogenous *H. minnesotensis* clade (Figs. 1, 2). This latter species is nematopathogenic, and has been isolated repeatedly from juvenile and mature individuals of tarsonemid and probably anoetid mites, reared in rotten wood of Norway spruce and black cherry in the laboratory (Bałazy et al. 2008). Single individuals have been found continuously in material from Bavarian spruce forests over a period of up to 2 years of rearing.

Hirsutella tydeicola found on *Tydeus* sp., *Phyllocoptes abaenus* and *Aceria phloeocoptes* on plum trees, is newly recorded from Poland and perhaps from Europe. In phylogenetic trees it is situated near the nematopathogenic *H. rhossiliensis*, but at conspicuous distance. We found no information on its artificial culturing, so we inserted mycelium of the obtained strains on SDA medium. The fungus grew slowly, attaining 2 cm colony diameter during 1 month. Aerial mycelium forms a hard and wrinkled layer of about 2–3 mm thick, in the centre elevated up to 5 mm of the surface, floccose and white with a slight navy-blue tint. The hyphae are evenly thick (1.3–3.8 μm in diameter). Phialides usually perpendicular or nearly so, protruding singly, 21–37 μm long, rarely attaining up to 50 μm; their basal parts are 3.8–5 μm thick, pretty abruptly narrowing near the half of their height into needle-like smooth necks, 0.5–0.7 μm thick at the tip. Phialospores fusiform, a little bent with the distal end subacute, 4.8–6.2 × 2–3.2 μm, thickest in the central part, set in twos stuck by mucus on the neck ends. Culture reverse brown with the stain diffusing to the medium at over 2 cm zone around the colony margin.

A much wider host range represents *H. nodulosa*, till now reported from insect larvae and several saproxylic or predacious mite species (Bałazy et al. 2008). Since some of this species' strains showed the ability to produce string-like or cylindrical synnemata of the phialides unlike those protruding on their mononematous hyphae (Fig. 3), we selected them for comparative molecular analysis (work in progress). An acaropathogenic strain of this fungus isolated from a tarsonemid mite displays closest genetic affinity to *H. aphidis* from the aphid *Dysaphis plantaginea* (bootstrap support 98%, posterior probabilities 1.0). The closest teleomorphic state for both is *Cordyceps cochlidiicola*, whereas *H. vermicola* and *H.* cf. *brownorum* are placed in the same clade (bootstrap support 96%, posterior probabilities 1.0).

Similar polyphagous aspects are suspected in *H. haptospora* and *H. rostrata*. The first was originally isolated from uropodid mites found in ant nests (*Formica polyctena*), but morphologically identical strains were found on digamasellid mite species (mostly *Dendrolaelaps*) and sciarid midge larvae inhabiting subcortical galleries of cambiophagous insects on Scots pine logs and branches in Poland (Notecka Forest). Recently it was also found on a sciarid larva in subcortical detritus of *Mimosa* wood near Alès, in southern France. Contrary to the easily isolated strains from midges, the fungus on mites appeared

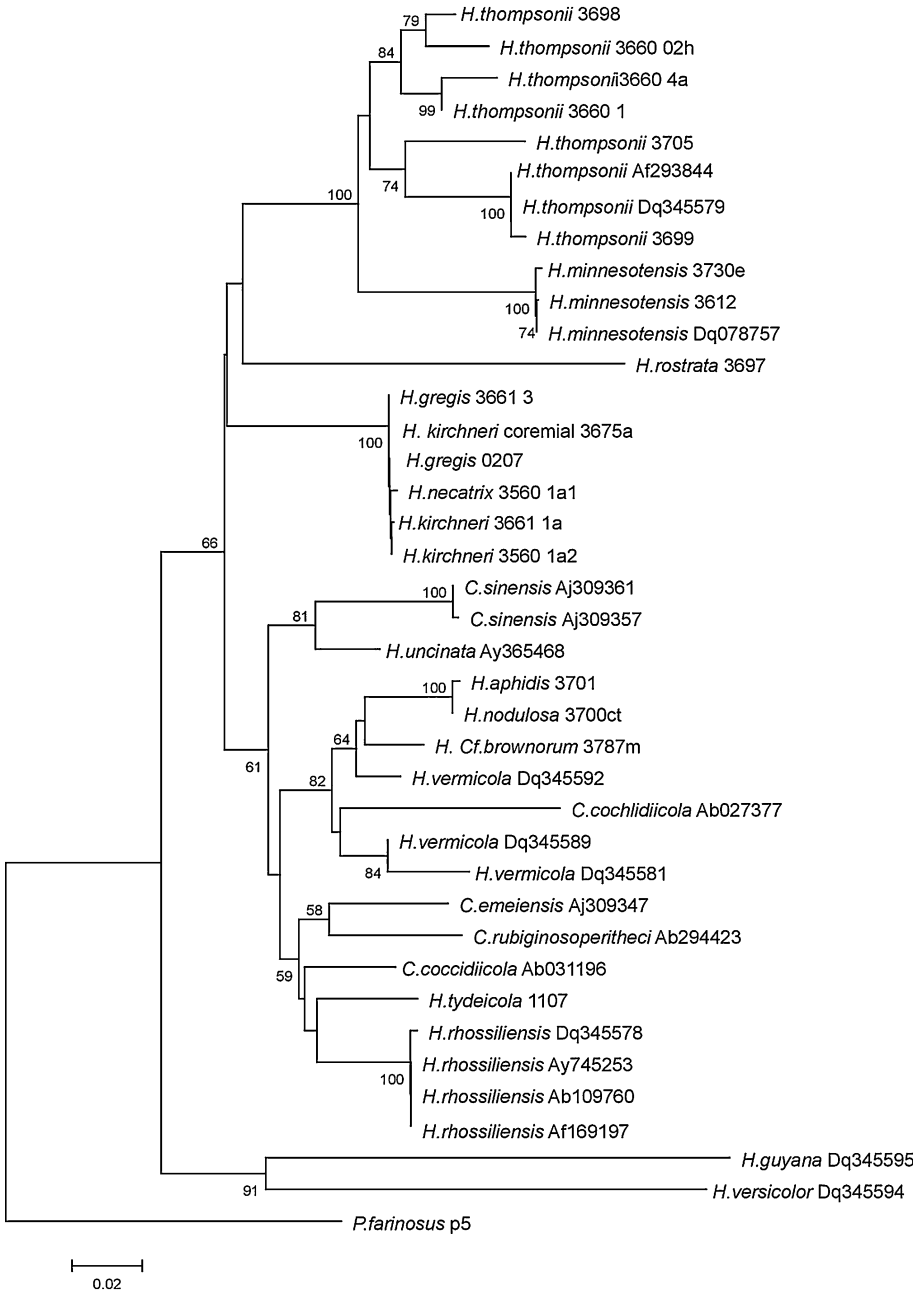

Fig. 1 Phylogenetic tree of *Hirsutella* and *Cordyceps* species obtained with Neighbor Joining method (Kimura, two parameters model). Numbers below the branches are bootstrap percentage values based on 10,000 replicates

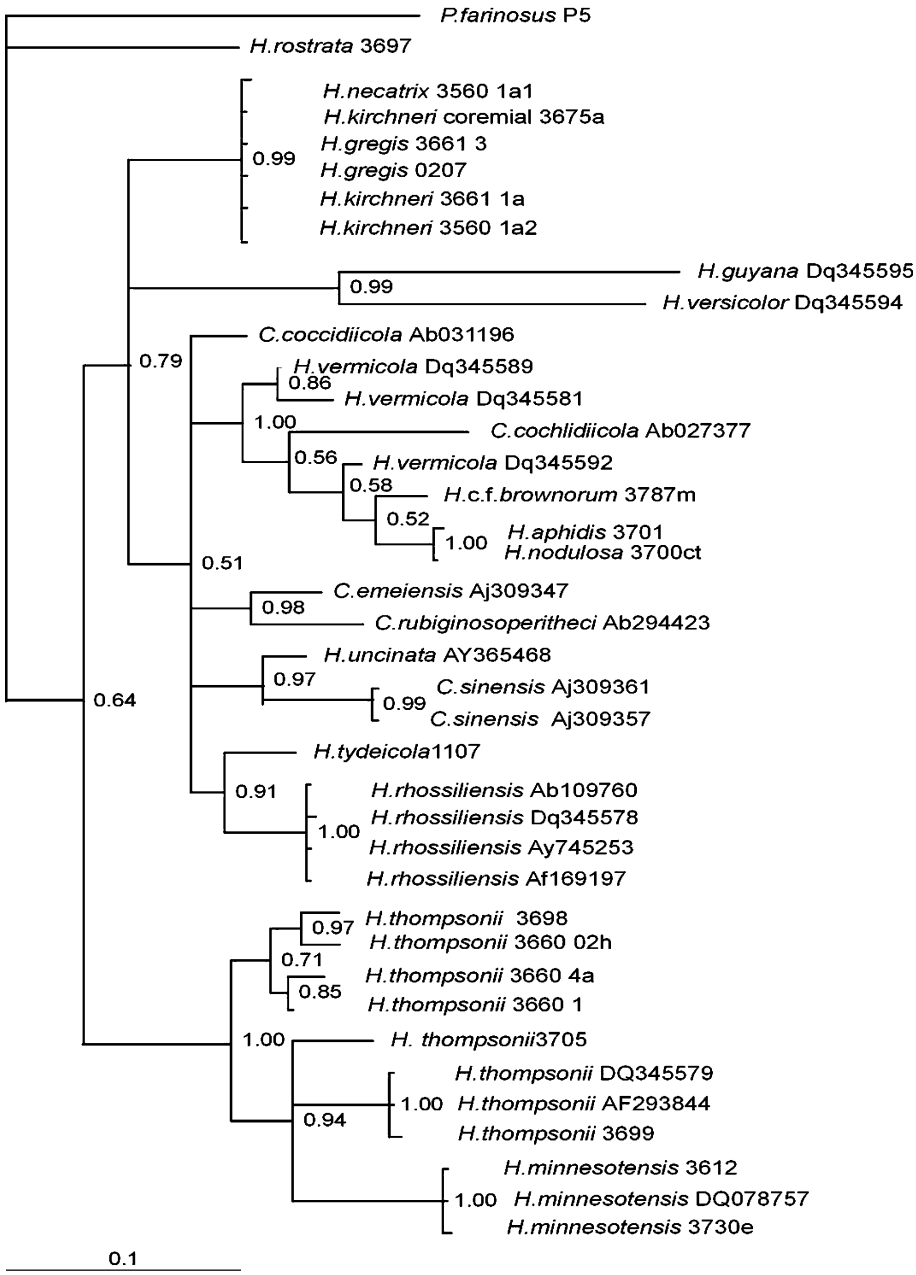

Fig. 2 Phylogeny (consensus tree) of *Hirsutella* and *Cordyceps* species derived from the ITS region data set using Bayesian analysis. Bayesian posterior probabilities are given on nodes

recalcitrant for culture isolation, which hinders a comparison of their genetical markers. *H. rostrata*, the species common on dead mites found in bark beetle galleries, has lately been found on a beetle larva, but attempts to artificially infect birch bark beetle (*Scotylus*

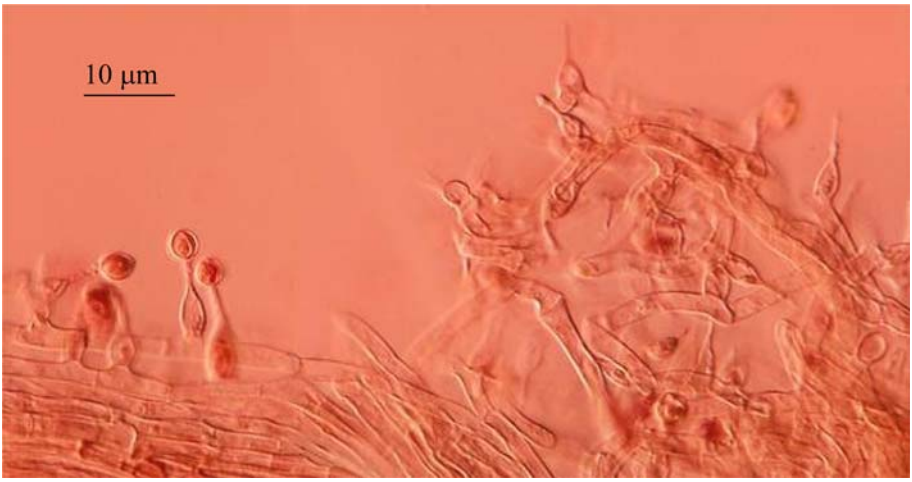

Fig. 3 *Hirsutella nodulosa*, sporulation on synnemata

ratzeburgi) larvae and codling moth (*Cydia pomonella*) caterpillars with its spores failed (Bałazy et al. 2008).

A fungus close to *H. brownorum* often occurs on instars of tarsonemids and, supposedly, acarid, anoetid and parasitid larval forms (seldom also adults) in rotting cambium among the galleries of bark beetle and sciarid larvae under the bark of coniferous trees. However, the shape of its phialospores and its hyphal diameter hardly match the original description of *H. brownorum* (Minter and Brady 1980). The only culture obtained yet allowed us to establish its position in the phylogenetic tree between *H. nodulosa* and *H. vermicola* (Figs. 1, 2). We apply for it the designation *H.* cf. *brownorum* until its nomenclatural position is solved, which can be difficult because of the scarcity of original material of *H. brownorum*. The fungus seems to be widely distributed and it affected over 50–60% of juvenile mites in some bark and wood laboratory rearings. Mycelium on host mites is rather scarce, with delicate hyphae (2–2.8 µm thick) protruding mostly from mouthparts and tarsal segments of the legs, procumbent among the cambium fibres and insect excrements at 3–6 mm distance from the host body. Phialides usually 12–25 long (extreme 32 µm), with the basal ampulliform part 6–12 × 4–5 µm, with single or bi- to four-furcate thin necks of the terminal parts delicately twisted. They protrude singly or oppositely, sometimes in groups of three, laterally from the hyphae in rather distant intervals ca. 70–150 µm. Phialoconidia of somewhat asymmetrical outline 4.5–5.8 × 2.8–3.5 µm, smooth, with a small wart-like projection on the distal end directed to the side; the spores are produced singly or in twos on the necks; on furcate or polyphialidic sporogenous cells normal conidia developed only on one neck, on the second one and further they were underdeveloped or not produced at all. The culture was obtained only once on SDEYA agar, and difficult to isolate; at first mycelium was delicate, white, non-sporulating, and velvety. After enrichment with additional egg yolks consecutive secondary subcultures were growing a little faster and in some of them single conidiogenous cells with very weak sporulation were produced.

Although we investigated entomopathogenic fungi in broad context, the common polyphagous insect pathogens, e.g., *Beauveria*, *Metarhizium*, *Paecilomyces* (or *Isaria* as proposed by Sung et al. (2007)) or the spider-pathogenic *Gibellula* species, did not or

rarely appear on mites. Some higher infection rates appeared in cases of a few *Lecanicillum* species, but only in favorable humidity conditions and at simultaneous high prevalence rates of aphid or scale insects diseases, which seem to be the primary sources of infective material. Among the most common of these pathogens, *L. muscarium* appeared in autumnal months on *Stachys sylvatica* leaves infested by *Cryptomyzus ribis* aphids, but the infections of mites were sporadical. More *Lecanicillium* and *Simplicillium* strains were isolated from mites from the subcortical communities, but their pathogenicity to mites needs to be verified experimentally. Apart from the most common forms close to *Lecanicillium* cf. *lecanii*, also *L. psalliotae*, *L. polymorphum* and *Simplicillium lanosoniveum* were isolated repeatedly. Although some *Lecanicillium* strains isolated from mites showed micromorphological features identical to isolates from insects, their cultures could differ considerably in macromorphological aspects, e.g., mycelium texture, growth intensity, medium staining and others. This suggests that the biological diversity of these organisms is greater than mentioned in recent monographs (Zare and Gams 2001). In two grass samples strains of *Haptocillium* were obtained from dead eriophyids. Mainly in autumn, *Cladosporium* spp. were also isolated from dead mites, though their pathogenicity to mites and insects has as yet been documented weakly.

The constant component of fungal communities of eriophyid mites feeding on grasses is *Ramularia ludoviciana*. Its pathogenic abilities to eriophyids have been confirmed lately in trials by R. Miętkiewski (unpublished). As most acaropathogenic fungi it begins to appear in mid-summer and peak mite mortality falls in October and the first half of November.

Description of two new *Hirsutella* species

Two of the obtained *Hirsutella* forms could not be identified, so we decided to give their full morphological characteristics as new species. Unfortunately, numerous and careful trials to isolate cultures on artificial media failed.

Hirsutella danubiensis Tkaczuk, Bałazy et Wegensteiner, spec. nov. (Fig. 4a, b)

Fungus acaropathogenicus. Mycelium in acaris copiosum, album ex longis et aequoangustis hyphis crassitate 2.5–5.0 µm constans, cum septis in spatia 15–55 µm collocatis. Hyphae ex acarorum mortuorum corporibus radiate excrescunt et circiter eorum sub Potentilla anserina foliis extenduntur. Phialides multae, tenuiconicales ad basim 2.5–3.7 crassae, 35–62.5 µm longae (longitudine media 48.5 µm), modulate in summum exile collum, angustatum ad 0.8–1 µm crassum, nonnumaquam furcillatum, cuius superficies minimum aspera, attenuantur. Phialoconidia parva, dimensionibus 4.5–6.6 × 2.0–2.7 µm (in medio 5.2 × 2.4 µm), instar citri sinensis fructus segmentorum, in apicali parte paulo fortius attenuata, bilateraliter symmetrica, singula vel bina in summis collis formantur.

In acaris mortuis Tetranychidarum (Tetranychus urticae) ex Potentilla anserina foliis in Danubii fluminis valle prope Vindobonam die 12 mensis Augusti anno 2007 collectis. Holotypus: specimen numero 1208 designatum, in collectione Universitatis Podlasiensis in Siedlce.

Hirsutella danubiensis Tkaczuk, Bałazy et Wegensteiner, sp. nov. (Fig. 4a, b)

Mycelium abundant dirty-white of long equally-narrow hyphae 2.5–5.0 µm, with the septa distributed in uneven distances 15–55 µm, protruding radially from dead mites and

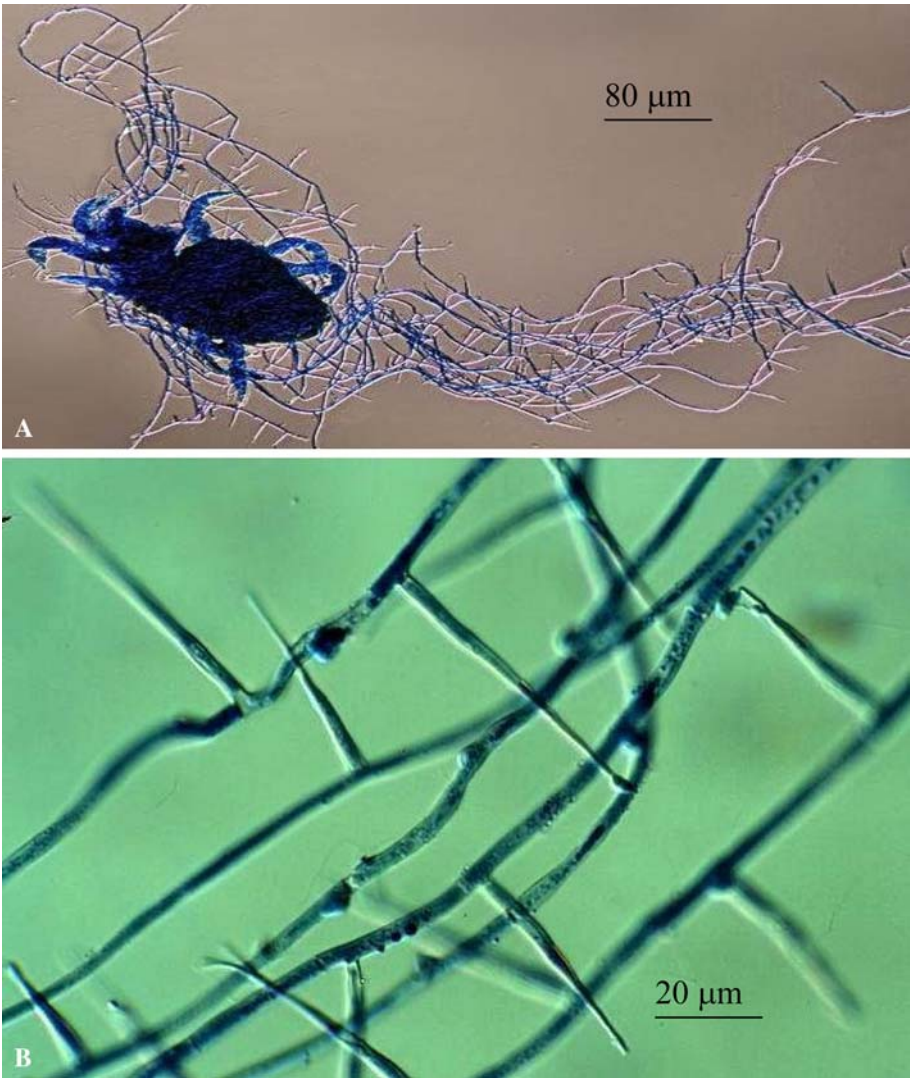

Fig. 4 *Hirsutella danubiensis* sp. nov. **a** Mycelium on spider mite *Tetranychus urticae*, **b** fialides and conidia

outspread around them on the leaf surface. Phialides numerous, very long, arising singly at right angles from hyphae, 2.5–3.7 μm wide at the base, 35–62.5 μm long, (average 48.4 μm), sometimes forked, slender, and from approximately 2/3 of their total height gradually tapering into a slightly warted equally narrow neck, about 0.8–1 μm thick, on the summit. Phialoconidia small, 4.5–6.6 × 1.9–2.7 μm of the shape of orange segment, more strongly narrowed at the distal end and bilaterally symmetrical, produced and persisting singly or stuck in twos by a thin layer of mucus on the top of necks.

On dead *Tetranychus urticae* (Tetranychidae) collected on *Potentilla anserina* leaves in the Danube river valley near Vienna (Austria), in August 2007. The specimen nr 1207 in

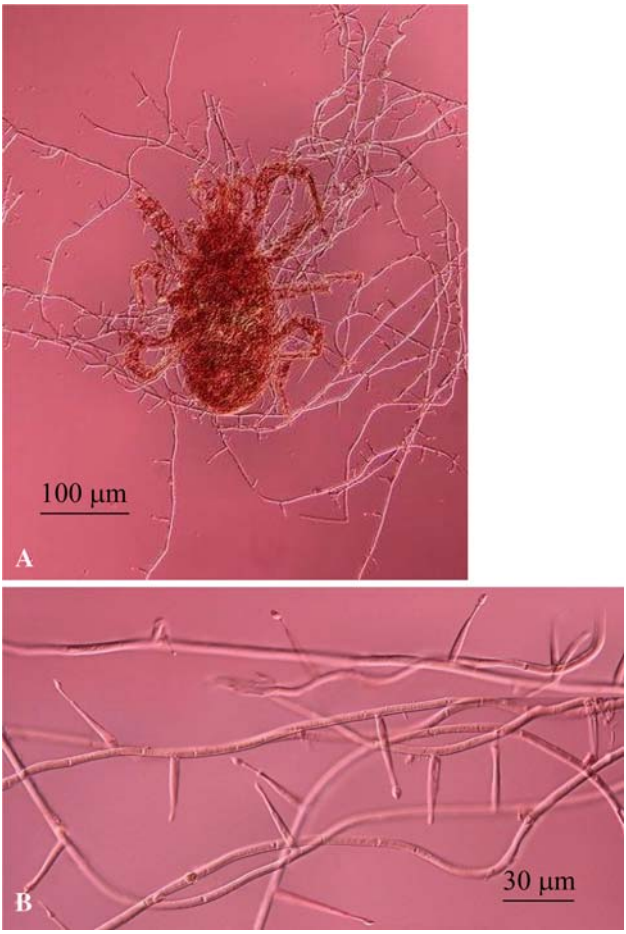

Fig. 5 *Hirsutella vandergeesti* sp. nov. **a** Mycelium on phytoseid mite, **b** hyphae, phialides and phialospores

the collection of the Department of Plant Protection of the University of Podlasie in Siedlce, found on 12 August 2007 is designed as a holotype. *Hirsutella danubiensis* could be easily distinguished from the other *Hirsutella* species producing small conidia, and by its very long phialides of general narrowly conical appearance, without conspicuous basal distension. Etymology: related to the Danube valley as the site of its occurrence.

Hirsutella vandergeesti Bałazy, Miętkiewski et Tkaczuk, spec. nov. (Fig. 5a, b)

Fungus acarorum pathogenicus. Acari mortui corpus textis hypharum, e cellulis elongatis vel ovoidaeis, 2–6 µm crassis constantium implent. Hyphae irregulares ramos formant et in basis partibus coxarum atque chelicarum inhaerescunt. Hyphis aerinis copiose increscentibus cellulae mycelii intra hospitis corpus ovoideae aut subglobosae diametro 4–7 µm, continent mycelii hyphalis reliquias, cuius cellulae in cruribus elongatae sunt. Hyphae aerinae aequotenues, crassitudine 3–4 (–4.5) µm, exigue ramosae, ad longitudinem

3–4 mm radiate excrescunt, maxime inter scutum dorsale et ventrale atque circum partes buccales, anales et inter basis particula coxarum. Hyphae perveniunt ad longitudinem 2.5–3 mm, plerumque circum acaros mortuos mycelii canoalbi texta densa formantes. Parietes hypharum externi clarofusci superficie polita, septa hyalina, paululum distincta, regulariter in spatia 10–18 μm collocantur. Phialides tenuiconicales, maxime crassae ad basim, ex hyphis directe, aliquando in hypharum finibus paulo oblique excrescunt. Quarum dimensiones (27–) 30–37 (–43) × 4–4.5 μm ad basim, usque ad circa 4/5 suae longitudinis totaliter recte ad crassitudinem 1.5–2 μm attenuantur. Adeo paries clarofuscus et crassus phialides protegit. In phialidis apice tenuissimus (∼0.5 μm), achromaticus processus plasmaticus, 4–7 μm longus invenitur, in cuius apice phialospora formatur. Raro ex una phialide duo colla crescunt, quorum alterum saepe – sed non semper – brevius est. Raro etiam collum supra phialidis segmentum crassoparietalis furcatam formam habet, quamquam duarum sporarum formatio in collis bifurcatis rarissime notabatur. Phialoconidia parva dimensionibus (4.0–) 4.2–5.5 × 2.1–2.5 (–3.0) μm, instar fructus citri sinensis segmentorum, in parte apicali magis attenuata, saepe cum fine paululum acuto, bilateraliter symmetrica, fere semper singula et mucotecta formantur.

In acaris mortuis Phytoseiidarum (Amblyseius sp. Neoseiulus, Seiulus sp., Typhlodromus sp.), et Tarsonemidarum (Tarsonemus lacustris) ex Stachydis sylvaticae foliis collectis, in silva humida Saint Gobin prope Cessières, in Francogallia, Septembri et Octobri mensibus anno 2007. Folia ab insectis Aphidarum et Coccodearum atque ab acaris Tetranychidarum indefinitis, quae non sunt infecta, inhabitantur. Holotypus: specimen numero 4200 g designatum, in collectione mycologica Insituti Agrariae et Silvestris Oecologiae Academiae Scientiarum Polonorum Posnaniae depositum.

Hirsutella vandergeesti Bałazy, Miętkiewski et Tkaczuk sp. nov. (Fig. 5a, b)

Acaropathogenic fungus; the body of a dead mite filled with hyphae consisting of elongated or oval cells 2–6 μm thick, irregularly branched and penetrating also into basal parts of legs and chelicers. During the period of the abundant growth of aerial mycelium, the cells of the internal hyphae take ovoid or subglobose forms 4–7 μm in diameter and steadily disappear with age, apart from some elongated cells only in legs. Aerial hyphae equally narrow 3–4 (–4.5) μm thick, poorly branched and up to 3–4 mm long, grow radially around the mite between dorsal and ventral discs, a little more abundant at the mouth parts and between the basal segments of legs. Outer walls of the hyphae light brownish, smooth; septa hyaline, distributed regularly in distances of 10–18 μm. Phialides narrowly conical, thickest at the base, protruding perpendicularly, sometimes somewhat obliquely in the terminal parts of hyphae. Their measurements are (27–) 30–36 (–43) × 4–4.5 μm at the base, converging rectilinearly up to the height of approximately 4/5 of their total length where they attain the thickness of 1.5–2 μm. Up to this point they are covered with a thick light-brownish wall as in hyphae and never forking. The apical part of each phialide forms a very thin (0.5 μm) and thin walled, colourless plasmatic outgrowth 4–7 μm long, with a phialosphore at its tip. Very seldom two such necks grow on one phialide, one of them almost always shorter. The formation of two normal phialospores on forked necks only very rarely observed. Phialoconidia small, (4–) 4.2–5.5 × 2.1–2.5 (–3.0) μm, shaped like an orange segment, more strongly narrowed at the distal end and ventrally sinuous beneath subapiculate tip. The spores are covered with a thin layer of mucus.

On dead mites of the families Phytoseiidae (*Amblyseius* sp., *Neoseiulus*, *Seiulus* sp., *Typhlodromus* sp.) and Tarsonemidae (*Tarsonemus lucustris*), collected on *Stachys*

sylvatica leaves in the flood plain forest St. Gobin near Laniscourt (France), in September and October 2007. The plants were infested by aphids, coccids and tetranychid mites, which were never found to be infected by this fungus. Specimen nr. 4200 g in the collection of the Research Centre for Agricultural and Forest Environment, found on 1 September 2007, is designated as holotype. Etymology: to honour Dr. Leo Van der Geest's merits in acarology.

Conclusions

Fungal diseases of mites occurring on plants, under bark (in feeding galleries of cambio-xylophagous insects), and in decayed wood are widespread in (semi)natural habitats. The fungal pathogens of mites are closely related to most insect pathogenic fungi but only few species are capable to infect both insects and mites. Among five reported representatives of the order of Entomophthorales only one pleophagous species, *C. coronatus*, appears capable to infect hosts of both groups, whereas from among 13 acaropathogenic *Hirsutella*-species, classified as hyphomycetous clavicipitalean anamorphs of ascomycetes only for two (*H. nodulosa* and *H. rostrata*), single insect species have so far been reported as hosts. Alternatively, from the common and typically entomopathogenic anamorphs only two species of the genus *Paecilomyces* were found on single mites within this study.

A preliminary attempt to determine the affinity of particular acaropathogenic species of *Hirsutella* by the analysis of ITS region sequences of the genomic DNA showed two distinct groups infecting some phytophagous eriophyid, tarsonemid and tetranychid mites. The first is formed by three very close species, *H. gregis*, *H. kirchneri* and *H. necatrix*; the second group, containing *H. thompsonii* and its variety *H. t.* var. *synnematosa*, appears more variable. Other species are scattered singly in dendrograms, neighboring with the entomopathogenic (*H. nodulosa*, *H.* cf. *brownorum*) or nematopathogenic (*H. thompsonii*, *H. tydeicola*) clades, except *H. rostrata* which is clearly separated. Two newly described species, basing on the differences in morphology (*H. danubensis* and *H. vandergeesti*), appeared too fragile for culture isolation, therefore could not be included in ITS sequencing. Currently *Lecanicillium*, *Simplicillium* and allied taxa are subjected to insect and mite pathogenicity bioassays, as well as morphological analysis including exact biometrics and successive nucleic acid sequencing.

The majority of the recorded acaropathogenic fungi affecting mites on plants appear from about mid-summer, increasing in density till the first frost. Some of them, e.g., species inhabiting subcortical insect galleries and those hibernating on branches or in buds and excrescences (zoocecidia) on twigs, may be collected for laboratory studies all through the year. Resting spores of *Neozygites* species may be collected from fruit tree branches. The species *H. kirchneri* and *Ramularia ludoviciana* can also be found on grasses during winter, under the snow.

Fungal acaropathogens on plants appear characteristically in very small patches, distributed randomly over the area of potential host distribution. Though the local incidence rates may be high, e.g., *H. thompsonii*, *H. kirchneri* or *N. abacaridis* on eriophyids feeding on grasses or plum leaves, this does not seem to have much effect on the general distribution and density of their host mites. In fresh subcortical insects' feeding grounds, mycosed mites were seen only singly, and their density increases usually after 2–3 weeks of rearing.

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Entomopathogenic fungi against South American tick species

Éverton Kort Kamp Fernandes · Vânia Rita Elias Pinheiro Bittencourt

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Abstract Ticks are parasites of serious concern for humans, domesticated animals and wild animals. Despite scientific advances, in South America the principal control method for ticks is the use of chemical acaricides. Indiscriminate use of these products causes environmental pollution, food contamination and development of tick resistance to acaricides. In vitro studies and field trials have demonstrated that pathogenic fungal isolates not only cause mortality of many tick species, but also reduce subsequent generations due to effects on their reproductive efficacy. Accordingly, this review presents results of several studies which were conducted in South America. Furthermore, it outlines current information on fungal pathogens of ticks and discusses the need to develop and implement effective strategies for use of entomopathogenic fungi to control ticks in the near future.

Keywords Fungal acaricides · Biological control · *Beauveria bassiana* · *Metarhizium anisopliae* · *Anocentor nitens* · *Amblyomma cajennense* · *Rhipicephalus* (*Boophilus*) *microplus* · *Rhipicephalus sanguineus*

Introduction

Ticks are obligate blood-sucking Arachnids that feed on vertebrates. In South America, four tick species [*Amblyomma cajennense* (Fabricius, 1787), *Anocentor nitens* (Neumann, 1897), *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887) and *R. sanguineus* (Latreille, 1806)] are widely distributed geographically and found on domestic and wild animals. These species are grouped in the family Ixodidae. They are hard bodied, highly fecund, biologically diverse, and economically devastating.

É. K. K. Fernandes
Department of Biology, Utah State University, Logan, UT 84322-5305, USA

V. R. E. P. Bittencourt (✉)
Departamento de Parasitologia Animal, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ 23890-000, Brazil
e-mail: vaniabit@ufrrj.br

These four species of ticks are responsible for several problems. *A. cajennense* can transmit pathogens to several animal species, including pathogens that cause severe zoonotic infections, such as Rocky Mountain Spotted Fever, caused by *Rickettsia rickettsii* (Dias and Martins 1939). *A. nitens* transmits several equine disease etiological-agents, including babesiosis, and also causes skin damage that facilitates bacterial infection and myiasis (Pfeifer et al. 1995). *R.* (*B.*) *microplus*, known as the cattle tick, causes annual economic damages estimated at 2 billion dollars per year in Brazil due to slower development of infested animals, mortality, decreased milk production, leather production losses, etc. (Grisi et al. 2002). *R.* (*B.*) *microplus* can also transmit pathogens that cause diseases such as babesiosis and anaplasmosis (Horn and Arteche 1985). *R. sanguineus* is an important vector of *Babesia*, *Ehrlichia*, *Hepatozon* and *Rickettsia* to domesticated and wild dogs (Conceição-Silva et al. 1988; Walker et al. 2000).

In practice, ticks are mostly controlled by chemical acaricides. However, biological control is quickly becoming one of the more attractive approaches to tick management. Several entomopathogenic fungi are naturally associated with ticks and some have demonstrated high virulence under laboratory conditions. Of all the fungal genera and species that have been tested, *Metarhizium anisopliae* and *Beauveria bassiana* have demonstrated the highest virulence; and therefore, these are the most investigated entomopathogenic fungi regarding their potential for the control of tick species worldwide.

Several previous reviews describe generally tick biological control using various pathogens in various locations, as listed by Samish and Rehacek (1999) and Samish et al. (2004). This review, however, focuses on the use of entomopathogenic fungi to control important tick species from Southern America, and summarizes findings and perspectives of several authors attempting to develop effective strategies for biological control of ticks under tropical environmental conditions.

Laboratory assays and field trials

Laboratory assays using entomopathogenic fungi are commonly used in studies on biological control of ticks. A laboratory assay is an important test to verify the virulence of a fungal pathogen. It considers mortality and other data (e.g., egg production, egg hatchability, longevity) that might reduce the growth rate of tick populations in the field. However, comparing assay results is difficult due to variations in methods and use of different fungal isolates. Experiments under laboratory conditions (i.e. controlled temperature, humidity, day-light period) present more consistent results than similar experiments under natural conditions, laboratory assays are expected to indicate the most virulent isolates, and therefore those with greatest potential for biological control.

Many in vitro studies have used entomopathogenic fungi against eggs (Table 1), larvae (Table 2), nymphs, adults (Table 3) and engorged females (Table 4) of several tick species that occur in South America. In general, larva is the stage most susceptible to fungal infection. The results indicate that certain fungal species are promising alternatives to chemical acaricides for tick control. Various isolates of *M. anisopliae* and *B. bassiana* demonstrated the strongest pathogenicity to all stages of all four tick species, suggesting that they have high potential as biocontrol agents of ticks.

In most field trials, conidial suspensions are sprayed directly onto ticks or on pasture plants to control tick infestations (Table 5). As expected, the mortality levels in field trials were lower than those observed under laboratory conditions. This suggests that, under commonly occurring environmental conditions, conidia may not germinate or

Table 1 Tick eggs treated in vitro with conidial suspensions of entomopathogenic fungi

Study	Association between fungus and tick[a]	Results[b]
Bittencourt et al. (1994a)	*Metarhizium anisopliae* and *Rhipicephalus* (*Boophilus*) *microplus*	(1) Increased EIP (29.4–39.5 days at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (23.6–23.8 days); (2) increased EHP (10.3–15.0 days, at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (6.8 days).
Bittencourt et al. (1994b)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Decreased EH (66.0–3.3% at 10^5–10^9 conidia ml^{-1}, respectively) compared to control (91.3–94.0%); (2) LC_{50}: 1.35–5.0 × 10^6 conidia ml^{-1} depending on isolate.
Bittencourt et al. (1996)	*Beauveria bassiana* and *R.* (*B.*) *microplus*	(1) Decreased EH (86.6–20.0% at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (93.3%); (2) LC_{50}: 2.46–2.49 × 10^7 conidia ml^{-1} depending on isolate; (3) increased EIP (22.3–30.6 days at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (21.3–22.0 days); (4) increased EHP (6.3–9.6 days at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (5.3–5.7 days).
Souza et al. (1999)	*B. bassiana* and *Amblyomma cajennense*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) decreased EH (0.33%) compared to control (100%); (2) LC_{50}: 3.2–38 × 10^7 conidia ml^{-1} depending on isolate; (3) EIP and EHP not different from control.
	M. anisopliae and *A. cajennense*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) decreased EH (1.6–0%) compared to control (100%); (2) LC_{50}: 3.2–11 × 10^7 conidia ml^{-1} depending on isolate; (3) EIP and EHP not different from control.
Carneiro et al. (1999)	*B. bassiana* and *Anocentor nitens*	(1) EH (66.4–82.5% at 10^5–10^8 conidia ml^{-1}, respectively) not different from control (82.1%); (2) decreased EIP compared to control (25.1 days); (3) decreased EHP (5.4–4.1 days at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (6.6 days); (4) increased larva mortality (>85%) compared to control (3%), 10 days after hatching.
Paião et al. (2001a)	*B. bassiana* and *R.* (*B.*) *microplus*	(1) Decreased EH (64.7–33.9% at 10^6–10^8 conidia ml^{-1}, respectively) compared to control at day 20 after treatment.
Onofre et al. (2001)	*M. anisopliae* var. *anisopliae* and *R.* (*B.*) *microplus*	(1) Decreased EH (60.5–35.7% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (90.0–89.4%) at day 20 after treatment.
	M. anisopliae var. a*cridum* and *R.* (*B.*) *microplus*	(1) Decreased EH (65.0–19.5% at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (95.0–81.2%) at day 20 after treatment.
Fernandes et al. (2003)	*B. bassiana* and *R.* (*B.*) *microplus*	(1) Decreased EH (68.0–30.0% at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (80.0–96.0%); (2) the conidial suspension with the highest concentration (10^8 conidia ml^{-1}) decreased EHP (3.2–2.8 days) compared to control (6.3–6.5 days).
Fernandes et al. (2004)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Decreased EH (83.0–24.0% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (79.0–94.0%).

Table 1 continued

Study	Association between fungus and tick[a]	Results[b]
Polar et al. (2005a)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Decreased EH (15% at 10^8 conidia ml^{-1}) compared to control (92%); (2) increased EHP (19.8 ± 0.5 days at 10^8 conidia ml^{-1}) compared to control (14.8 ± 0.5 days).
	Simplicillium lamellicola and *R.* (*B.*) *microplus*	(1) EH (92% at 10^8 conidia ml^{-1}) not different from control (92%); (2) decreased EHP (12.7 ± 0.4 days at 10^8 conidia ml^{-1}) compared to control (14.8 ± 0.5 days).
	Isaria farinosa and *R.* (*B.*) *microplus*	(1) Decreased EH (78% at 10^8 conidia ml^{-1}) compared to control (92%); (2) EHP (14.9 ± 0.6 days at 10^8 conidia ml^{-1}) not different from control (14.8 ± 0.5 days).
	M. anisopliae and *Rhipicephalus sanguineus*	(1) Decreased EH (8% at 10^8 conidia ml^{-1}) compared to treated (with 0.2% Tween 80 solution) and untreated controls (66 and 67%, respectively); (2) EH higher in *R.* (*B.*) *microplus* (12%) than in *R. sanguineus* (8%); (3) increased EHP (16.7 ± 0.3 days at 10^8 conidia ml^{-1}) compared to treated (with 0.2% Tween 80 solution) and untreated controls (9.7 ± 0.1 and 9.6 ± 0.1 days, respectively).
Prette et al. (2005)	*B. bassiana* and *R. sanguineus*	(1) Decreased EH (12.1–0.7% at 10^7–10^9 conidia ml^{-1}, respectively) compared to control (98.3–95.0%) at day 20 after treatment.
Melo et al. (2006)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) decreased EH (19.0 ± 14.5%) compared to control (86.0 ± 11.4%); (2) conidial suspensions with highest concentrations (10^7 and 10^8 conidia ml^{-1}) decreased EHP (4.6 ± 0.8 and 5.3 ± 1.8 days, respectively) compared to control (8.8 ± 1.3 and 8.4 ± 0.7 days).
Angelo et al. (2007)	*Lecanicillium* sp. and *R.* (*B.*) *microplus*	(1) EH not different from control.

Studies are ordered according to year of publication

[a] Fungus was applied as an aqueous conidial suspension, unless specified differently

[b] EIP (egg incubation period) = days before hatch; EHP (egg hatchability period) = first to last day of hatch; EH (egg hatchability) = % hatch

penetrate the cuticle; and/or they may cause only sub-lethal levels of infection (Samish et al. 2004), probably due to conditions such as temperature and humidity variations, solar radiation, and host microclimatic factors, including skin temperature, chemical composition of animal skin secretions and animal skin microflora (reviewed by Polar et al. 2005c). To circumvent these problems, appropriate formulations can be devised to protect the conidia, thereby enhancing their ability to germinate and initiate the cuticle penetration process (Bittencourt et al. 2002).

When selecting isolates for tick control, in addition to considering virulence, high tolerance to environmental conditions should be also evaluated. Heat and cold are two very important limiting environmental factors. Furthermore, solar radiation negatively affects

Table 2 Tick larvae treated in vitro with conidial suspensions of entomopathogenic fungi

Study	Association between fungus and tick[a]	Results
Bittencourt et al. (1994b)	*Metarhizium anisopliae* and *Rhipicephalus* (*Boophilus*) *microplus*	(1) Increased mortality (29.3–97.3% at 10^5–10^9 conidia ml^{-1}, respectively) compared to control (6.7–7.3%) at day 10 after treatment; (2) LC_{50}: 2.0–5.6 $\times 10^6$ conidia ml^{-1} depending on isolate.
Bittencourt et al. (1996)	*Beauveria bassiana* and *R.* (*B.*) *microplus*	(1) Increased mortality (18.8–88.0% at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (13.0–16.5%) at day 10 after treatment; (2) LC_{50}: 6.8–10.1 $\times 10^6$ conidia ml^{-1} depending on isolate.
Barbosa et al. (1997)	*B. bassiana* and *Rhipicephalus sanguineus*	(1) Increased mortality of engorged larvae (35.0–100% at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (24.0%) at day 30 after treatment; (2) decreased survival of nymphs (24% and 11%, at 10^4 and 10^6 conidia ml^{-1}, respectively) compared to control (100%) at day 15 after ecdysis.
Monteiro et al. (1998)	*B. bassiana* and *R. sanguineus*	(1) Increased mortality (60.0–90.0% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control at day 10 after treatment; (2) LC_{50}: 4.7–12 $\times 10^5$ conidia ml^{-1} depending on isolate (at 27 ± 1°C); (3) LC_{50}: 2.2–21 $\times 10^5$ conidia ml^{-1} depending on isolate (at room temperature).
	M. anisopliae and *R. sanguineus*	(1) Increased larva mortality (13.3–96.0% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control at day 10 after treatment; (2) LC_{50}: 1.3–14 $\times 10^4$ conidia ml^{-1} depending on isolate (at 27 ± 1°C); (3) LC_{50}: 7.7 $\times 10^4$–4.3 $\times 10^6$ conidia ml^{-1} depending on isolate (at room temperature).
Souza et al. (1999)	*B. bassiana* and *Amblyomma cajennense*	(1) Increased mortality (40.0–100% at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (10.0–15.0%) at day 10 after treatment; (2) LC_{50}: 7.9 $\times 10^5$–3.9 $\times 10^7$ conidia ml^{-1} depending on isolate.
	M. anisopliae and *A. cajennense*	(1) Increased mortality (43.3–96.7% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (0–23.3%) at day 10 after treatment; (2) LC_{50}: 1.8–6.5 $\times 10^5$ conidia ml^{-1} depending on isolate.
Paião et al. (2001a)	*B. bassiana* and *R.* (*B.*) *microplus*	(1) Increased mortality (19.6–60.7% at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (13.2%) at day 20 after treatment.
Samish et al. (2001)	*B. bassiana* and *R. sanguineus*	(1) Increased mortality (7.2 ± 1.8–8.2 ± 2.6% at 10^7 conidia ml^{-1}) compared to control (1.8 ± 2.6%) at day 7 after treatment.
	B. bassiana and engorged larvae of *R. sanguineus*	(1) No mortality at day 10 after treatment (10^7 conidia ml^{-1}); (2) decreased emergence of nymphs (79.5 ± 7.6% at 10^7 conidia ml^{-1}) compared to control (91.8 ± 9.2%) at day 16 after treatment; (3) no mortality of emerged nymphs at day 21 after treatment (10^7 conidia ml^{-1}).
	M. anisopliae var. *anisopliae* and *R. sanguineus*	(1) Increased mortality (69.3 ± 8.8–95.9 ± 3.9% at 10^7 conidia ml^{-1}) compared to control (1.8 ± 2.6%) at day 7 after treatment.

Table 2 continued

Study	Association between fungus and tick[a]	Results
	M. anisopliae var. *anisopliae* and engorged larvae of *R. sanguineus*	(1) Increased mortality (49.1 ± 16.1–82.6 ± 13.0% at 10^7 conidia ml^{-1}) compared to control (0%) at day 10 after treatment; (2) decreased emergence of nymphs (11.4 ± 4.9–7.3 ± 5.9% at 10^7 conidia ml^{-1}) compared to control (91.8 ± 9.2%) at day 16 after treatment; (3) increased mortality of emerged nymphs (36.8 ± 3.2–75.3 ± 4.4% at 10^7 conidia ml^{-1}) compared to control (0%) at day 21 after treatment.
	M. anisopliae var. *acridum* and *R. sanguineus*	(1) Increased mortality (8.8 ± 3.1–9.8 ± 4.0% at 10^7 conidia ml^{-1}) compared to control (1.8 ± 2.6%) at day 7 after treatment.
	M. anisopliae var. *acridum* and engorged larvae of *R. sanguineus*	(1) No mortality at day 10 after treatment (10^7 conidia ml^{-1}); (2) decreased emergence of nymphs (83.9 ± 7.1–81.5 ± 3.8% at 10^7 conidia ml^{-1}) compared to control (91.8 ± 9.2%) at day 16 after treatment; (3) increased mortality of emerged nymphs (6.3 ± 3.4–13.3 ± 6.3% at 10^7 conidia ml^{-1}) compared to control (0%) at day 21 after treatment.
	Isaria fumosorosea and *R. sanguineus*	(1) Increased mortality (6.5 ± 1.2% at 10^7 conidia ml^{-1}) compared to control (1.8 ± 2.6%) at day 7 after treatment.
	I. fumosorosea and engorged larvae of *R. sanguineus*	(1) No mortality at day 10 after treatment (10^7 conidia ml^{-1}); (2) emergence of nymphs (88.7 ± 3.8–88.1 ± 4.1% at 10^7 conidia ml^{-1}) not different from control (91.8 ± 9.2%) at day 16 after treatment; (3) no mortality of emerged nymphs at day 21 after treatment (10^7 conidia ml^{-1}).
Fernandes et al. (2003)	*B. bassiana* and *R.* (*B.*) *microplus*	(1) Increased mortality (10.0–96.0% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (0%) at day 10 after treatment.
Fernandes et al. (2004)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Increased mortality (11.0–94.0% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (0%) at day 10 after treatment.
Polar et al. (2005a)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Decreased average survival time (9.3 ± 0.4 days at 10^8 conidia ml^{-1}) compared to control (11.8 ± 0.8 days).
	Simplicillium lamellicola and *R.* (*B.*) *microplus*	(1) Average survival time (12.3 ± 0.5 days at 10^8 conidia ml^{-1}) not different from control (11.8 ± 0.8 days).
	Isaria farinosa and *R.* (*B.*) *microplus*	(1) Average survival time (15.4 ± 0.6 days at 10^8 conidia ml^{-1}) not different from control (11.8 ± 0.8 days).
	M. anisopliae and *R. sanguineus*	(1) Increased average survival time (14.6 ± 0.3 days at 10^8 conidia ml^{-1}) compared to treated control (with 0.2% Tween 80 solution) (12.6 ± 0.4 days), but not to untreated control (14.1 ± 0.4 days).
Prette et al. (2005)	*B. bassiana* and *R. sanguineus*	(1) Decreased larva ecdysis (33.7–4.6% at 10^7–10^8 conidia ml^{-1}, respectively, with no significant differences among treatments) compared to control (93.4% and 94.1%).

Table 2 continued

Study	Association between fungus and tick[a]	Results
Fernandes et al. (2006)	*B. bassiana* and *R.* (*B.*) *microplus*	(1) Increased mortality (16.0–100% at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (10%) at day 10 after treatment; (2) high virulence variability among 50 isolates, LC_{50}: $<10^4$ to $>10^8$ conidia ml^{-1} depending on isolate.
Bahiense et al. (2006)	*M. anisopliae* (aqueous conidial suspension associated with deltamethrin) and a pyrethroid-resistant strain of *R.* (*B.*) *microplus*	(1) in general, all *M. anisopliae* isolates in association with deltamethrin had higher mortality rates than treatments using either fungus or pyrethroid alone at lower or equivalent doses used in the association, at day 10 after treatment; (2) LC_{50}: Deltamethrin: 10.8 ppm (4.5–25.8 ppm), and deltamethrin + Ma 10^8 conidia ml^{-1}: 6.0×10^{-9} ppm (5.8×10^{-39} to -1.1×10^{23} ppm).
Melo et al. (2006)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Conidial suspensions with highest concentration (10^8 conidia ml^{-1}) increased mortality (97.0 ± 4.8 and 99.0 ± 3.1%) compared to control (1.0 ± 0.1 and 2.0 ± 1.2%) at day 10 after treatment.
Angelo et al. (2007)	*Lecanicillium* sp. and *R.* (*B.*) *microplus*	(1) Increased mortality compared to control at day 20 after treatment; (2) LC_{50}: 1.16×10^6 conidia ml^{-1}.

Studies are ordered according to year of publication

[a] Fungus was applied as an aqueous conidial suspension, unless specified differently

survival of entomopathogenic fungi in the field. The UV-B portion of sunlight appears to be directly related to reduced survival of entomopathogenic conidia in insolated field environments (Fargues et al. 1997). Therefore, several studies have made efforts to identify isolates with high tolerance to heat, cold and ultraviolet radiation (UV-A and UV-B) (Fargues et al. 1996; Braga et al. 2001, 2002; Rangel et al. 2004, 2005; Fernandes et al. 2007, 2008), and these isolates may hold promise for use in biological control settings with high insolation, such as those found in tropical environments in South America.

Isolation of entomopathogenic fungi from naturally infected ticks

Studies have reported that several tick species are naturally infected by pathogenic fungi. In Brazil, 24.5% of the engorged female of *R.* (*B.*) *microplus* ticks collected from soil were infected with *B. bassiana* and 10% with *M. anisopliae* (Costa et al. 2002), and 22% of the *R. sanguineus* nymphs were infected with fungi distributed among five genera, some of which saprophytes (Guerra et al. 2001). Engorged females of *R.* (*B.*) *microplus* naturally infected with *B. bassiana* were also detected in Argentina (Posadas et al. 2005). This study isolated *B. bassiana* from ticks and soil samples, and concluded that this entomopathogenic fungus not only infects this tick species, but also is found in soil of endemic areas.

As reviewed by Samish et al. (2004), the percentage of ticks naturally infected by fungi varies considerably according to the stage and species of ticks and also the ecological conditions at the sample sites. The isolation of indigenous entomopathogenic fungi is important for developing isolates that avoid the introduction of new (exotic) fungal isolates for tick biological control in certain environments. Moreover, indigenous isolates may be

Table 3 Tick nymphs and adults treated in vitro with conidial or blastospores suspensions of entomopathogenic fungi

Study	Association between fungus and tick[a]	Results
Reis et al. (2001)	*Beauveria bassiana* and unfed nymphs of *Amblyomma cajennense*	(1) Increased mortality (29–73% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (14%) at day 15 after treatment; (2) LC_{50}: 1.7–18 × 10^8 conidia ml^{-1} depending on isolate.
	Metarhizium anisopliae and unfed nymphs of *A. cajennense*	(1) Increased mortality (32–45% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (14%) at day 15 after treatment; (2) LC_{50}: 5.3–28 × 10^{10} conidia ml^{-1} depending on isolate.
	B. bassiana and fed nymphs of *A. cajennense*	(1) Increased mortality (31–51% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (17%) at day 15 after treatment; (2) LC_{50}: 2.7 × 10^{11}–7.6 × 10^{22} conidia ml^{-1} depending on isolate.
	M. anisopliae and fed nymphs of *A. cajennense*	(1) Increased mortality (36–65% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (17%) at day 15 after treatment; (2) LC_{50}: 5.1 × 10^8–1.0 × 10^{13} conidia ml^{-1} depending on isolate.
	B. bassiana and unfed adults of *A. cajennense*	(1) Increased mortality (47–79% at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (23%) at day 15 after treatment; (2) LC_{50}: 1.1–3.3 × 10^8 conidia ml^{-1} depending on isolate.
	M. anisopliae and unfed adults of *A. cajennense*	(1) Increased mortality (49–76% at 10^7–10^8 conidia ml^{-1}, respectively) compared to control (23%) at day 15 after treatment; (2) LC_{50}: 1.4–4.3 × 10^8 conidia ml^{-1} depending on isolate.
Samish et al. (2001)	*B. bassiana* and unfed nymphs of *Rhipicephalus sanguineus*	(1) Mortality (6.4 ± 4.4–11.3 ± 6.9% at 10^7 conidia ml^{-1}) not different from control (13.4 ± 3.7%) at day 7 after treatment.
	B. bassiana and engorged nymphs of *R. sanguineus*	(1) Mortality (12.5 ± 9.6–17.5 ± 9.6% at 10^7 conidia ml^{-1}) not different from control (10.0 ± 2.5%) at day 14 after treatment; (2) emergence of adults (72.5 ± 12.6–82.5 ± 5.0% at 10^7 conidia ml^{-1}) not different from control (75.0 ± 7.1%) at day 25 after treatment; (3) no mortality of emerged adults at day 30 after treatment (10^7 conidia ml^{-1}).
	M. anisopliae var. *anisopliae* and unfed nymphs of *R. sanguineus*	(1) Increased mortality (47.7 ± 8.7–92.2 ± 15.6% at 10^7 conidia ml^{-1}) compared to control (13.4 ± 3.7%) at day 7 after treatment.
	M. anisopliae var. *anisopliae* and engorged nymphs of *R. sanguineus*	(1) Increased mortality (57.5 ± 17.1–60.0 ± 8.2% at 10^7 conidia ml^{-1}) compared to control (10.0 ± 2.5%) at day 14 after treatment; (2) decreased emergence of adults (10.0 ± 8.2–2.5 ± 3.0% at 10^7 conidia ml^{-1}) compared to control (75.0 ± 7.1%) at day 25 after treatment; (3) increased mortality of emerged adults (62.5 ± 27.9–100% at 10^7 conidia ml^{-1}) compared to control (0%) at day 30 after treatment; (4) up to 100% mortality of adults was observed at days 7–10 after emergence.
	M. anisopliae var. *anisopliae* and unfed adults of *R. sanguineus*	(1) Increased mortality of male (22.5 ± 1.2–100%) and female ticks (20.0 ± 1.5–100%) at 10^7 conidia ml^{-1} compared to control (5.0 ± 1.5%) at day 21 after treatment.

Table 3 continued

Study	Association between fungus and tick[a]	Results
	M. anisopliae var. *acridum* and unfed nymphs of *R. sanguineus*	(1) Increased mortality (22.5 ± 3.0–47.7 ± 8.7% at 10^7 conidia ml^{-1}) compared to control (13.4 ± 3.7%) at day 7 after treatment.
	M. anisopliae var. *acridum* and engorged nymphs of *R. sanguineus*	(1) Increased mortality (27.5 ± 5.0% at 10^7 conidia ml^{-1}) compared to control (10.0 ± 2.5%) at day 14 after treatment; (2) decreased emergence of adults (42.5 ± 9.6% at 10^7 conidia ml^{-1}) compared to control (75.0 ± 7.1%) at day 25 after treatment; (3) increased mortality of emerged adults (33.3 ± 20.4–59.3 ± 9.1% at 10^7 conidia ml^{-1}) compared to control (0%) at day 30 after treatment.
	M. anisopliae var. *acridum* and unfed adults of *R. sanguineus*	(1) Increased mortality of male (6.7 ± 1.4–15.0 ± 1.5%) and female ticks (13.3 ± 1.5–15.0 ± 6.5%) at 10^7 conidia ml^{-1} compared to control (5.0 ± 1.5%) at day 21 after treatment.
	Isaria fumosorosea and unfed nymphs of *R. sanguineus*	(1) Mortality (12.8 ± 5.0–16.1 ± 5.2% at 10^7 conidia ml^{-1}) not different from control (13.4 ± 3.7%) at day 7 after treatment.
	I. fumosorosea and engorged nymphs of *R. sanguineus*	(1) Mortality (10.0 ± 8.2–15.0 ± 5.8% at 10^7 conidia ml^{-1}) not different from control (10.0 ± 2.5%) at day 14 after treatment; (2) emergence of adults (72.5 ± 15.0–75.0 ± 5.8% at 10^7 conidia ml^{-1}) not different from control (75.0 ± 7.1%) at day 25 after treatment; (3) no mortality of emerged adults at day 30 after treatment (10^7 conidia ml^{-1}).
Kirkland et al. (2004)	*B. bassiana* (aqueous conidial or blastospores suspension) and adults of *R. sanguineus*	(1) Conidial or blastospores suspensions with highest concentration (10^8 cells ml^{-1}) caused significant mortality, but required at least 14 days of infection; (2) ≈40% of overall mortality occurred within 14–21 days post-infection.
	B. bassiana and nymphs of *R. sanguineus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) resulted in >60% mortality within 14 days, and almost 100% mortality within 21 days post-infection; (2) nymphs were much more susceptible to fungal infection and subsequent mortality than adults.
	M. anisopliae and adults of *R. sanguineus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) caused significant mortality (≈20%), but required at least 14 days of infection; (2) ≈40% mean mortality was observed 28 days post-infection.
	M. anisopliae and nymphs of *R. sanguineus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) resulted in almost 60% mortality within 7 days, and almost 100% mortality within 21 days post-infection; (2) nymphs were much more susceptible to fungal infection and subsequent mortality than adults.
Prette et al. (2005)	*B. bassiana* and fed nymphs of *R. sanguineus*	(1) Decreased nymphs ecdysis (16.6–0% at 10^8–10^9 conidia ml^{-1}, respectively, with no significant differences among treatments) compared to control (93.3–100%).

Studies are ordered according to year of publication

[a] Fungus was applied as an aqueous conidial suspension, unless specified differently

Table 4 Engorged female ticks treated in vitro with conidial suspensions of entomopathogenic fungi

Study	Association between fungus and tick[a]	Results[b]
Bittencourt et al. (1992)	*Metarhizium anisopliae* and *Rhipicephalus* (*Boophilus*) *microplus*	(1) Decreased EP; (2) decreased EH; (3) %C: 38.5–97.4% (10^4–10^8 conidia ml^{-1}); (4) LC_{50}: 1.1–2.2 × 10^6 conidia ml^{-1} depending on isolate.
Bittencourt et al. (1994a)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Increased EIP (26.9–37.0 days at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (22.4–24.8 days); (2) increased EHP (9.8–13.2 days at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (6.9–7.0 days); (3) decreased OP (6.3–1.3 days at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (11.6–11.4 days); (4) decreased EPI (38.5–4.7% at 10^4–10^8 conidia ml^{-1}, respectively) compared to control (54.9–55.4%).
Bittencourt et al. (1997a)	*Beauveria bassiana* and *R.* (*B.*) *microplus*	(1) Decreased OP (10.5–10 days at 10^5–10^8 conidia ml^{-1}, respectively, with no significant differences among treatments) compared to control (11.2 days); (2) conidial suspension with highest concentration (10^8 conidia ml^{-1}) increased EIP (24.7 days) compared to control (22.9 days); (3) decreased REI (34.1–18.4% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (50.5–53.5%); (4) decreased NI (40.6–22.9% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (60.6–64.4%).
Bittencourt et al. (1997b)	*B. bassiana* and *R.* (*B.*) *microplus*	(1) Decreased EH (39.2–20.2% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (82.7–88.2%); (2) decreased RE (19.3–5.9% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (44.7–45.8%); (3) %C: 4.4–86.7% (10^4–10^8 conidia ml^{-1}); (4) LC_{50}: 2.2–870 × 10^6 conidia ml^{-1} depending on isolate.
Frazzon et al. (2000)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Several isolates (at 10^8 conidia ml^{-1}) caused 100% mortality at day 14 after treatment; (2) isolates, after previous passage in ticks, induced 100% mortality within 4 and 7 days after treatment.
Paião et al. (2001a)	*B. bassiana* and *R.* (*B.*) *microplus*	(1) Decreased OP (11.4–6.6 days at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (12.7 days); (2) decreased EH (74.5–68.2% at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (78.5%); (3) decreased ER (952.5–436.0 at 10^6–10^8 conidia ml^{-1}, respectively) compared to control (1107.1); (4) conidial suspension with highest concentration (10^8 conidia ml^{-1}) decreased EPI (54.2–42.6%) compared to control (57.6%); 5) PRER: 13.9–64.5% at 10^6–10^8 conidia ml^{-1}, respectively.
Onofre et al. (2001)	*M. anisopliae* var. *anisopliae* and *R.* (*B.*) *microplus*	(1) %C: 3.5–60.0% (10^4–10^8 conidia ml^{-1}); (2) decreased RE (18.6–11.0% at 10^5–10^8 conidia ml^{-1}, respectively) compared to control (27.6–27.8%); (3) LC_{50}: 1.4–2.3 × 10^7 conidia ml^{-1} depending on isolate.
	M. anisopliae var. *acridum* and *R.* (*B.*) *microplus*	(1) %C: 10.6–75.9% (10^4–10^8 conidia ml^{-1}); (2) decreased RE (21.3–5.3% at 10^5–10^8 conidia ml^{-1}) compared to control (29.3–42.9%); (3) LC_{50}: 3.4–4.2 × 10^5 conidia ml^{-1} depending on isolate.

Table 4 continued

Study	Association between fungus and tick[a]	Results[b]
Samish et al. (2001)	*M. anisopliae* var. *anisopliae* and *Rhipicephalus sanguineus*	(1) Increased mortality (66.7 ± 11.1–100% at 10^7 conidia ml^{-1}) compared to control (16.6 ± 4.7%) at day 14 after treatment; (2) mortality exceeded 50% at day 9 after treatment; (3) decreased number of eggs were laid (17.3 ± 30.0–0 mg/female at 10^7 conidia ml^{-1}) compared to control (153.0 ± 24.0 mg/female) at day 14 after treatment.
	M. anisopliae var. *acridum* and *R. sanguineus*	(1) Mortality (22.2 ± 12.8–22.2 ± 6.5% at 10^7 conidia ml^{-1}) not different from control (16.6 ± 4.7%) at day 14 after treatment; (2) decreased number of eggs were laid (93.3 ± 35.0–55.0 ± 6.3 mg/female at 10^7 conidia ml^{-1}) compared to control (153.0 ± 24.0 mg/female) at day 14 after treatment.
Polar et al. (2005a)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) decreased AST (5.2 ± 0.1 days) compared to control (19.0 ± 0.5 days); 2) 100% mortality was observed within 6 days after treatment; 3) AST was shortened as concentration increased from 1.0×10^7 conidia ml^{-1} (14.9 ± 0.4 days) to 5.0×10^8 conidia ml^{-1} (9.4 ± 0.3 days).
	Simplicillium lamellicola and *R.* (*B.*) *microplus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) decreased AST (11.8 ± 0.4 days) compared to control (19.0 ± 0.5 days).
	Isaria farinosa and *R.* (*B.*) *microplus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) did not reduce AST (17.3 ± 0.6 days) compared to control (19.0 ± 0.5 days).
	M. anisopliae and *R. sanguineus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) decreased AST (10.3 ± 0.3 days) compared to treated (with 0.2% Tween 80 solution) and untreated controls (17.9 ± 0.1 and 17.7 ± 0.1 days, respectively); (2) results suggest that *M. anisopliae* (at 10^8 conidia ml^{-1}) was more pathogenic to *R* (*B.*) *microplus* (AST = 8.8 ± 0.3 days) than to *R. sanguineus* (AST = 10.3 ± 0.3 days).
Polar et al. (2005b)	*M. anisopliae* (aqueous or oil-based conidial suspension at 10^8 conidia ml^{-1}) and *R.* (*B.*) *microplus*	(1) Aqueous conidial suspension decreased AST (8.4 ± 0.4 days) compared to control (13.4 ± 0.5 days); (2) coconut oil formulation decreased AST (4.6 ± 0.2 days) compared to control (9.6 ± 0.8 days); (3) liquid paraffin formulation decreased AST (5.5 ± 0.4 days) compared to control (11.8 ± 0.8 days); (4) 10% coconut oil + emulsifiable adjuvant oil (EAO) formulation decreased AST (11.9 ± 0.5 days) compared to control (15.2 ± 0.6 days); (5) 10% liquid paraffin + EAO formulation decreased AST (4.4 ± 0.1 days) compared to control (15.2 ± 0.8 days); (6) 10% liquid paraffin + EAO and coconut oil formulations produced lower AST compared to aqueous conidial suspension; (7) liquid paraffin formulation did not differ from aqueous formulation; (8) 10% coconut oil + EAO formulation caused highest AST.
Polar et al. (2005c)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Decreased AST (6.7 ± 0.3 and 7.5 ± 0.3 days) compared to control (9.6 ± 0.1 days) at 28°C bioassay temperature; (2) decreased (7.1 ± 0.4 days) and no significant reduction (9.1 ± 0.2 days) of AST compared to control (9.5 ± 0.1 days) at temperature comparable to cattle surface (31–35°C, 12 h cycle).

Table 4 continued

Study	Association between fungus and tick[a]	Results[b]
Melo et al. (2006)	*M. anisopliae* and *R.* (*B.*) *microplus*	(1) Conidial suspension with highest concentration (10^8 conidia ml^{-1}) decreased OP (2.6 ± 1.4 and 2.7 ± 3.1 days) compared to control (9.2 ± 2.3 and 10.1 ± 2.2 days); (2) decreased EP (0.041–0.013 g at 10^7–10^8 conidia ml^{-1}, respectively) compared to control (0.125 and 0.116 g); (3) decreased EH (20.0 ± 8.9% at 10^8 conidia ml^{-1}) compared to control (72.5 ± 27.6 and 73.3 ± 19.4%); (4) decreased NI (26.8 ± 10.6% at 10^8 conidia ml^{-1}) compared to control (66.7 ± 19.4%); (5) decreased RE (11.0 ± 6.6 and 5.6 ± 3.9% at 10^8 conidia ml^{-1}) compared to control (52.2 ± 15.5 and 47.98 ± 18.0%).
Reis et al. (2007)	*B. bassiana* or *M. anisopliae* conidia (formulated in emulsifiable concentrate plus polymerized cellulose gel) and *R. sanguineus*	(1) %C: 86.7% (*B. bassiana*); (2) %C: 80.1% (*M. anisopliae*).
Angelo et al. (2007)	*Lecanicillium* sp. and *R.* (*B.*) *microplus*	(1) Decreased OP; (2) decreased NI; (3) decreased EPI; (4) increased EIP.
Posadas and Lecuona (2007)	*B. bassiana* and *R.* (*B.*) *microplus*	(1) 45–60% reduction of EP at 10^8 conidia ml^{-1} compared to control; (2) decreased EH (46–69%) at 10^8 conidia ml^{-1}.
Leemon and Jonsson (2008)	*M. anisopliae* (aqueous or oil-based conidial suspension) and *R.* (*B.*) *microplus*	(1) Mortality varied form 0% to 100% depending on isolate and concentration of aqueous conidial suspension at day 6 after treatment; (2) 100% mortality at day 2 after exposure to conidia in an oil-based formulation (10^8 conidia ml^{-1}), while 100% mortality was observed at day 5 after exposure to conidia of the same isolate in an aqueous suspension with the same concentration (control presenting 0% mortality).

Studies are ordered according to year of publication

[a] Fungus was applied as an aqueous conidial suspension, unless specified differently

[b] OP (Oviposition Period) = number of days laying eggs; EP (Egg Production) = weight of egg mass; EPI (Egg Production Index) = [weight of egg mass (g)/initial weight of engorged female (g)] × 100; EIP (Egg Incubation Period) = days before hatch; EHP (Egg Hatchability Period) = first to last day of hatch; EH (Egg Hatchability) = percentage of hatch; AST (Average Survival Time); NI (Nutrient Index) = [weight of egg mass (g)/initial weight of engorged female (g) − residual weight of engorged female (g)] × 100; RE (Reproductive Efficiency) = [weight of egg mass (g)/initial weight of engorged female (g)] × EH; REI (Reproductive Efficiency Index) = [weight of egg mass (g)/initial weight of engorged female (g)] × 100; ER (Estimated Reproduction) = [weight of egg mass (g)/initial weight of engorged female (g)] × EH × 20,000 (20,000 is a constant corresponding to the estimated number of larvae from 1 g of eggs); PREP (Percent Reduction of Estimated Reproduction) = [ER (Control) − ER (Treated) / ER (Control)] × 100; %C (Percentage of Control) = RE (Control) − RE (Treated)/RE (Control) × 100

better adapted to the natural conditions (e.g., tolerance to heat, cold activity, UV-radiation) of their geographical origins, suggesting that these isolates could reach higher efficacy in tick population control (Braga et al. 2001; Fernandes et al. 2007, 2008).

The virulence of *B. bassiana* and *M. anisopliae* isolates obtained by Costa et al. (2002) from naturally infected *R.* (*B.*) *microplus* was evaluated by Fernandes et al. (2003, 2004, 2006), and several isolates were highly virulent to *R.* (*B.*) *microplus* eggs and larvae;

Table 5 In vivo use of entomopathogenic fungi conidial suspensions to control ticks

Study	Association between fungus and tick[a]	Results[b]
Castro et al. (1997)	*Metarhizium anisopliae* to control *Rhipicephalus* (*Boophilus*) *microplus* on cattle held in pens	(1) Decreased number of females parasitizing cattle: 43.7 and 43.3 engorged females/day (at 10^7 and 10^8 conidia ml^{-1}, respectively) compared to control (109 engorged females/day); (2) EC: 50.4 and 54.8% at 10^7 and 10^8 conidia ml^{-1}, respectively; (3) PREP: 22.2% and 31.8% at 10^7 and 10^8 conidia ml^{-1}, respectively.
Corrêia et al. (1998)	*M. anisopliae* to control *R.* (*B.*) *microplus* on cattle held in pens	1) no effect was observed on number of engorged females parasitizing the cattle; 2) fungal growth and sporulation were observed in 91.7% of engorged females collected from cattle at day 1 after fungal application (10^8 conidia ml^{-1}); 3) engorged females collected at days 1 and 3 after application (10^7 or 10^8 conidia ml^{-1}) presented reduction up to 52% in EP.
Bittencourt et al. (1999a)	*M. anisopliae* to control *R.* (*B.*) *microplus* on cattle in the field	(1) A conidial suspension at 10^8 conidia ml^{-1} was used: no significant PE was observed; (2) engorged females collected at days 1 and 7 after treatment decreased REI (27.2% and 30.5%) compared to control (45.2% and 42.4%), respectively; (3) those collected at day 1 decreased NI (44.7%) compared to control (75.5%); (4) those collect at day 7 decreased EIP (23.1 days) compared to control (26.0 days); (5) reduced weight of females (0.15 g) compared to control (0.27 g) at day 14 after treatment.
Bittencourt et al. (2002)	*Beauveria bassiana* formulated in a polymerized cellulose gel to control *Anocentor nitens* on infested horses in the field	(1) PE above 50% between days 4 and 25 after treatment; (2) aqueous conidial suspension only or cellulose polymerized gel only caused PE <20%.
Monteiro et al. (2003)	*B. bassiana* to control *A. nitens* on cattle ears; cattle were held in pens, ears were protected with a cloth cover	(1) PE: 70.1% (10^8 conidia ml^{-1}).
Bittencourt et al. (2003)	*M. anisopliae* to control *R.* (*B.*) *microplus* larvae on pasture plants	(1) 1st bioassay: PE(2) was 17.8% (10^7 conidia ml^{-1}) and 17.4% (10^9 conidia ml^{-1}) at day 15 after treatment; (2) 2nd bioassay: PE(2) was 22.5% (10^7 conidia ml^{-1}) and 52.2% (10^9 conidia ml^{-1}) at day 15 after treatment; (3) 3rd bioassay: PE(2) was 37.8% (10^7 conidia ml^{-1}) and 53.7% (10^9 conidia ml^{-1}) at day 21 after treatment; (4) total PE(2) was 25.8% (10^7 conidia ml^{-1}) and 40.0% (10^9 conidia ml^{-1}) after 3 bioassays; (5) results suggest that conidia might persist in the pasture increasing efficiency of treatments.
Basso et al. (2005)	*M. anisopliae* to control *R.* (*B.*) *microplus* larvae on pasture plants infested with engorged females	(1) %C varied from 86.9% to 94.08% (10^8 conidia ml^{-1}), from day 35 to 48 post-infestation; (2) in general, in each collection, fewer larvae survived on *Cynodon* spp. pastures than on *Brachiaria brizantha* pastures; (3) results suggest that efficacy of control was influenced by pasture plant type.
Polar et al. (2005c)	*M. anisopliae* was pre-soaked for 24 h and weekly sprayed on cattle to control *R.* (*B.*) *microplus* in the field	(1) A conidial suspension at 10^8 conidia ml^{-1} was used: after 3 weeks, average tick density was reduced (8.5 ± 0.6 and 19.1 ± 0.6 ticks/host) (10^8 conidia ml^{-1}) compared to control (29.6 ± 0.6).

Table 5 continued

Study	Association between fungus and tick[a]	Results[b]
Bahiense et al. (2007)	*M. anisopliae* only or combined with deltamethrin to control a pyrethroid strain of *R.* (*B.*) *microplus* on cattle held in pens	(1) Mean mortality rate of ticks observed for 28 days after treatment was 38.5% (animals sprayed with 25 ppm deltamethrin solution), 32.5% (animals sprayed with conidial suspension only, 10^8 conidia ml^{-1}), and 30.9% (animals sprayed with conidial suspension combined with deltamethrin).
Alonso-Diaz et al. (2007)	*M. anisopliae* to control *R.* (*B.*) *microplus* on cattle in the field	(1) Animals were sprayed with conidial suspension every 15 days. More than 45% PE was observed a) at day 3 and 5 after the 1st fungal application; b) at day 7 and 14 after the 2nd fungal application; (2) PE higher than 70% a) at day 1, 3, 5 and 7 after 3rd fungal application; b) at day 5, 7 and 14 after the 4th fungal application.

Studies are ordered according to year of publication

[a] Fungus was applied as an aqueous conidial suspension, unless specified differently

[b] EP (Egg Production) = weight of egg mass; EIP (Egg Incubation Period) = days before hatch; NI (Nutrient Index) = [weight of egg mass (g)/initial weight of engorged female (g) − residual weight of engorged female (g)] × 100; REI (Reproductive Efficiency Index) = [weight of egg mass (g)/initial weight of engorged female (g)] × 100; ER (Estimated Reproduction) = [weight of egg mass (g)/initial weight of engorged female (g)] × EH × 20,000 (20,000 is a constant corresponding to the estimated number of larvae from 1 g of eggs); PREP (Percent Reduction of Estimated Reproduction) = [ER (Control) − ER (Treated)/ ER (Control)] × 100; EC (Efficacy of Control) = [1 − number of engorged females on treated animals after fungal application × number of engorged females on control animals 3 days before application of control solution/number of engorged females on treated animals 3 days before fungal application × number of engorged females on control animals after application of control solution] × 100; %C (Percentage of Control) = [1 − number of engorged females on treated animals/number of engorged females on control animals] × 100; PE (Percentage of Efficacy) = [average of engorged female number in control groups − average of engorged female number in treated groups/average of engorged female number in control groups] × 100; PE(2) (Percentage of Efficacy) = [average of live larvae collected in control groups − average of live larvae collected in treated groups/average of live larvae collected in control groups] × 100

however, their virulence was not higher to ticks than some other isolates obtained from different arthropod orders. Therefore, the isolates obtained from *R.* (*B.*) *microplus* are not necessarily the most virulent isolates to their original host.

Infection mechanism

The dynamics of the infection process of *M. anisopliae* on *R.* (*B.*) *microplus* (Bittencourt et al. 1995, 1999b; Dutra et al. 2004; Leemon and Jonsson 2008) and *R. sanguineus* (Garcia et al. 2004, 2005) has been described. Also, the infection process of *B. bassiana* has been described for *R. sanguineus* (Kirkland et al. 2004a), and *B. bassiana* and *B. amorpha* on *R.* (*B.*) *microplus* (Campos et al. 2005).

Bittencourt et al. (1995) reported that *M. anisopliae* was detected and isolated from *R.* (*B.*) *microplus* hemolymph at day 2 post-inoculation, and at day 3 and 4 post-inoculation the fungus was detected and isolated from internal organs. No evidence of the fungus in natural cavities was detected by histological analysis. Four years later, Bittencourt et al. (1999b) reported the attachment of *M. anisopliae* (isolate ESALQ 959) conidia to the

cuticle of *R.* (*B.*) *microplus* engorged females, followed by germination, and enlargement of the germ tube to form the appressorium.

Arruda et al. (2005) reported that *M. anisopliae* invades *R.* (*B.*) *microplus* by a process which involves adhesion of conidia to the cuticle, conidia germination, formation of appressoria and penetration through the cuticle. Adhesion of conidia was observed 24 h post-infection, and germination started on the tick's cuticle. Conidia differentiate to form appressoria and infection pegs exert mechanical pressure, and secrete hydrolytic enzymes for cuticle penetration. Massive penetration was observed after 72 h, and hyphae emerged from the cuticle to start the conidiogenesis process 96 h post-inoculation.

The majority of *M. anisopliae* conidia germinated on *R. sanguineus* 18 h after inoculation. The fungus then penetrated through the tick cuticle after 48 h. After fungal penetration, the principal alteration observed was the lyses of intestine and leakage of intestinal content to hemocoel. Mortality was observed after 96 and 120 h post-infection, and sporulation was detected after 120–144 h (Garcia et al. 2004).

These studies indicate that *M. anisopliae* penetration into *R.* (*B.*) *microplus* and *R. sanguineus* occurs through the cuticle, as previously described on other arthropod species (Alves 1998). The penetration process of the fungi through the cuticle involves secretion of enzymes such as proteases and chitinases, and is assisted by mechanical processes of the appressorium infection peg (Charnley and St. Leger 1991). After penetration, the fungus invades the internal organs, produces mycotoxins, and kills the host (Kaaya et al. 1991). However, tick species may display differential susceptibility to entomopathogenic fungi due to fungistatic compounds present in the epicuticle of certain tick species (Kirkland et al. 2004b).

Beauveria bassiana and *M. anisopliae* secrete toxic metabolites during the infection process that contribute to the establishment and progression of disease (Roberts 1981; Alves 1998). Accordingly, an oxalate secreted by *B. bassiana* is presumed to act synergistically with other factors in promoting pathogenesis to ticks (Kirkland et al. 2005). Also, *B. bassiana* and *B. amorpha* produce subtilisin-like proteases and chitinases in the presence of *R.* (*B.*) *microplus* tick cuticle (Campos et al. 2005). *M. anisopliae* genes encoding proteins such as a subtilisin-like protease and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were detected in the presence of *R.* (*B.*) *microplus* cuticle, suggesting that these genes may be involved in *M. anisopliae* pathogenicity. GAPDH is predicted to play a role in adhesion to tick cuticle in early stages of appressorium development and attachment (Dutra et al. 2004).

In general, tick mortality is related to the conidial concentration. According to Kaaya et al. (1996), an increased number of conidia on the ticks' cuticle leads to increased mortality rate. Possibly, there is 'cooperation' (mass action) between neighboring germinating conidia on the arthropod cuticle (Zhioua et al. 1997).

Formulation

Several entomopathogenic organisms need to be ingested to infect their arthropod host; however, the penetration of pathogens via oral ingestion is not feasible for arthropods that are exclusively hematophagous. The fact that entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* penetrate arthropod cuticles means that conidial suspensions can be sprayed on ticks in the field to initiate infections and reduce pest populations. Conidial suspension formulation may improve field performance of conidia under adverse environmental conditions. For example, conidia in oil-based suspensions present higher

efficacy than aqueous conidial suspensions (Kaaya and Hassan 2000; Polar et al. 2005b; Leemon and Jonsson 2008) (see Table 4). Oil formulations have been used with ultra low volume spray technology, which proved effective probably due to improved promotion of conidial adhesion to the hydrophobic surfaces (Prior et al. 1988). Other studies have also demonstrated satisfactory results when conidia are formulated in a polymerized cellulose gel (Bittencourt et al. 2002; Reis et al. 2007) or polymerized cellulose gel with emulsifiable concentrate (Reis et al. 2007).

Formulation of conidial suspensions also may protect conidia against desiccation and ultraviolet radiation. As reviewed by Fargues et al. (1996), several UV protectants increased survival of *M. flavoviridae* conidia exposed to ultraviolet or simulated solar radiation under laboratory conditions. Inglis et al. (1995) also demonstrated that the use of UV-B protectants could increase conidial survival of *B. bassiana* under laboratory and field conditions. Therefore, correct formulation may greatly enhance the effectiveness of entomopathogenic fungi as biocontrol agents.

Immune system of ticks

As with vertebrates, the immune system of invertebrates recognizes invading foreign bodies as non-self, responding with cellular (hemocytic) and acellular (humoral) reactions (Ratcliffe et al. 1985; Ratcliffe and Rowley 1987).

Five classes of hemocytes have been identified in ticks: (1) prohemocytes are believed to be the essential stem cell from which all of the other types are formed, (2) plasmatocytes are believed to serve as phagocytes, exhibiting non-specific responses to corpuscular antigens, inert particles and foreign cells; in addition, a specific response to antigenic substances has been reported, (3) granulocytes type I and type II, which are involved in phagocytosis, also serve to encapsulate foreign material too large to be phagocitized, depositing substances on the particle surface which form membranes and effectively separate it from the tissue, while (4) spherulocytes and (5) oenocytoids perform roles not yet established in the tick circulatory system (reviewed by Sonenshine 1991). However, spherulocytes and oenocytoids seem to be related to the granular cell complex (Tanada and Kaya 1993), and possibly store antimicrobial peptides that are secreted following contact with foreign organisms (Goodman et al. 2005).

A well developed capability for phagocytizing microbes and other foreign organisms appears to exist in ticks, similar to many other arthropods (Sonenshine 1991). However, as has been also described in other arthropods, weak pathogens are encapsulated and destroyed, whereas virulent pathogens are capable of resist phagocytosis and encapsulation, and kill the host (Tanada and Kaya 1993). A similar phenomenon may occur in ticks infected with a virulent form of entomopathogenic fungi, such as *B. bassiana* and *M. anisopliae*.

The immune factors that occur in the plasma are the basis for humoral immunity. Humoral factors facilitate self/non-self recognition and activate defense reactions. It is important to note that no globulins similar to those of vertebrates have been detected in insects (Tanada and Kaya 1993) or ticks. In ticks, the presence of a hemolymph plasma lectin, a glycoprotein containing complex oligosaccharides, was described agglutinating mouse erythrocytes, and was suggested to serve as a recognition molecule (reviewed by Sonenshine 1991). Besides lectins, defensins, lysozyme and possibly other antimicrobial peptides are secreted into the hemolymph plasma to combat invasion by foreign organisms (Goodman et al. 2005; see also Kocan et al. 2008 and Hynes et al. 2008, in this issue). More studies are necessary to understand the role of hemolymph contents in humoral immunity.

Many studies on the tick immune system are limited in identifying and quantifying hemocytes, or in characterizing antimicrobial peptides (Sonenshine 1991; Carneiro and Daemon 1996; Pereira et al. 2001; Silva and Bittencourt 2006). The expression of four forms of defensin was observed in the intestine, fat body and reproductive tract of *Ornithodorus moubata* (Nakajima et al. 2001, 2002). In *R.* (*B.*) *microplus*, peptides that inhibit the growth of gram-positive bacteria and fungi were identified in intestine contents, hemolymph, ovaries of engorged females and eggs (Fogaça et al. 1999, 2006). It is not known, however, if expression of these peptides increases after infection.

Greater understanding of the immune responses of ticks to infection by entomopathogenic fungi would help elucidate the infection process, and this information might be useful in choosing appropriate entomopathogenic fungal isolates for biological control of ticks. Also, knowledge on the effects of formulation products may prove important in devising superior formulations for field use.

Biocontrol strategies

Based on the results reviewed in this study and the ecological conditions in South America, the best use of entomopathogenic fungi to control ticks would probably be to apply formulated conidia directly onto tick-infested animals. Although the spraying of conidial suspensions on pasture plants has reduced the population of tick larvae (see Table 5), the concentration of fungal inoculum used to control ticks in the field still remains high in comparison to those used for agriculture arthropod pests (Maniania et al. 2007). In order to effectively control the cattle tick, *R.* (*B.*) *microplus*, the application of bio-products on an entire pasture would not be economically feasible due to the large areas that free-range cattle occupy in Brazil and other South American countries. On the other hand, a system with pheromones and carbon dioxide delivery in the field could possibly attract ticks to a localized fungus-treated spot in the vegetation; however, further investigation is needed to improve this system (Maranga et al. 2003; Maniania et al. 2007).

Combinations of chemical acaricides and entomopathogenic fungi also have been studied, aiming for compatibility and synergism between them. In vitro studies have investigated the combination of pyrethroid with *M. anisopliae* (Camargo 1983; Bahiense et al. 2006); organophosphates and growth inhibitors (i.e., methoprene and diflubenzuron) with *M. anisopliae* (Mohamed et al. 1987); amitraz, organophosphate or pyrethroid with *M. anisopliae* (Paião et al. 2001b); imidacloprid or fipronil with *M. anisopliae* or *B. bassiana* (Moino and Alves 1998); pyrethroid with *B. bassiana* (Bahiense and Bittencourt 2004); and several pyrethroids and organophosphates, combined or not, or amitraz with *B. bassiana* (Wenzel et al. 2004). It is important to note that most of the studies evaluated the possible negative effects of chemical products on entomopathogenic fungi; however, Paião et al. (2001b), Bahiense and Bittencourt (2004) and Bahiense et al. (2006) evaluated the possible positive effect of the combination on *R.* (*B.*) *microplus* mortality. The results suggest that the combination of chemical acaricides with entomopathogenic fungi is potentially an important tool for integrated management of ticks.

Conclusion

Entomopathogenic fungi are the most promising of the currently available alternatives to chemical acaricides for tick control; especially since these organisms penetrate directly

through the tick cuticle, and therefore do not need to be ingested to initiate infection. In most cases, these fungi are able to infect all developmental stages of ticks, such as eggs, larvae, nymphs and adults. In addition, fungi might initiate natural epizootic outbreaks (Alves 1998), and where environmental conditions (e.g., temperature, humidity, solar radiation) are appropriate, the ability of the fungi to multiply and spread is an important advantage. The genetic variability among fungal isolates is another advantage, in that simple assays may detect the most virulent isolates, level of host specificity, and tolerance to field conditions.

Unfortunately, most field trials performed to date have reported rather low efficacy of fungi for the control of tick populations in South America, with the exception of *A. nitens* control on cattle ears using *B. bassiana* formulated in a polymerized cellulose gel (Bittencourt et al. 2002). Only a few field trials have been conducted in South America, and in most cases, a simple aqueous conidial suspension was used. In Brazil, an acaricide needs to achieve 95% efficacy to be commercialized (Ministério da Agricultura, Pecuária e Abastecimento 1997). Therefore, studies on formulation are required to improve conidial performance under environmental conditions. Furthermore, structured control programs need to be developed, and include information about the application method, appropriate season for applications, and periodicity of treatments. These programs may vary with tick species due to different biological behavior and geographic region. Future field trials should consider these aspects.

In addition, future studies should consider (1) the association of chemical and biological products in integrated management programs, (2) the impact of biological control agents on non-target arthropods, (3) the efficacy of biological control programs in different environments and ecosystems, with special consideration of temperature and humidity variation, and vegetation, (4) safety to animals and humans, (5) the infection process of fungi on ticks, and the immune response of ticks to the fungal infection, (6) the establishment of companies with capacity for mass production of conidia and formulation of the final product, (7) the improvement of the shelf life of the biological product, and (8) the education of consumers.

Tick biological control offers several advantages over currently available chemical acaricides, including lower costs; and therefore, there are increased efforts in South America to develop new biological products. Unfortunately, regulations for microbial pesticides in many South American countries are poorly developed and/or virtually not enforced, and thus have impeded the development of quality biological control products. However, while more research is necessary, entomopathogenic fungi have great promise as alternatives to current tick control methods, and they could alleviate many of the current environmental and health concerns that come with the present-day methods.

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Pathogenicity and thermotolerance of entomopathogenic fungi for the control of the scab mite, *Psoroptes ovis*

M. Lekimme · C. Focant · F. Farnir · B. Mignon · B. Losson

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Abstract *Psoroptes ovis* is responsible for a highly contagious skin condition, both in sheep and cattle. This parasite has a marked economical impact in the sheep and cattle industry. Biological control is considered as a realistic alternative to chemotherapeutic control. Laboratory experiments were carried out to evaluate the pathogenicity and the thermotolerance of twelve isolates of entomopathogenic fungi from four genera (*Beauveria* Vuillemin, *Metarhizium* Sorokin, *Paecilomyces* Bainier and *Verticillium* Nees). The pathogenicity was evaluated by the survival of *P. ovis* females after exposure to 10^6 to 10^8 conidia ml^{-1} in humidity chambers. Results revealed intra- and interspecies differences. All isolates with the exception of *B. bassiana* IHEM3558 and *V. lecanii* MUCL8672 induced 50% mortality within 2 days at the highest concentration. At this concentration the entire mite population became infected with all isolates but *B. bassiana* IHEM3558; however, only four isolates gave rise to 100% infected cadavers at the lowest concentration. The thermotolerance of each isolate was evaluated by measuring its growth on an artificial medium kept between 25 and 37.5°C. All isolates were able to grow up to 30°C but only two, *M. anisopliae* IHEM18027 and *Paecilomyces farinosus* MUCL18885, tolerated temperatures up to 35°C. These two isolates could be considered as good candidates for further use as biopesticide taking into account their virulence and thermotolerance. Other critical factors linked with the implementation of this type of biocontrol in *P. ovis* infected animals are discussed.

Keywords *Psoroptes ovis* · Biological control · Entomopathogenic fungi · Temperature · Virulence

M. Lekimme (✉) · C. Focant · B. Mignon · B. Losson
Laboratory of Parasitology and Pathology of Parasitic Diseases B43, Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20, 4000 Liege, Belgium
e-mail: mireille.lekimme@ulg.ac.be

F. Farnir
Department of Functional Sciences, Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20, 4000 Liege, Belgium

Introduction

Psoroptes ovis (Hering) is an ectoparasitic mite responsible for a severe debilitating dermatitis in cattle, sheep, goats and rabbits. Until now, control of psoroptic scab is based on the use of chemical acaricides. Nevertheless treatment failures and environmental considerations have led to the development of alternative approaches, particularly the use of entomopathogenic fungi (Smith et al. 2000; Brooks and Wall 2001, 2005; Brooks et al. 2004; Lekimme et al. 2006a; Abolins et al. 2007). Because infection by entomopathogenic fungi such as *Beauveria* and *Metarhizium* species results from direct penetration of the tegument without any requirement for ingestion, these organisms often display wide host ranges. There is a growing literature dealing with their virulence and use in insects, ticks and other members of the class Arachnida (Kaaya and Munyinyi 1995; Chandler et al. 2000; Samish 2000; Scholte et al. 2004; Polar et al. 2008, this issue). Additionally inter- and intraspecific differences in the pathogenicity were observed in different arthropod species (Ferron and Diomandé 1969; Daoust and Roberts 1982; Barson et al. 1994; Frazzon et al. 2000; Kaaya and Hassan 2000; Onofre et al. 2001; Samish et al. 2001; Darwish and Zayed 2002; Gindin et al. 2002; Liu et al. 2002; Scholte et al. 2004; Shi and Feng 2004).

Recently interesting results were obtained in *Psoroptes* mites using *B. bassiana* and *M. anisopliae* in in vitro bioassays (Smith et al. 2000; Brooks and Wall 2001; Lekimme et al. 2006a). To abate the development of psoroptic mange, 50% of the mite population must be killed every 2 days (Wall et al. 1999). Another important parameter is the proportion of infected mites reached. These two factors were unfortunately not achieved with the isolates evaluated by these authors at a concentration of 10^8 conidia ml^{-1}. It seemed thus interesting to test various isolates belonging to different genera and species in order to identify a highly virulent one. Moreover the high temperature prevailing at the skin surface of the hosts could limit the use of these fungi for the control of *P. ovis*. Indeed the temperature at the skin level varies between 31 and 37°C in sheep, and between 30 and 35°C in cattle (Brooks et al. 2004; Polar et al. 2005). *Beauveria bassiana* and *M. anisopliae* are known to have their optimum of growth at 25 and 30°C, respectively (Hallsworth and Magan 1999). However, differences can be observed between isolates with respect to thermotolerance. This could be related with their geographical origin (Fargues et al. 1997; Vidal et al. 1997; Davidson et al. 2003; Devi et al. 2005; Rangel et al. 2005; Quesada-Moraga et al. 2006).

The aim of the present study was thus to investigate in twelve entomopathogenic fungal isolates the in vitro pathogenicity against *P. ovis* and the thermotolerance. These isolates belonged to four genera and originated from temperate and tropical areas.

Materials and methods

Mites

Psoroptes mites were isolated from the ears of chronically infested rabbits maintained at the laboratory of the Faculty of Veterinary Medicine, Liège, Belgium. Adult females were collected from freshly removed scabs (maximum 1 h) and directly transferred into conidial or control suspensions.

Fungi

Twelve strains of five entomopathogenic fungal species belonging to four genera were cultured on Sabouraud Dextrose Agar + Yeast (SDAY; Agar 20 g, D-glucose 20 g,

Neopeptone 10 g, Yeast Extract 10 g l^{-1} in deionised water) or Potato Dextrose Agar (PDA; Difco) for 2 weeks at 27°C. Isolates were provided by the IHEM (Institut d'Hygiène et d'Epidémiologie-Mycologie, Brussels, Belgium) or the MUCL (Mycothèque de l'Université de Louvain-La-Neuve, Belgium) collections. Isolates from temperate regions were *Beauveria bassiana* IHEM18747 (SDAY) and IHEM3558 (SDAY), *Metarhizium anisopliae* IHEM1552 (SDAY) and IHEM1639 (SDAY), *Metarhizium brunneum* MUCL9645 (PDA), *Paecilomyces farinosus* MUCL18885 (PDA), and *Verticillium lecanii* MUCL8672 (PDA). Isolates from tropical origin were *B. bassiana* MUCL104 (SDAY), MUCL38502 (SDAY), and MUCL39817 (SDAY), *M. anisopliae* IHEM18027 (SDAY), and *Paecilomyces fumosoroseus* MUCL15122 (PDA).

Conidia were collected by scraping the colony with a loop in sterile 0.03% PBS-Tween-20 solution (PBST). The conidial suspension was pipetted from the plate and the concentration was evaluated using a haemocytometer. Dilutions ranging from 10^8 to 10^6 conidia ml^{-1} were obtained in the same buffer.

Pathogenicity evaluation

Twenty *P. ovis* adult females were immersed for 5 min in the various concentrations of conidial suspensions or in PBST alone (controls) and then transferred into handling chambers. These chambers consisted of 3.5 cm Petri dishes filled with 1% agarose (Lekimme et al. 2006b). The handling chambers were sealed with parafilm and placed at 27°C in an incubator exposed to the artificial illumination of the lab during working periods. Mites were examined daily and dead mites (absence of motility) were transferred, after washing in 0.2% sodium hypochlorite and distilled water for 30 s, into a 96-well plate filled with 1% agarose. This plate was left at 27°C and checked every day for the apparition of fungal hyphae on the mite body. Four bioassays were carried out with each isolate and LT_{50} (time to 50% mortality) and the number of infected mites were calculated. Entomopathogenic activity of the fungi was confirmed by examining histological sections of infected cadavers. Classical protocol for formalin fixation and paraffin embedding was used and 5 μm sections were stained with Periodic Acid Schiff (PAS; Sigma-Aldrich).

Temperature profiles

Ten microlitres of the 10^6 conidia ml^{-1} suspension were inoculated onto the centre of an SDAY or PDA plate. Plates were incubated in quadruplicates at temperatures of 25, 27.5, 30, 32.5, 35 and 37.5°C. Ten days after inoculation, the diameter of each colony was measured (cm). Five measurements were made for each plate and the average was calculated. This assay was repeated four times at all temperatures for all strains.

Statistical analysis

Data were submitted to a two way analysis of variance (SAS software, version 6.12, Cary, NC, USA). For the pathogenicity evaluation, the LT_{50} (dependent variable) was compared between the 12 fungal strains and the three concentrations (independent variables); for the temperature profiles, the colony diameter was the dependent variable while the fungi and the temperatures were the independent ones. Normality was evaluated using the Shapiro–Wilk test. Although the data were not distributed normally (with or without transformation), the effects observed were so highly significant ($P < 0.0001$) that normality

departures should not affect the conclusions. Post-hoc multiple range tests consisted in pre-planned contrasts.

Results

Evaluation of the pathogenicity

The susceptibility of *P. ovis* varied significantly according to the isolate tested ($P < 0.0001$) (Fig. 1). For all isolates a dose responsive effect could be observed ($P < 0.0001$). Only four isolates were able to infect 100% of the mites at all concentrations: *M. anisopliae* IHEM18027, *M. brunneum* MUCL9645, *P. farinosus* MUCL18885, and *V. lecanii* MUCL8672. Four isolates were able to infect 100% of the mites at 10^8 and 10^7 conidia ml^{-1}: *B. bassiana* IHEM18747 and MUCL38502, and *M. anisopliae* IHEM1552 and IHEM1639. Three isolates infected 100% of the mites only at the highest concentration: *B. bassiana* MUCL104 and MUCL39817, and *P. fumosoroseus* MUCL15122. Finally, *B. bassiana* IHEM3558 failed to infect all the mites at all concentrations (80–85% infected mites).

Verticillium lecanii MUCL8672 and *B. bassiana* IHEM3558 killed 50% of the mite population more slowly than the other isolates ($P < 0.001$ and $P < 0.002$, respectively) while the former led to 100% of infection. In contrast, *M. anisopliae* IHEM18027 was significantly more effective than the other isolates ($P < 0.0001$).

Histological sections confirmed the presence of fungal hyphae inside the body of the infected mites.

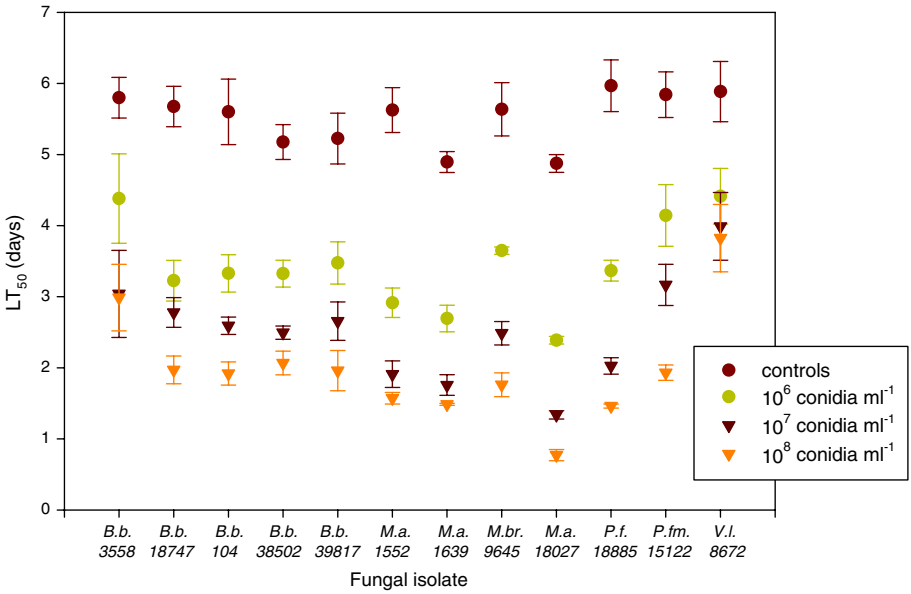


Fig. 1 Mean time to get 50% mortality (LT_{50}) (±SE) of adult females *Psoroptes ovis* after exposure to 10^6, 10^7 or 10^8 conidia ml^{-1} of twelve isolates of entomopathogenic fungi

Evaluation of the temperature profiles

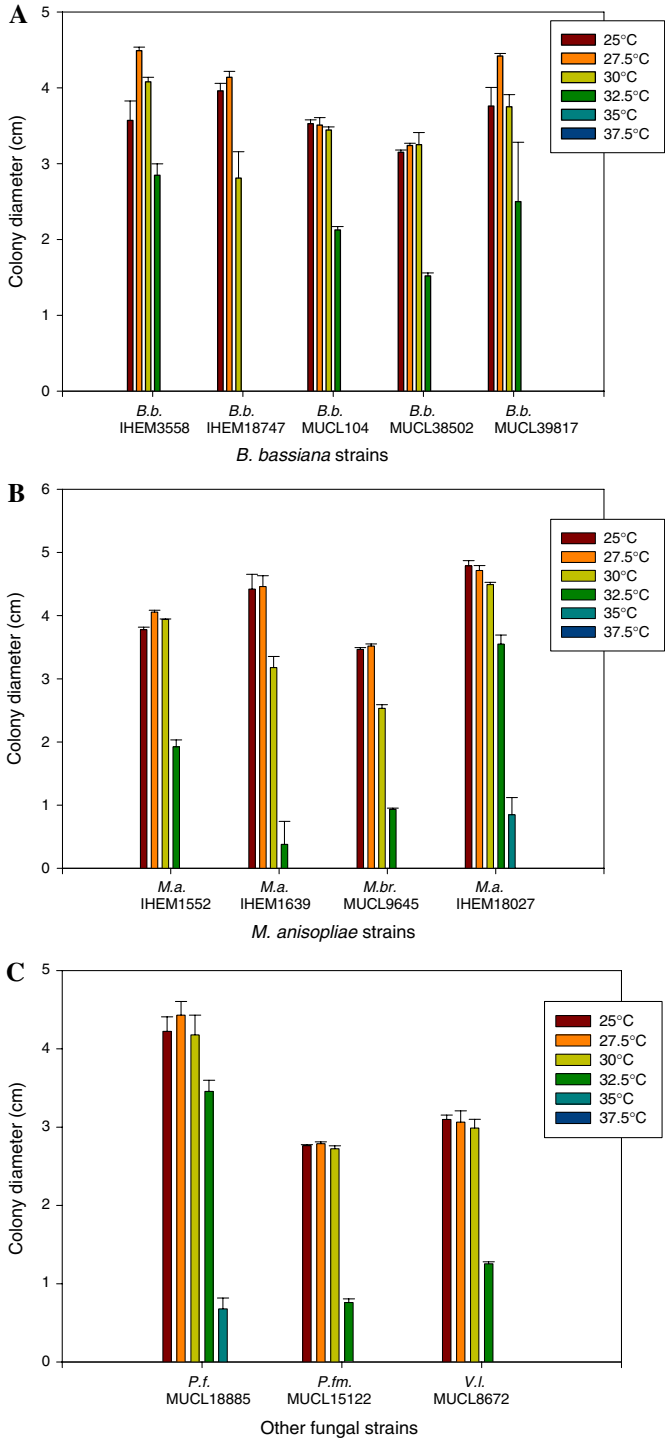
The growth of the various isolates was significantly affected by temperature ($P < 0.0001$) (Fig. 2) and there was a significant difference in colony size between the twelve isolates ($P < 0.0001$). The growth was higher at 25, 27.5 and 30°C. The interaction between isolates and temperature was also significant with a nearly perfect suitability with the model ($r^2 = 0.99$).

Temperate and tropical strains of *B. bassiana* and *M. anisopliae* were compared (Fig. 2a, b). The analysis of the results showed that the temperature profiles of temperate and tropical strains of *B. bassiana* did not differ globally ($P = 0.22$) (Fig. 2a). In contrast in *M. anisopliae* (*M. brunneum* was considered a synonym of *M. anisopliae*, following Tulloch (1976)) the difference between temperate and tropical strains was significant ($P < 0.0001$) and only the tropical strain *M. anisopliae* IHEM18027 grew at 35°C (Fig. 2b). For the other isolates, no comparison could be made but it is noteworthy that also the temperate isolate *P. farinosus* MUCL18885 was able to grow at 35°C (Fig. 2c).

Discussion

The use of entomopathogenic fungi for the control of psoroptic mange was first evaluated by Smith et al. (2000). The major advantage of the fungal pathogens is that they infect their hosts directly through the tegument. *Psoroptes ovis* is almost completely unsclerotised which facilitates the penetration of the hyphae. Until now, only two species of entomopathogenic fungi have shown lethal activity against this mite, i.e. *M. anisopliae* (Smith et al. 2000) and *B. bassiana* (Lekimme et al. 2006a). Two parameters are important for the control of psoroptic mange with fungi. The first one is the rate of killing the target (50% of dead mites every 2 days, according to Wall et al. 1999). This study demonstrated that several other species of entomopathogenic fungi have a lethal activity against *Psoroptes* mites. All the isolates but two (*V. lecanii* MUCL8672 and *B. bassiana* IHEM3558) gave high rates of killing, considered able to impede the development of mite populations. Among the fungi tested, *M. anisopliae* IHEM18027 seems particularly interesting with an LT_{50} of 0.7 days at 10^8 conidia ml^{-1} and a rate of infection of 100%. Additionally a 100% infection rate was also recorded at a concentration of 10^6 conidia ml^{-1}. The proportion of infected mites is also a crucial factor. Indeed, Brooks and Wall (2001, 2005) and Lekimme et al. (2006a) have shown that the infection can be transferred from infected cadavers or surfaces to healthy mites. This aspect is important to ensure a lasting effect to the product. In *P. ovis*-infested cattle the parasite burden is usually very high and the mites have numerous contacts with each other. Therefore the entomopathogenic fungi could spread very easily on the host and in the herd. Indeed movements of the infected arthropods are the most efficient way of dispersion for the entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* (Meyling and Eilenberg 2007). This way of infection has an additional advantage as it allows the use of a lower concentration of conidia and, consequently, easier and cheaper mass production.

The infection process was not really submitted to a defined photoperiod; nevertheless the dishes received the artificial light of the lab during the working periods. Some authors showed indeed that virulence, germination or conidial production on cadavers could be affected or not, depending on the fungal species by the applied photoperiod (Tang and Hou 2001; Hiroki et al. 2005). In the present study the germination of the fungi tested did not show any alteration when incubated in the dark. Standard bioassays investigating the


A
Colony diameter (cm)
0
1
2
3
4
5
25°C
27.5°C
30°C
32.5°C
35°C
37.5°C
B.b. IHEM3558
B.b. IHEM18747
B.b. MUCL104
B.b. MUCL38502
B.b. MUCL39817
B. bassiana strains
B
Colony diameter (cm)
0
1
2
3
4
5
6
25°C
27.5°C
30°C
32.5°C
35°C
37.5°C
M.a. IHEM1552
M.a. IHEM1639
M.br. MUCL9645
M.a. IHEM18027
M. anisopliae strains
C
Colony diameter (cm)
0
1
2
3
4
5
25°C
27.5°C
30°C
32.5°C
35°C
37.5°C
P.f. MUCL18885
P.fm. MUCL15122
V.l. MUCL8672
Other fungal strains

◀**Fig. 2** Mean colony diameters (cm) (±SE) 10 days after inoculation of *Beauveria bassiana* (**a**), *Metarhizium anisopliae* (**b**) and other species (**c**) maintained at 25, 27.5, 30, 32.5, 35 and 37.5°C ($n = 4$). No strains were able to grow at 37.5°C

pathogenicity of entomopathogenic fungi on *P. ovis* are usually performed at 25–27°C, the optimum temperature for both the mites and the fungi in vitro. However, isolates with an optimal growth under these conditions may not be suitable for application on cattle skin where the temperature is higher (30–35°C) as shown by Polar et al. (2005). Consequently the isolates were tested for their growth on artificial medium at temperatures ranging from 25 to 37.5°C. It appeared that all strains were capable of growth up to 30°C and all but one (*B. bassiana* IHEM18747) up to 32.5°C. However optimum growth was recorded between 25 and 30°C. These results are in agreement with other studies (Hallsworth and Magan 1999; Davidson et al. 2003; Iskandarov et al. 2006; Quesada-Moraga et al. 2006). Only two isolates, *M. anisopliae* IHEM18027 and *P. farinosus* MUCL18885, tolerated a temperature of 35°C, and none were able to grow at 37.5°C. A comparison between tropical and temperate strains of *B. bassiana* failed to show a significant difference in the aptitude to grow at high temperatures. These results are similar to those obtained by Fargues et al. (1997) where an association between thermotolerance and geographic origin was not found. For *M. anisopliae*, a marked difference was found, in contradiction with the observations of Rangel et al. (2005). However it is noteworthy that only one tropical strain was included in the present study. These differences could be explained by the hosts from which the fungi originated. In fact, isolates originating from acridids were generally more heat tolerant because the temperatures can reach 47.4°C in this family of insects (Rangel et al. 2005). However several of the isolates used in this study were isolated from the soil and it is not possible to draw any conclusion.

In addition, the skin temperature on cattle fluctuates during the day (Polar et al. 2005). Fortunately, these authors demonstrated that isolates which are more thermotolerant are more likely to exhibit high level of pathogenicity under conditions reflecting the thermal characteristics of the cattle coat. Brooks et al. (2004) also observed that thermotolerant *M. anisopliae* isolates gave greater proportion of infection in *P. ovis* when compared to isolates with a lower optimal temperature. Thus the next step should consist of an in vivo bioassay to verify that the isolates able to grow in vitro at temperatures ranging from 30 to 35°C are able to germinate, infect the mites and sporulate on the body surface of cattle in presence of diurnal temperature fluctuations. A recent study performed on sheep showed that three isolates of *M. anisopliae* and one specific isolate of *B. bassiana* can infect *P. ovis* in vivo (Abolins et al. 2007) while the temperature prevailing at the sheep skin surface usually ranged between 31 and 37°C (Brooks et al. 2004). In their study, Abolins et al. (2007) have also demonstrated the absence of fungal inhibitors in the fleece of the sheep. A saprophytic microflora can in fact be present on the tegument of the arthropods or on the hair of the host and stimulate or suppress the germination of the conidia in vivo (Schabel 1978; Fargues 1981; Polar et al. 2008).

Other abiotic important parameters include relative humidity and UV radiations (Luz and Fargues 1997; Magalhães and Boucias 2004; Polar et al. 2005; Rangel et al. 2005; Iskandarov et al. 2006; Fernandes et al. 2007). High relative humidity is generally considered to be necessary for conidial germination but contradictory data can be found concerning this parameter (Lord 2005). In the case of cattle psoroptic mange, the relative humidity at the skin surface is high. Furthermore, it is likely that the conidia formulation would play a crucial role in the infection process by maintaining a high humidity level

(hydrophilic excipient) or decreasing the dependency on moisture (oil formulations) (Brooks et al. 2004). The susceptibility of the conidia to UV radiations should not be a problem in *P. ovis* infected cattle. In fact, cattle mange is a disease occurring mainly during the winter, when the animals are maintained indoors and not exposed to UV radiations. In the case the conidia would still be present when the cows are turned out, the solar radiations could inactivate them which could prelude the dissemination of the fungus to non-target organisms.

From the present study, two isolates could be considered as potential control agents for psoroptic mange: *M. anisopliae* IHEM18027 and *P. farinosus* MUCL18885. Because of the availability of mass production procedures for the former (no commercial biopesticide based on the use of *P. farinosus* exists to the authors' knowledge; see also Kabaluk and Gazdik 2004) and the better activity/thermotolerance observed in vitro, *M. anisopliae* IHEM18027 will be used in the near future.

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Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on *Varroa* mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health

William G. Meikle · Guy Mercadier · Niels Holst · Vincent Girod

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Abstract Bee colonies in southern France were treated with conidia (asexual spores) from two strains of *Beauveria bassiana*, an entomopathogenic fungus. One strain was commercial (GHA) and the other had been isolated from *Varroa* mites in the region (Bb05002). Objectives were to evaluate treatment effect on colony weight, adult bee mass, capped brood, and on *Varroa* fall onto sticky boards. Treatments included conidia formulated with either carnauba or candelilla wax powder, candelilla wax powder alone, or control; in two treatment groups formulation was applied a second time after one week. Treatment did not affect colony health. Colonies treated twice with Bb05002 conidia and carnauba wax powder had significantly higher mite fall compared to colonies treated with blank candelilla wax powder. The proportion of fallen mites that were infected in both conidia treatments was higher than controls for 18 days after the second treatment. The number of fungal propagules on the bees themselves remained elevated for about 14 days after the second treatment. These results were compared to published results from previous experiments with regard to infection duration.

Keywords *Apis mellifera* · *Varroa destructor* · *Beauveria bassiana* · Biopesticide · Formulation

W. G. Meikle (✉) · G. Mercadier
European Biological Control Laboratory, USDA–ARS, Campus International de Baillarguet, CS 90013 Montferrier sur Lez, St. Gely du Fesc, 34988 Montpellier, France
e-mail: wmeikle@ars-ebcl.org

N. Holst
Faculty of Agricultural Sciences, Department of Integrated Pest Management, University of Aarhus, Flakkebjerg, 4200 Slagelse, Denmark

V. Girod
ADAPRO LR-CRALR, Maison des Agriculteurs, Mas de Saporta CS 30012, Lattes, 34875 Montpellier, France

Introduction

Varroa destructor Anderson and Trueman, one of the most serious pests of honeybees (*Apis mellifera* L.) (Hymenoptera: Apidae) (Chandler et al. 2001; Martin 1998), weaken larvae and adults by feeding on haemolymph, transmitting diseases, and inducing deformities (Chandler et al. 2001; Martin 2001). *Varroa* infestations have been largely responsible for the almost complete elimination of feral colonies in the U.S. (Rinderer et al. 2001). The use of entomopathogenic fungi has been considered a promising alternative to chemical miticides (Chandler et al. 2001), and fungal isolates of several species have been tested (James et al. 2006; Kanga et al. 2003, 2006; Meikle et al. 2006, 2007, 2008; Shaw et al. 2002). Meikle et al. (2006) reported the discovery of several isolates of *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycota: Hyphomycetes) from *Varroa* mites collected from honeybee colonies in southern France. Collecting fungal isolates from either the target environment and/or even the target pests themselves is intended to increase the probability of finding the best adapted isolates. For example, an isolate of *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycota: Hyphomycetes), collected from cadavers of *Ornithacris cavroisi* (Finot) (Orthoptera: Acrididae) in Niger, was found in tests to be well-adapted to the high temperatures and low humidities of the region and was subsequently incorporated into a biopesticide against the desert locust *Schistocerca gregaria* Forskål (Orthoptera: Acrididae) (Cherry et al. 1999; Lomer et al. 1997). The high temperatures and humidities of beehives, among other factors, can present similar challenges to entomopathogenic fungi.

Meikle et al. (2007, 2008) used a *B. bassiana* isolate, collected from *Varroa*, in field experiments where it significantly increased *Varroa* fall and was found on the surfaces of bees and infecting fallen *Varroa* mites for at least a week after a single treatment. The use of an entomopathogenic fungus in an insect colony involves some risk. *Beauveria bassiana* is known to have a broad host range (Tanada and Kaya 1993) and Meikle et al. (2006) reported *B. bassiana* infections of bee pupae that had been exposed to treated *Varroa* mites in laboratory bioassays. However, Jaronski et al. (2004) and Meikle et al. (2008) found that a single application of *B. bassiana* did not have measurable negative impact on colony health or survivorship. Meikle et al. (2008) observed that while a single application significantly increased mite fall, it did not have sufficient negative impact on *Varroa* densities to be considered as a complete control strategy.

Entomopathogenic fungi are usually applied in the form of conidia, which are asexual, non-motile spores (Burges 1998), and the conidia are often combined with other materials in order to stabilize the conidia during storage, facilitate application, protect the conidia, and enhance conidia activity (Jones and Burges 1998). Carnauba wax, obtained from *Copernicia cerifera* or *C. prunifera*, is hydrophobic and inert, with no nutritive value for the conidia, and is permitted as a food additive (U.S. Code of Federal Regulations, Title 21, part 184, Sect. 1978) so it poses no honey contamination problems in the U.S. Formulation with carnauba wax has been tested in previous studies and no negative effects have been observed with respect to honeybee colony health (Meikle et al. 2007, 2008). Meikle et al. (2008) also formulated conidia with wheat flour, which is different from wax powders because it can be used as a food source for germinating conidia and is therefore not an inert ingredient (Burges 1998). Meikle et al. (2008) found that the impact of the wheat flour formulation on mite fall was significantly less than conidia combined with carnauba wax powder. Treating hives with a powder, such as powdered sugar or pollen, is known to provoke mite fall (Fakhimzadeh 2001; Macedo et al. 2002), but 99% of the effect occurs within 18 h (Fakhimzadeh 2001).

The main objective in this study was to evaluate the impact of two successive applications of conidia of entomopathogenic fungi on colony health and on *Varroa* mite fall. Two isolates of *B. bassiana* were used: isolate 05002 (NRRL 30976) and strain GHA (Laverlam International, Butte MT; Technical Grade Active Ingredient lot number 03-04-1C/1D). Two kinds of wax powder were also compared as formulation ingredients: carnauba wax, as used in previous experiments, and candelilla wax, which is obtained from *Euphorbia antisyphilitica* and *Pedilanthus pavonis* and is also safe as a human food ingredient (U.S. Code of Federal Regulations, Title 21, part 184, Sect. 1976). Here we measured colony growth rates per week, total adult bee weight and the amounts of sealed brood and honey. The use of growth rates, which are independent of colony size, was intended to facilitate comparison of these results with other studies. *Varroa* mite fall and the proportion infected mites were measured as in Meikle et al. (2007).

Materials and methods

Preparation of formulation

Cultures of *B. bassiana* isolate Bb05002 were grown on Sabouraud dextrose agar with yeast (SDAY) (Goettel and Inglis 1997) for a minimum of 15 days. Conidia were harvested by scraping the surface of the cultures onto glass petri dishes with a metal spatula, and placing the petri dishes in a crystallizing dish containing silica gel for 20–24 h at room temperature for drying.

Four formulations were prepared: carnauba wax powder and conidia from *B. bassiana* isolate 05002 ("Bb05002 + carnauba"); carnauba wax powder and conidia from *B. bassiana* isolate GHA ("BbGHA + carnauba"), candelilla wax powder and 05002 conidia ("Bb05002 + candelilla") and candelilla wax powder alone ("candelilla alone"). Since carnauba wax powder alone had been tested in previous experiments (see Meikle et al. 2007, 2008), it was not tested here. All formulations were prepared on 8 May. The per colony dose of Bb05002 + carnauba and BbGHA + carnauba consisted of 1.0 g conidia of the respective isolate mixed with 9.0 g carnauba wax powder (Strahl & Pitsch Inc., West Babylon, NY, USA) and 0.05 g hydrated silica (Hi-Sil-233, Pittsburgh Plate Glass, Pittsburgh, PA, USA) as a flow agent. The per colony dose of Bb05002 + candelilla consisted of 1.0 g conidia of isolate 05002 mixed with 9.0 g candelilla wax powder (Strahl & Pitsch Inc., West Babylon, NY, USA) and 0.05 g hydrated silica. The per colony dose of candelilla alone consisted of 9.0 g candelilla wax powder and 0.05 g silica. All formulations were mixed using a food processor (Valentin Mini Chopper, SEB, Dijon, France). The density of colony-forming units (cfu) per g formulation was determined at the time of colony treatment by plating three sub-samples of the formulation diluted in distilled water and Tween 80 (Merck, Munich, Germany) onto potato-dextrose agar, and counting the number of colonies 96 h after plating. Formulated and unformulated conidia were stored in a refrigerator at 4°C.

Field experiment

In April 2007, 26 honeybee colonies were selected for the field experiment. The colonies were part of an apiary of 52 colonies near Lattes, in southern France. The bee colonies were kept in painted, 10-frame, wooden Langstroth brood boxes (56 l capacity) with telescoping lids and with screens underneath the frames and queen excluders on top of the

brood box. On 17 April one sticky board (31 × 42 cm, Mann Lake Ltd, Hackensack, MN, USA) was placed under each colony. The boards were replaced on 24 April and every 3–4 days thereafter with fresh boards. All mites adhering to the used boards were counted, and 40-mite samples were taken from each board and plated on water agar (6.0 g/l) with chloramphenicol (0.4 g/l). If a board had 40 or fewer mites, all mites were plated. Plated mite samples were incubated at 23°C, examined for sporulation after 15 days, and the proportion of sporulating cadavers was calculated (hereafter referred to as the "proportion infected mites"). Temperature loggers (Thermachron iButton, Dallas Semiconductor, Sunnyvale, CA, USA) were placed in seven hives at the center of the queen excluder on the top of the brood box to record internal temperature hourly starting the day of treatment, and another logger was placed nearby in the shade to record ambient temperature.

On 2 May and at seven-day intervals until 8 June (after which hives were moved and the experiment stopped) each hive was weighed using a portable electronic balance with a precision of 50 g (OHaus Corporation model Champ CQ100L, Pine Brook, NJ, USA). On 9 May, and again on 27 June, each hive was opened and each hive part (i.e. brood box, lids, colony base, and frames after shaking them free of bees) was weighed using a smaller portable electronic balance with a precision of 1 g (Kern & Sohn model 12 K 1 N, Balingen, Germany). Digital photographs were taken of each side of each frame using a 3.3 megapixel camera (Nikon Coolpix 990, Tokyo, Japan). The hive was then reassembled, and one super containing nine frames with wax foundation was weighed and placed on top of each colony. On 27 June, the super was also weighed.

Five colonies were selected for each treatment group except the untreated control, which had 6. Colonies were randomly assigned treatments, but treatments were occasionally re-assigned to distribute treatments evenly. Colonies in the Bb05002 + carnauba, BbGHA + carnauba and candelilla alone treatments were treated on 10 and 17 May. Colonies in the 05002 + candelilla treatment were treated only once, on 10 May. For each colony treatment, a plastic laboratory wash bottle (Nalge Nunc International, Rochester, NY) was filled with a single dose of preparation, the hive lid and super removed, the formulation blown between all brood box frames by squeezing the wash bottle, and the super and lid replaced.

To calculate colony and adult bee weight, hive weight was divided into a "non-colony" part, consisting of the hive pieces, e.g., brood box, lids, super, hive base, and 10 empty frames with foundation comb, and the "colony" part, consisting of the adult bees, brood, honey, pollen and wax (other than foundation comb). Adult bee weight was calculated as the difference between the sum of the weights of all the hive parts and the observed hive weight. The non-colony weight was calculated as the total weight of all the hive parts except brood box frames, plus the weight of 10 empty frames, or about 2.87 kg (Meikle et al. 2008). Colony weight was calculated by subtracting the non-colony component from the total hive weight. The area of sealed brood and sealed honey per frame was estimated from the photographs using ArcView 3.0 (Environmental Systems Research Institute, Redlands, CA, USA). Brood areas were inspected closely for any signs of fungal infection. Colony entrances were inspected for unusually large numbers of dead bees.

On 2, 9, 11, 15, 18, 22 and 25 May, and on 1 and 8 June, samples of approximately 15 bees were collected from within each hive into plastic boxes in the field, the boxes placed in a cooler with ice packs, and the boxes transferred to a freezer. Two subsamples of five bees each were removed from bags of three colonies per treatment (the same colonies were always used) for a total of 30 subsamples. Each subsample was placed in a 50 ml plastic centrifuge tube and vortexed for 3 min. in 10 ml of a 0.1% aqueous solution of Tween 80. Aliquots of 20 and 100 µl of the resulting suspension from each subsample were spread

onto each of three petri dishes containing potato dextrose agar with chloramphenicol (0.4 g/l); thus six plates for each subsample. The dishes were incubated for at least 14 days at 23°C, and the number of *B. bassiana* cfu were counted in the plates with 20 μl of solution; when cfu densities became low, cfu were counted on the 100-μl plates.

Statistical analysis

Data were analyzed using SAS (SAS Institute, Inc., Cary NC, USA) software. Repeated measure analyses of variance were conducted for a linear mixed model using PROC MIXED of SAS (Littell et al. 1996) with either mite fall (log transformed), or the proportion infected mites (arcsine square-root transformed) as the response variable and with three fixed effects: treatment, date and their interaction ($\alpha = 0.05$). The covariance matrix of both response variables was inspected for patterns and residual plots were assessed visually for variance homogeneity. Colony number was incorporated as a random effect. The degrees of freedom were calculated using the Satterthwaite method. Analyses were designed to maximize the degrees of freedom for detection of differences among treatments. Insignificant main effects were excluded from the model but if the interaction was significant both main factors were retained. Post hoc contrasts of the least squares means differences were conducted for all significant factors, using Bonferroni adjustment for the t-value probability. Because excess formulation on sticky boards immediately after treatment may cause spurious infection data, the 1st sample after treatment was excluded from analysis. Daily intrinsic natural rates of increase, *r*, were calculated for colony weight by dividing the observed value by the value for the previous sampling occasion and then dividing the logarithm of that ratio by the number of days between the two measurements (7). The *r* values for total adult bee weight and brood surface area were calculated in a similar manner by dividing the post treatment value (5 June) by the pretreatment value (9 May), and then dividing the logarithm of that ratio by the number of days between these two dates (27).

Results

Cfu density at time of treatment was 3.70×10^{10} cfu/g for the Bb05002 + carnauba formulation, 1.79×10^{10} cfu/g for the Bb05002 + candelilla formulation and 1.72×10^{10} cfu/g for the BbGHA + carnauba formulation. Average temperature at the top interior of the brood boxes was 30.0°C (average minimum = 21.4°C and average maximum = 37.8°C).

In the analysis of colony weight r values, treatment ($F_{4,116} = 4.72$, $P = 0.0014$), and date ($F_{4,116} = 25.29$, $P < 0.0001$) were significant factors, but their interaction was not ($P = 0.870$) (Fig. 1). Colony growth in the candelilla alone treatment was significantly greater than the Bb05002 + carnauba treatment ($t_{116} = 3.26$, $P = 0.0144$) and the control treatment ($t_{116} = 4.10$, $P = 0.0008$). Starting the day before treatment, the average total weight gain (s.e.) for colonies treated with Bb05002 + carnauba was 4.8 kg (0.8); for those treated with BbGHA + carnauba was 6.8 kg (2.4); for those treated with Bb05002 + candelilla 6.0 kg (2.3); for those treated with candelilla alone was 11.6 kg (1.8); and for the control colonies was 2.7 kg (4.8). One colony in the candelilla alone group gained 17.8 kg, exceeding by 5.3 kg the next highest colony weight gain; removing that datum reduced the treatment average to 10.0 kg. Treatment with conidia did not significantly affect the changes in surface areas of sealed brood ($P = 0.905$); overall, brood

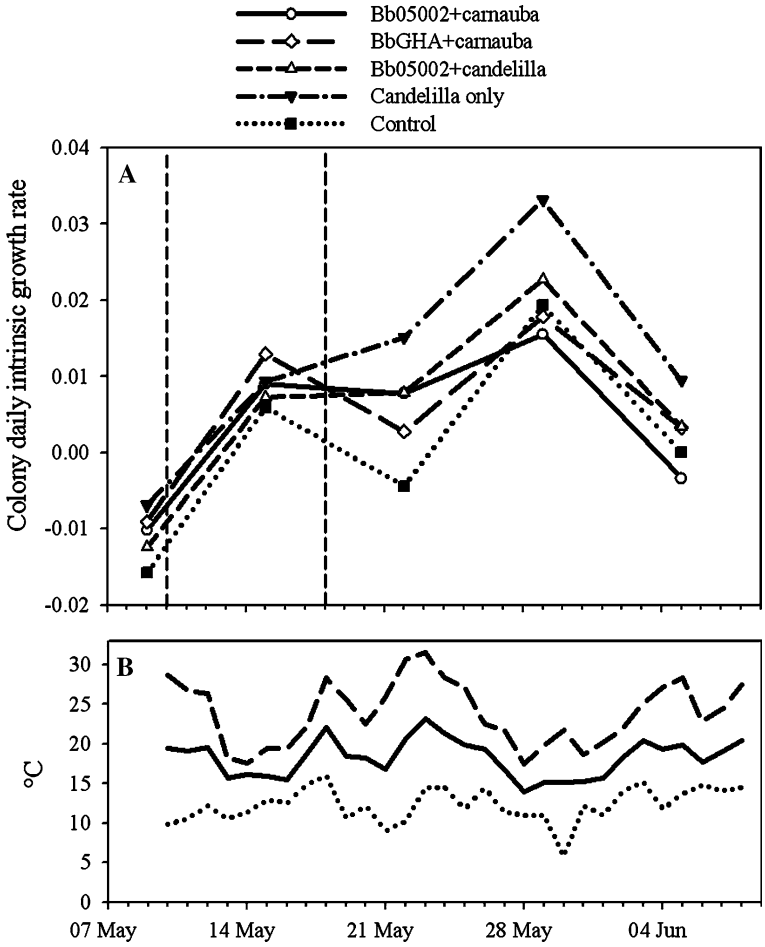

Fig. 1 Daily intrinsic growth for bee colonies treated with *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder (2 treatments), *B. bassiana* isolate GHA conidia + carnauba wax powder (2 treatments), isolate 05002 conidia + candelilla wax powder (1 treatment), candelilla wax powder alone (2 treatments), and untreated control in an experiment conducted in May 2007 near Lattes in southern France. (**a**) Average daily intrinsic rates of increase, r, of bee colonies; (**b**) Daily minimum (dotted line), maximum (dashed line) and average temperature (solid line) (°C). Vertical dashed line shows treatment dates

surface area declined on average by about 1.3% during the course of the experiment. Brood loss was 6.2% (6.0) in the Bb05002 + carnauba treatment, 1.8% (2.2) in the BbGHA + carnauba treatment, 0.6% (0.5) in the candelilla alone treatment and 2.2% (4.0) in the control treatment while hives in the Bb05002 + candelilla treatment gained an average of 2.1% (2.5). No infected brood were observed in any photographs. Treatment did not significantly affect total adult weights ($P = 0.460$); average total adult weights increased by 0.32 kg (0.62) in the Bb05002 + carnauba treatment, by 0.92 kg (0.41) in the BbGHA + carnauba treatment, by 0.24 kg (0.43) in the Bb05002 + candelilla treatment, and by 1.67 kg (0.21) in the candelilla alone treatment; hives in the control treatment lost an average of 0.27 kg (0.81).

In the analysis of mite fall, treatment was significant ($F_{2,245} = 4.94$, $P = 0.0008$) but neither date ($P = 0.999$) nor treatment × date ($P = 0.999$) were (Fig. 2). Post hoc contrasts showed that mite fall in hives treated with 05002 + carnauba was significantly higher than in hives treated with candelilla powder alone ($t_{245} = 4.35$, $P = 0.0002$). No other contrasts were significant. Treatment ($F_{4,160} = 62.45$, $P < 0.0001$), date ($F_{7,160} = 7.50$, $P < 0.0001$) and treatment x date ($F_{28,160} = 1.77$, $P = 0.0150$) were all significant factors in explaining the proportion of infected mites. In post hoc contrasts treatment was a significant factor (P always <0.0001) for all dates through 1 June. Treatment was not significant for mites collected on 5 June, but it was again significant ($P = 0.0027$) for mites collected on 8 June.

The proportion of infected mites in the treatments Bb05002 + carnauba and BbGHA + carnauba were significantly different from zero for all days (P always <0.0001); the same result held for the treatment Bb05002 + candelilla except that the probability varied between <0.0001 and 0.0339 (Fig. 3). Among the hives not treated with spores, the only occasion on which the proportion of infected mites rose to significance occurred the candelilla alone treatment for mites collected on 5 June. At least one infected mite was found in three of the experimental hives during the three weeks before application. Infected mites were also found in hives not treated with conidia, probably due to bee drift or robbing. The infection rate after the second treatment appeared to remain

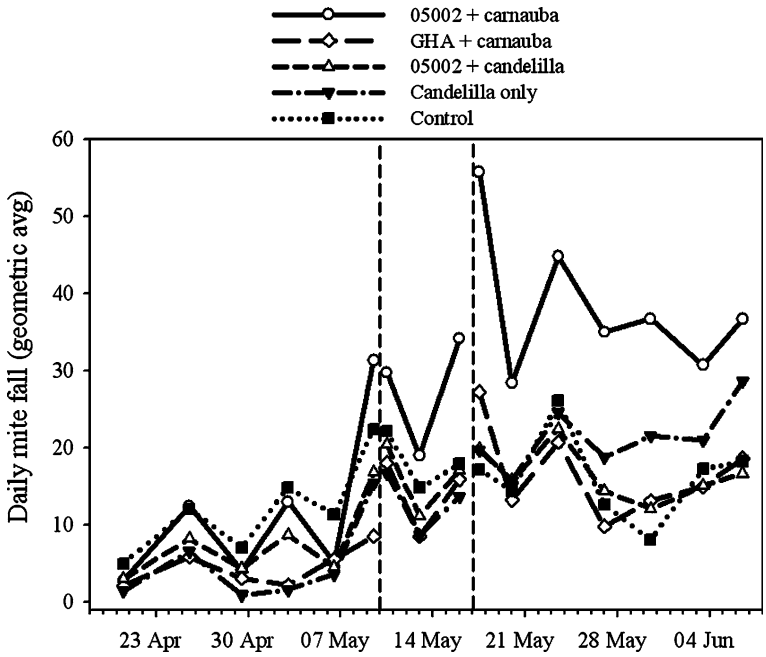

Fig. 2 Daily fall (geometric average of x + 0.01) of mites onto sticky boards placed under bee colonies treated with *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder (2 treatments), *B. bassiana* isolate GHA conidia + carnauba wax powder (2 treatments), isolate 05002 conidia + candelilla wax powder (1 treatment), candelilla wax powder alone (2 treatments), and untreated control in an experiment conducted in May 2007 near Lattes in southern France. Vertical dashed line shows treatment dates

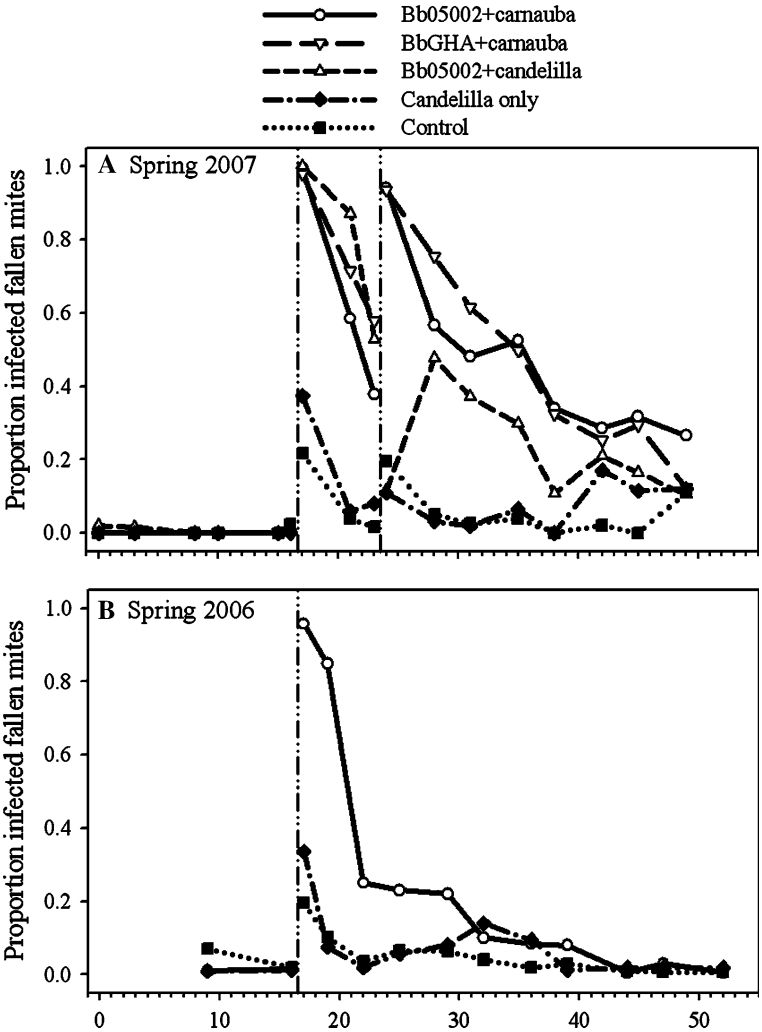

Fig. 3 Proportion of fallen mites that were found to be infected by *B. bassiana* among bee colonies in two experiments near Lattes in southern France. (**a**) colonies treated in Spring, 2007, with *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder (2 treatments), *B. bassiana* isolate GHA conidia + carnauba wax powder (2 treatments), isolate 05002 conidia + candelilla wax powder (1 treatment), candelilla wax powder alone (2 treatments), and untreated control. (**b**) colonies treated in Spring 2006, with one treatment of either *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder or carnauba wax powder alone, and untreated control. Data from Meikle et al. (2007). Vertical dashed line shows treatment days

elevated for a longer time than in hives treated with a single application of Bb05002 + carnauba in Spring, 2006.

The analysis of cfu density was conducted in the same manner as for the proportion infected mites. No *B. bassiana* cfu were observed in any bee samples before application. Treatment ($F_{4,70} = 42.99$, $P < 0.0001$), date ($F_{6,70} = 43.11$, $P < 0.0001$) and treatment × date ($F_{24,70} = 5.59$, $P < 0.0001$) were significant overall (Fig. 4). After

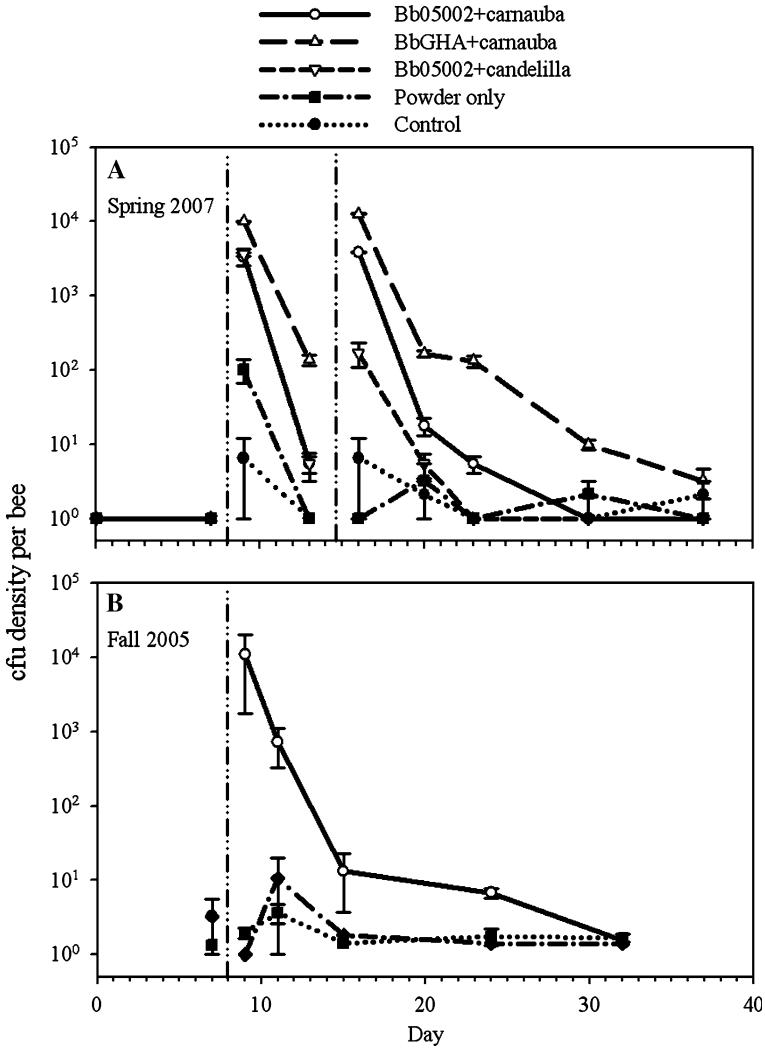

Fig. 4 Density of colony-forming units (cfus) per bee in two experiments in southern France (log scale). (**a**) colonies treated in Spring, 2007, with *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder (2 treatments), *B. bassiana* isolate GHA conidia + carnauba wax powder (2 treatments), isolate 05002 conidia + candelilla wax powder (1 treatment), candelilla wax powder alone (2 treatments), and untreated control. (**b**) colonies treated in Fall 2005, with one treatment of either *B. bassiana* isolate 05002 conidia formulated with carnauba wax powder or carnauba wax powder alone, and untreated control. Vertical dashed line shows treatment days. Data from Meikle et al. (2007)

application, treatment was a significant factor on all days (P varied from <0.0001 to 0.0245) until the samples collected on 1 June, after which treatment was no longer significant. The day after the 1st application, 11 May, cfu densities in the Bb05002 + carnauba ($t_{70} = 10.54$, $P < 0.0001$), BbGHA + carnauba ($t_{70} = 12.81$, $P < 0.0001$) and Bb05002 + candelilla ($t_{70} = 11.33$, $P < 0.0001$) were all significantly different from 0 but in the next sample four days later only the cfu density in the BbGHA + carnauba treatment was significant. In the samples collected just after the 2nd

application on 18 May, all three treatments using *B. bassiana* were significant but by the next sample only the BbGHA + carnauba treatment was significant; it remained so until the last sample on 8 June. The decline in cfu density per bee was very similar to that observed in Fall, 2005, in hives treated with a single application of Bb05002 + carnauba.

Discussion

The goals of this study were to evaluate the effects of two applications of *B. bassiana* conidia, with two different strains and formulated with two kinds of wax powder, on bee colony health and on *Varroa* mite fall. The experiment was conducted in the spring, when brood densities and foraging activity were high and the sensitivity of hives to a perturbation likewise high. While hives are generally not treated against *Varroa* in the spring in southern France (Meikle et al. 2008), this period was chosen because a negative treatment effect would be expected to have a quantitatively greater impact on adult bee and brood populations and/or colony food stores and weight gain. We found no negative effect of application of entomopathogenic fungi on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood, and colony survivorship. Colony growth among all groups was lowest immediately after application, but this was likely due to food consumption prior to a nectar flow. Colony growth increased among all groups thereafter. No treatment differences were observed in either total adult weight change or changes in the amounts of sealed brood or honey. Little impact of *B. bassiana* application in beehives has been observed in similar studies elsewhere (Jaronski et al. 2004; Meikle et al. 2008). While some workers (e.g. Kanga et al. 2003) have collected and plated dead adult bees in an effort to measure bee mortality due to mycosis, this was not done here because it was felt the data would be difficult to interpret properly. As observed here, and by Meikle et al. (2007) and Kanga (2003), adult bees retain *B. bassiana* cfus on their bodies for days and even weeks after application. Since the length of time between a given bee's death, its ejection from the hive and its collection by the researcher is unknown, the presence of sporulation on these cadavers, even with surface sterilization, would not reliably indicate whether the fungus killed the bee.

Treatment was a significant factor in explaining average daily mite fall, and colonies treated with Bb05002 + carnauba had significantly higher mite fall than those treated with candelilla powder alone. However, average mite falls in treated hives were always higher than both controls and candelilla powder alone within the time frame of this study so fungal treatment did not lower mite densities as they were measured here. Mite fall in hives treated with isolate GHA was not significantly different than the control hives. These results should not be considered an indication that GHA could not be effective in this context because the number of replicates per treatment was too low for a definitive answer. Davidson et al. (2003) found that BbGHA was highly virulent against *Varroa* mites in lab bioassays. Further work with different isolates is needed to determine the role that isolate characteristics play in the field control of *Varroa* mites. The powder + conidia formulation apparently distributed well in hives; the cfu densities per bee found here were similar to those found in an earlier experiment by Meikle et al. (2007) as well as by Kanga et al. (2003) who used at least 40 g spores per hive.

While large numbers of fallen mites were infected with *B. bassiana*, they clearly did not all die due to infection. As observed by Meikle et al. (2007, 2008) a mite with viable conidia on its cuticle may fall for other reasons, and in the 3–4 days between board replacement conidia could germinate on the dead or dying mite, resulting in a false positive

because the fungus did not cause the mite to drop. *Beauveria bassiana* conidia grow readily on cadavers (Tanada and Kaya 1993), and surface sterilization of the mites would reliably remove only some of those false positives - those less than 1–2 days old (the time needed by the fungus to establish itself within a mite). Given the low probability of false negatives (in which a mite dies from fungal infection but the cadaver does not sporulate), the proportion of infected mites should be considered upper-bound estimates of the true percentage of infection, and not necessarily related to how well the treatment works against *Varroa*. However, these data can be considered indicators for the presence of viable *B. bassiana* propagules in the hive.

Conidia formulated with candelilla wax was not measurably different, in terms of cfu per bee or proportion infected mites, from those formulated with carnauba wax. This supports the hypothesis that properties the two waxes have in common, such as being hydrophobic and lipophilic, are those properties that are important as formulation ingredients. The group treated once with Bb05002 + candelilla wax powder did distinguish itself in one important regard: although those hives were only treated once, the proportion of infected mites increased significantly in a manner very similar to the hives in the two groups that were treated twice with fungal formulation. Of the 26 hives in the experiment, only seven hives increased in terms of proportion infected mites between 22 and 25 May, that is, between five and eight days after hives in the Bb05002 + carnauba and BbGHA + carnauba groups were treated a second time. Of those seven hives, one was in the BbGHA + carnauba group (increase of 9%), one in the control group (increase of 7%) and the remaining five comprised all the hives in the Bb05002 + candelilla group, with an average increase of 36% (s.e. = 0.06) and a range of 25–59%. The likelihood that the five hives with by far the largest increases in infection rate would randomly turn up in the same treatment group is low: <0.001. That bees with *B. bassiana* propagules on their bodies, as well as infected mites, were found in that treatment group indicate that those bees must have visited treated hives and returned to their colonies. While this could result from bees robbing treated hives, or bee drift, why this would occur among all the hives of one treatment and essentially none of the hives in other treatments is curious.

Spore viability over time was not directly measured in the hives but it is likely that *B. bassiana* can survive there. Aerial conidia are known to tolerate high temperatures (Burges 1998). Although brood mass temperatures range from 33–36°C (Southwick 1991; Winston 1987), temperatures in broodless areas tend to be lower (Simpson 1961). The average temperature on top of the queen excluder between the brood box and the super in this experiment was 30.0°C. Meikle et al. (2008), in an experiment conducted in the same location the previous year but later in the spring (thus with higher ambient temperatures), recorded temperatures at the bottom of the brood box from 30–32°C. Temperatures in this range present little problem for either survivorship or germination of *B. bassiana* conidia. Davidson et al. (2003) observed growth in all seven of their *B. bassiana* isolates at 30°C and in five of those isolates at 35°C, and Fargues et al. (1992) observed growth in all three isolates at 32°C and one of those at 35°C. The isolate used here germinated at 34°C (Meikle et al. 2008), so apparently conditions in much of the hive would not have prevented conidium survivorship or germination. Using a simulation model of conidium longevity (Meikle et al. 2003) based on the relationship between r.h. and conidium moisture content described by Hong et al. (2002), at 35°C the half lives of the eight *B. bassiana* isolates described in Hong et al. (2001) were estimated to be 43–135 d at 40% r.h. and 4–13 d at 70% r.h.

The proportion infected mites in the colonies treated with conidia was significantly higher than in controls for about 18 days after application. Meikle et al. (2008) reported

significantly higher infection rates for less than a week after a single application, while Meikle et al. (2007) observed higher infection more than a month after single applications in two experiments conducted in the fall. Infection half life would have been affected by colony dynamics, weather conditions, and their interaction. A large emergence of young bees would dilute cfu density among bees and mites and thus shorten infection half life as it was measured here.

In these experiments no impact on colony health was observed after two successive applications of formulation containing *B. bassiana* conidia. Two applications of the biopesticide increased mite fall relative to the blank powder treatment but did not reduce mite fall during the four weeks between the first application and the end of the experiment treatment. Future experiments will include more replicates per treatment, to better distinguish any treatment effects, and other measures of mite density, such as number of mites per adult bee, in addition to mite fall onto sticky boards. The results thus far are encouraging, but further work is clearly needed concerning conidia dosage, number of applications, and the ecology of entomopathogenic fungi within the beehive under ambient conditions and colony age structures.

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Topically applied myco-acaricides for the control of cattle ticks: overcoming the challenges

Perry Polar · Dave Moore · Moses T. K. Kairo · Adash Ramsubhag

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Abstract In the absence of commercially viable and environmentally friendly options, the management of cattle ticks is heavily dependent on the use of chemical acaricides. Due to recent advances in production, formulation and application technology, commercial fungus-based biological pesticides (myco-insecticides, myco-acaricides) are becoming increasingly popular for the control of plant pests; however, they have not been used against animal ectoparasites. The literature clearly demonstrates that entomopathogenic fungi are pathogenic to ticks under laboratory conditions. Pasture applications have also shown promise while experiments on topical application have had variable results. These results suggest that major research hurdles still exist especially for the latter. Although literature on ticks and their interactions with entomopathogenic fungi exists, there is not a clear understanding on how this can be influenced by the microenvironment of the cattle skin surface. This paper critically reviews pathogen, tick target and host skin microenvironmental factors that potentially affect pathogenicity of the applied entomopathogen. Factors influencing the route of infection for topically applied myco-acaricides are also reviewed. Major researchable constraints and recommendations are identified and prioritized. In particular, there is the need for basic studies to understand the interaction of entomopathogenic fungi with the components of the skin microenvironment, to identify suitable strains, and to develop improved formulations to overcome the various challenges.

P. Polar (✉)
CABI, Caribbean and Latin America, Curepe, Trinidad and Tobago
e-mail: p.polar@cabi.org

D. Moore
CABI Europe-UK, Bakeham Lane, Egham, Surry TW 20 9TY, UK

M. T. K. Kairo
Center for Biological Control, Florida A&M University, 310 Perry-Paige South, Tallahassee, FL 32307, USA

A. Ramsubhag
Faculty of Science and Agriculture, The University of the West Indies, St. Augustine, Trinidad and Tobago

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Introduction

Ticks are obligate, intermittent, ectoparasites of terrestrial vertebrates, and all species are exclusively haematophagous in all feeding stages (Klompen et al. 1996). Blood loss due to feeding of adult female ticks can result in reduction of live weight gain of cattle (Pegram and Oosterwijk 1990), dry matter intake and milk yield (Jonsson et al. 1998). Approximately 10% of the currently known tick species act as vectors of a broad range of pathogens such as those that cause theileriosis, heartwater, babesiosis and anaplasmosis. Ticks may also cause toxic conditions (e.g., paralysis, toxicosis, irritation and allergy) and direct damage to the skin, due to their feeding behaviour (Latif 2003; Jongejan and Uilenberg 2004). Worldwide, there are numerous species of hard ticks (Ixodidae) within eighteen genera (Barker and Murrell 2004) (Fig. 1). However, the most economically important species fall within the following genera: *Amblyomma*, *Dermacentor*, *Ixodes* and *Rhipicephalus*, including the recently subsumed subgenus *Boophilus* (Barker and Murrell 2004; Kettle 1995). Ticks are likely to become increasingly important due to climate change. Cumming and Van Vuuren (2006) predicted that climate conditions in Africa and the rest of the world will become more suitable for African ticks with 68 of 73 tick species studied estimated to expand in population. The areas under threat include Australia, Latin America, parts of Asia and Europe, the oceanic islands and other countries in similar latitude.

Ticks are intrinsically difficult to control. They lay numerous eggs, resulting in high numbers of host-seeking first instars. In addition different species have at least one or more developmental stage present in the environment, actively seeking hosts or alternative hosts for feeding (Kettle 1995). The introduction of "exotic" livestock breeds, of North

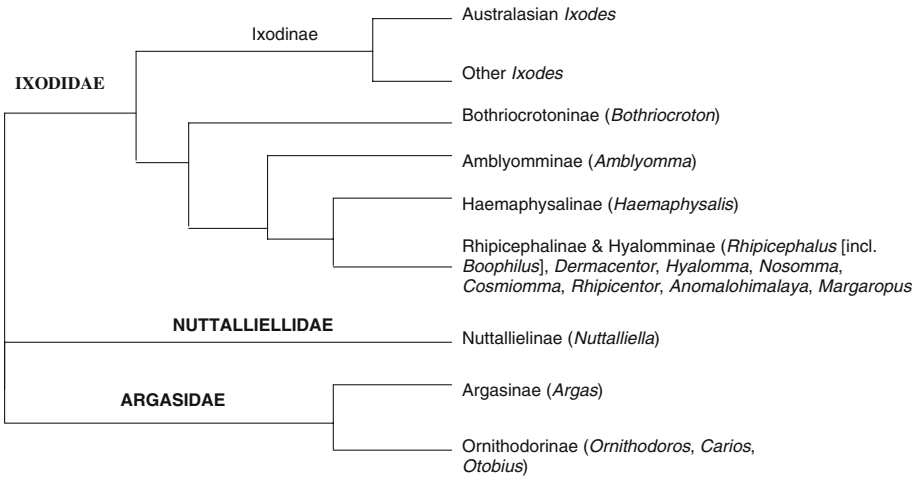

Fig. 1 Working hypothesis of the phylogeny of the subfamilies of ticks (Suborder: Ixodida) based on Barker and Murrell (2004)

American and European origin, with limited natural immunity into many tropical and subtropical areas, has made ticks one of the major constraints to development of a livestock industry in these areas (Graf et al. 2004). Until the middle of the twentieth century, the major acaricides used for tick control were arsenic derivatives, which had low efficacy and residual effect and were highly toxic to cattle (Graf et al. 2004). Over time, a range of acaricides including organochlorines, organophosphates, carbamates, amidines and pyrethroids were developed for tick control. However, use of theses acaricides has resulted in the development of resistance, accumulation of toxic residues in milk and meat, negative effects on the environment and humans, and high production costs (Beugnet and Chardonnet 1995; Jonsson 1997; Latif and Jongejan 2002).

Development of resistance is common in any tick control programme which utilizes acaricides (George et al. 2004). Ticks, such as *Rhipicephalus* (*Boophilus*) *microplus* Canestrini often develop multiple acaricide resistance, which is particularly problematic, since few effective alternative acaricides are available and these are often more expensive (Jonsson 1997). The most effective method to slow the rate of development of resistance is to reduce the number of pesticide treatments to a minimum (George et al. 2004). This can be achieved through the use of geographic isolation (clean zones, quarantine areas, control zones), acaricidal management (prophylactic, threshold or opportunistic) and vaccination (Nari 1995; Latif and Jongejan 2002). Although the use of biological control as an alternative to chemical pesticide application or as a resistance management tool is popular against crop pests, it has not been effectively developed for management of ectoparasites such as ticks. Samish and Rehacek (1999) discussed the potential of using predators, parasitoids and biological pesticides for the control of ticks and concluded that the most promising biological control agents were likely to be entomopathogenic fungi (*Beauveria* and *Metarhizium* spp.), nematodes of the family Steinernematidae and Heterorhabditidae, and birds such as the oxpecker. (For convenience, the term 'entomopathogenic' is used to describe the fungi that kill insects as well as arachnids.)

Biological pesticides based on entomopathogenic fungi (myco-insecticides, myco-acaricides) are well suited to situations where chemical pesticides have been banned or are being phased out, but particularly where resistance to conventional pesticides has developed (Butt et al. 2001). Entomopathogenic fungi invade their arthropod host by penetration through the cuticle using physical and chemical means, and cause death through a combination of actions, which can include depletion of nutrients, physical obstruction, invasion of organs or toxicosis (Inglis et al. 2001; Zimmermann 2007). Therefore, development of resistance is unlikely due to the multiple modes of action. Further, myco-insecticides/myco-acaricides, relative to chemical pesticides, are environmentally benign (Whipps and Lumsden 2001), less expensive to bring to market and capable of imparting more effective control in certain situations (Kooyman et al. 1997; Langewalde et al. 1999).

This review critically examines the current status of knowledge on the control of ticks using myco-acaricides. It also identifies the challenges associated with topical application of myco-acaricides to cattle and potential avenues for improvement.

Status of knowledge on the control of ticks using myco-acaricides

Myco-acaricides have been shown to have the ability to kill ticks under laboratory conditions (Gindin et al. 2002; Polar et al. 2005a), and in recent times there have been efforts to translate these successes to the field. Two strategies for the application of

myco-acaricides against ticks currently being investigated are pasture application (off host) and topical application (on host) to cattle. Research on pasture application has produced promising results. For instance, in studies with *Metarhizium anisopliae* (Metschnikoff) Sorokin, Kaaya et al. (1996) noted substantial mortality of different developmental stages of *Rhipicephalus appendiculatus* Neumann within 5 weeks after application: larvae (100%), nymphs (76–95%) and adults (36–64%). In a similar experiment using *M. anisopliae* and *Beauveria bassiana* (Balsamo), high mortality of larvae (100%), nymphs (80–100%) and adults (80–90%) of *R. appendiculatus* and *Amblyomma variegatum* Fabricius was also achieved (Kaaya and Hassan 2000). Application of *M. anisopliae* and *B. bassiana* to pasture once a month for 6 months reduced the number of *R. appendiculatus* on cattle by 92% and 80%, respectively (Kaaya and Hassan 2000). Commercially, a *M. anisopliae* isolate F52 is registered in the United States as a broad spectrum biological pesticide for the control of ticks, flies, gnats, root weevil and grubs in greenhouses and lawns (Environmental Protection Agency 2002). Pasture application has the potential to keep immature tick populations low and possibly produce epizootics when conditions are favourable for fungal development. Pasture application, however, may require the production of large quantities of conidia and regular application over large areas to achieve control.

A highly effective topically applied myco-acaricide for cattle may directly substitute for chemical acaricide applications. However, it is more likely to be used as a resistance management tool, means of control during the restricted entry intervals prior to slaughter or in organic livestock production. Additionally, relative to pasture application, a smaller quantity of conidia is likely to be used in a confined area (e.g., spray race or dip) and the efficiency of targeting is likely to be greater.

Research in this area has had variable results (Table 1). For instance, in pen trials, de Castro et al. (1997) reported 50–53% reduction of the *R. microplus* population using a single spray of *M. anisopliae* on cattle. On the other hand, Correia et al. (1998) did not observe any significant effect on tick populations in a similar experiment. In pen trials, Rijo-Camacho (1996), reported a 90% reduction in the *R. microplus* population after five weekly treatments with *B. bassiana* and *M. anisopliae* in comparison to only three weekly treatments with *Lecanicillium lecanii* (Zimmerman) Zare & W. Gams. (Note: the species in the genus *Verticillium* have dispersed into other genera, primarily *Lecanicillium*.) However, weekly topical application of *L. lecanii* alone did not result in equivalent control relative to the chemical acaricide in field experiments although it was achieved with both topical and pasture application (Rijo-Camacho 1996). Polar et al. (2005b) and Alonso-Díaz et al. (2007) also reduced tick populations on cattle using weekly topical application of *M. anisopliae* isolates under field conditions. These reports have demonstrated the biological feasibility of using myco-acaricides, but the variability in performance implies that significant research hurdles have to be overcome before a commercial product for topical application to cattle or other domestic animals can be marketed.

Among the major problems previously associated with the use of myco-insecticides for the control of plant pest were: poor quality control standards, inconsistent levels of control and slow speed of kill (Jenkins and Grzywacz 2000; Whipps and Lumsden 2001). However, over the past 20 years knowledge of the biology and ecology of entomopathogenic fungi and host–pathogen interactions has improved (Inglis et al. 2001; Bateman 1997; Thomas 1999; Thomas et al. 1999) and protocols for the production of high quality inoculum have been established (Jenkins and Grzywacz 2000). In addition formulation and application technologies have evolved (Prior et al. 1988; Alves et al. 2002; Bateman and Alves 2000) and it has been shown that many pest situations do not necessarily require

Table 1 Summary of field and pen trial experiments using entomopathogenic fungi for the control of cattle ticks

Reference	Location	Fungus	Treatment	Results
Field trials				
Alonso-Díaz et al. (2007)	Mexico	*Metarhizium anisopliae* MA34	Two groups of ten Holstein × Zebu cattle (18 ± 4 months old) were each sprayed with 5 l of 10^8 conidia/ml *M. anisopliae* MA34 in 0.1% Tween 80 or the control solution every 15 days for four treatments.	Between the 2nd spray and the end of the experiment, the *Rhipicephalus microplus* (4.5–8.0 mm) population on cattle was reduced by 40–91.2%.
Polar et al. (2005b)	Trinidad	*M. anisopliae* IMI386697 and ARSEF3297	Three groups of ten Mixed Holstein cattle (12 months) were each sprayed with 2 l of 10^8 conidia/ml *M. anisopliae* IMI386697 and *M. anisopliae* ARSEF3297 in 2% Newman's Cropspray 11-E or the control solution weekly for 3 weeks.	The *R. microplus* (4.5–8.5 mm) population was reduced by *M. anisopliae* IMI386697 (72%) and *M. anisopliae* ARSEF3297 (36%) relative to the treated control. Confirmed fatal infection of *R. microplus* (4.5–8.5 mm) with IMI386697 (8.7%) and ARSEF3297 (2.7%) were low.
Rijo-Camacho (1996)	Cuba	*Verticillium lecanii* LBVI-2	Three groups of 20 Holstein cattle (approx. 9 months) were each sprayed with 5 l of 3–5 × 10^7 conidia/ml *V. lecanii* LBVI-2 in 1% Tween 80 weekly, the control solution weekly or sprayed with acaricide (cimiazol) biweekly for 3 months. The groups were divided and placed either on pasture sprayed with 600 l/ha at 5–9 × 10^7 conidia/ml (1 kg/ha) 1 week prior to entry of animals or unsprayed pasture.	Pasture treatment alone and pasture and cattle treatment with *V. lecanii* LBVI-2 produced equivalent *R. microplus* (all sizes) reduction as animal treated with the chemical acaricide. Pasture and chemical treatments produced lowest populations of *R. microplus*.

Table 1 continued

Reference	Location	Fungus	Treatment	Results
Pen trials				
Correia et al. (1998)	Brazil	*M. anisopliae* E9	Five groups of three European × Zebu cattle (14 months) were each sprayed with 5 l of 7.5×10^5, 7.5×10^6, 7.5×10^7 and 7.5×10^8 conidia/ml *M. anisopliae* E9 or the control solution (adhesive spreading agent).	No significant change in the *R. microplus* (>4 mm) population over 16 days after single spray.
de Castro et al. (1997)	Brazil	*M. anisopliae* 959	Three groups of three male Holstein × Zebu (134–178 kg) cattle were each sprayed with 5 l of 10^8 and 10^7 conidia/ml *M. anisopliae* 959 in Tween 80 or the control solution.	*R. microplus* (engorged) populations decreased with 10^8 conidia/ml (53.5%) and 10^7 conidia/ml (50.2%) relative to the control.
Rijo-Camacho (1996)	Cuba	*V. lecanii* BVI-2, *M. anisopliae* LBMa-NB and *Beauveria bassiana* LBBb-14	Three groups of five Jersey cattle (9 months old) were each sprayed with 5 l of 3–5 × 10^7 conidia/ml *V. lecanii* LBVI-2, *M. anisopliae* LBMa-NB and *B. bassiana* LBBb-14 in 0.1% Tween 80 every week for 5 weeks.	A 95% reduction of the *R. microplus* (all sizes) population occurred after three sprays of *V. lecanii* LBVI-2 and 5 sprays of *M. anisopliae* LBMa-NB and *B. bassiana* LBBb-14.

rapid kill, the hallmark of chemical pesticides. Despite this progress, little of this knowledge has been translated to the development of myco-acaricides for tick control.

Factors influencing pathogenicity of entomopathogenic fungi to ticks on host

Fungal pathogenicity to a target organism is determined by a variety of factors, including physiology of the host, physiology of the fungus and the environment (Inglis et al. 2001). Although significant information is available on ticks, entomopathogenic fungi, and their interaction (Kettle 1995; Bittencourt et al. 1995a, b, 1997; Chandler et al. 2000; Inglis et al. 2001) little is known about the significance of the skin surface micro-environment on this interaction. This section discusses key pathogen, target and microclimate factors which affect pathogenicity as well as factors relating to the route of infection which affect the ability of the target to obtain a lethal dose.

Pathogen factors

Fungal species

Fungi are a phylogenetically diverse group of eukaryotic organisms that are all heterotrophic, unicellular or hyphal and reproduce sexually or asexually. The current arrangement of the fungi recognises seven phyla (Hibbett et al. 2007): Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Glomeromycota, Neocallimastigomycota and Microsporidia. The Glomeromycota and the Neocallimastigomycota contain no entomopathogenic fungi. Chytridiomycota, Basidiomycota and Blastocladiomycota contain a few entomopathogenic species, but there are no reports of infection in the Acari (Chandler et al. 2000). Many Microsporidia are now considered entomopathogenic fungi.

The Ascomycota have a few species which infect ticks (Table 2) but they are generally unsuitable for myco-acaricide development. For example, *Scopulariopsis brevicaulis* (Saccardo) Bainier is found in soil, stored plant and animal products, insects and ticks (Samsinakova et al. 1974; Yoder et al. 2003; Polar 2007) and is known to cause onychomycosis (fungal infection of fingernails or toenails) in humans (Onions 1966). The yeast *Candida haemulonii* (van Uden & Kolipinski) Meyer & Yarrow was found to cause high pathogenicity in a laboratory colony of the tick *Ornithodorus moubata* Murray; however, this was due to contamination of the blood meal (Loosová et al. 2001).

The zygomycete subphylum Entomophthoromycotina contains important obligate entomopathogens such as *Conidiobolus*, *Entomophthora* and *Neozygites* which normally have narrow host ranges, often cause natural epizootics but are not easily grown in vitro (St. Leger and Screen 2001). Chandler et al. (2000) demonstrated that a number of isolates from these genera infect mites; however, only *Conidiobolus coronatus* (Constantin) Batko was reported to be isolated from a tick species, *Ixodes ricinus* L. This fungus is found in soil and decaying plant debris and is known to be pathogenic to a number of insect species (Kedra and Bogus 2006), but it is known to cause entomophytoramycosis (formation of tumours) in humans (Valle et al. 2001). The mould *Rhizopus thailandensis* (Zygomycete) has demonstrated experimental pathogenicity to *Rhipicephalus sanguineus* Latreille; however, under field conditions the performance was poor (Casasolas-Oliver 1991).

Table 2 Fungi associated with ticks

Fungal species	Ixodid species	Natural infection	Experimental infection
Ascomycota			
Alternaria sp.	*Rhipicephalus microplus*	Monteiro et al. (2004)	
	Rhipicephalus sanguineus	Monteiro et al. (2004)	
Candida haemulonii	*Ornithodoros moubata*	Loosová et al. (2001)	
Curvularia spp.	*R. microplus*	Monteiro et al. (2004)	
	R. sanguineus	Monteiro et al. (2004)	
Rhizopus thailandensis	*R. sanguineus*		Casasolas-Oliver (1991)
Scopulariopsis brevicaulis	*Dermacentor variablis*	Yoder et al. (2003)	
	Ixodes ricinus	Samsinakova et al. (1974)	
	R. microplus	Polar (2007)	
(ex-Deuteromycetes)			
Aspergillus sp.	*Rhipicephalus appendiculatus*	Mwangi et al. (1995) (in Chandler et al. 2000)	
Aspergillus flavus	*I. ricinus*	Cherepanova (1964) (in Kalsbeek et al. 1995)	
Aspergillus fumigatus	*Dermacentor marginatus*		Kolomeic (1950) (in Chandler et al. 2000)
	Hyalomma scupense		Kolomeic (1950) (in Chandler et al. 2000)
	I. ricinus	Cherepanova (1964) (in Kalsbeek et al. 1995)	
Aspergillus niger	*Dermacentor reticulatus*	Samsinakova et al. (1974)	
	D. marginatus	Samsinakova et al. (1974)	
	I. ricinus	Samsinakova et al. (1974)	
Aspergillus ochraceus	*R. microplus*	Polar (2007)	

Table 2 continued

Fungal species	Ixodid species	Natural infection	Experimental infection
Aspergillus parasiticus	*D. reticulatus*	Samsinakova et al. (1974)	
	D. marginatus	Samsinakova et al. (1974)	
	I. ricinus	Samsinakova et al. (1974)	
Aspergillus sydowii	*Amblyomma americanum*	Rogers (1989)	
Aspergillus tamarii	*R. microplus*	Polar (2007)	
Beauveria amorpha	*R. microplus*		Campos et al. (2005)
Beauveria bassiana	*A. americanum*		Kirkland et al. (2004)
	Amblyomma maculatum		Kirkland et al. (2004)
	Amblyomma variegatum		Kaaya et al. (1996)
	Anacentor nitens		Monteiro et al. (1998b)
	D. reticulatus		Samsinakova et al. (1974)
	D. marginatus		Samsinakova et al. (1974)
	I. ricinus	Kalsbeek et al. (1995); Samsinakova et al. (1974)	
	R. appendiculatus		Kaaya et al. (1996)
	Rhipicephalus decoloratus		Kaaya and Hassan (2000)
	R. microplus	Verissimo (1995) (in Chandler et al. 2000); da Costa et al. (2002)	Bittencourt et al. (1997)
	R. sanguineus		Monteiro et al. (1998a); Samish et al. (2001)
Beauveria brongniartii	*I. ricinus*	Kalsbeek et al. (1995)	
Fusarium sp.	*R. microplus*	Monteiro et al. (2004)	
	R. sanguineus	Lombardini (1953); Monteiro et al. (2004)	
Fusarium graminearum	*I. ricinus*	Samsinakova et al. (1974)	
	D. reticulatus	Samsinakova et al. (1974)	
	D. marginatus	Samsinakova et al. (1974)	

Table 2 continued

Fungal species	Ixodid species	Natural infection	Experimental infection
Fusarium napiforme	*R. microplus*	Polar (2007)	
Fusarium proliferatum	*R. microplus*	Polar (2007)	
Isaria farinosus	*I. ricinus*	Kalsbeek et al. (1995)	Polar (2007)
Isaria fumosoroseus	*I. ricinus*	Kalsbeek et al. (1995)	
	R. sanguineus		Samish et al. (2001)
Isaria tenuipes	*R. microplus*	Polar (2007)	
Lecanicillium lecanii	*I. ricinus*	Kalsbeek et al. (1995)	
Metarhizium anisopliae	*A. americanum*		Kirkland et al. (2004)
	A. maculatum		Kirkland et al. (2004)
	Amblyomma cajennense		Souza et al. (1999)
	A. variegatum		Kaaya et al. (1996); Kaaya and Hassan (2000)
	Rhipicephalus annulatus		Gindin et al. (2002)
	R. microplus	da Costa et al. (2002)	de Castro et al. (1997); Correia et al. (1998); Onofre et al. (2001); Bittencourt et al. (1994, 1995a, b); Polar et al. (2005a); Alonso-Díaz et al. (2007); Leemon and Jonsson (2008)
	Hyalomma excavatum		Gindin et al. (2002)
	Ixodes scapularis		Zhioua et al. (1997)
	R. appendiculatus		Kaaya et al. (1996); Kaaya and Hassan (2000)
	R. sanguineus		Monteiro et al. (1998a); Samish et al. (2001); Gindin et al. (2002); Polar et al. (2005a)

Table 2 continued

Fungal species	Ixodid species	Natural infection	Experimental infection
Metarhizium flavoviride var. *flavoviride*	*R. microplus*		Onofre et al. (2001)
	R. sanguineus		Samish et al. (2001)
Penicillium sp.	*R. sanguineus*	Monteiro et al. (2004)	
Penicillium citrinum	*A. americanum*	Rogers (1989)	
Penicillium insectivorum	*I. ricinus*	Cherepanova (1964) (in Kalsbeek et al. 1995)	
Simplicillium lamellicola	*R. microplus*		Polar et al. (2005a)
	R. sanguineus		Polar et al. (2005a)
	I. ricinus	Kalsbeek et al. (1995)	
Trichothecium roseum	*Argas persicus*	Koval (1974) (in Chandler et al. 2000)	
	D. marginatus	Koval (1974) (in Chandler et al. 2000)	
	I. ricinus	Koval (1974) (in Chandler et al. 2000)	
Zaygomycota[a]			
Conidiobolus coronatus	*I. ricinus*	Samsinakova et al. (1974)	

[a] Not a formal phylum, but used as a holding term for fungi to be placed correctly after research (Cannon and Kirk 2007)

A large group of fungi, which lost the ability to, or rarely does, produce sexual structures were formerly placed in the division Deuteromycota within the class Hyphomycetes (Inglis et al. 2001). Although both these terms are now obsolete with almost all the species placed at least presumptively within the Ascomycota, for purposes of this review the term Deuteromycete is retained, as it is one which insect pathologists are most familiar with. Many Deuteromycetes are facultative pathogens which generally have a broad host range but are commonly used in biological pesticides due to ease of mass-production and pathogenic properties (St. Leger and Screen 2001). Kalsbeek et al. (1995) stated that the Deuteromycetes are the only fungi which have been isolated from ticks and there are only a few exceptions to this general rule (Table 2). Some Deuteromycete genera which have been isolated from ticks are unsuitable for development as myco-acaricides due to safety reasons. For example, *Aspergillus* is known to cause respiratory diseases in humans, birds, domesticated animals and many other animal species (Smith 1989). Other groups are unsuitable as they are weak pathogens. For example, *Fusarium* is known to contain a complex of around three entomopathogenic species: *Fusarium coccophilum* (Desm.) Wollenweber & Reinking, *Fusarium larvarum* Fuckel and *Fusarium juruanum* P. Henn., which are strong pathogens of diaspidid scale insects (Evans and Prior 1990), but weak pathogens or saprothrops of the Acari (Chandler et al. 2000).

Common genera used in biological control of insects include *Metarhizium*, *Beauveria*, *Lecanicillium* and *Isaria* (Butt et al. 2001). (Note: Fungi of *Paecilomyces* were placed in the *Paecilomyces* section *Isarioidea* Samson and equated with *Isaria*.) These genera have also been shown to contain species which are strongly pathogenic to a range of tick species. *Beauveria bassiana* and *M. anisopliae* are the most commonly used species for experimental work, but there are only a few cases where they have been isolated from ticks (Table 2). *Lecanicillium* has only one entomopathogenic species, *L. lecanii*, which is reported to kill ticks, mites and other insects (Chandler et al. 2000). The 12 species currently placed in *Paecilomyces* section *Isarioidea* (although not all have corresponding names in *Isaria*) are facultative pathogens of insects (especially Coleoptera and Lepidoptera) and of these, only *Isaria farinosa* (Holmsk.) Fr. and *Isaria fumosorosea* Wize are reported to infect the Acari (Chandler et al. 2000). However, Polar (2007) has recently isolated *Isaria tenuipes* Peck from *R. microplus*.

Host specificity of isolate

Registration of any fungal isolate for commercial use requires documentation on host specificity, in order to assess potential impacts on non-target organisms including safety to humans. The host range of individual isolates within a species may be quite variable. The tick pathogenic isolate *M. anisopliae* ARSEF3297 demonstrated varied levels of pathogenicity to several non-target orders such as Orthoptera (*Coscineuta virens* Thunberg), Hymenoptera (*Anagyrus kamali* Moursi, *Polistes* sp.), Coleoptera (*Callosobruchus maculatus* Fabricius) and Hemiptera (*Maconellicoccus hirsutus* Green) but was not pathogenic to Araneae (spiders of the family Mecicobothiidae) or other Hymenoptera (*Azteca* spp.) (Polar 2007). Ginsberg et al. (2002) similarly found broad physiological host range characteristics with a tick pathogenic *M. anisopliae* isolate. This isolate exhibited pathogenicity to mole crickets (*Acheta domesticus* L.) and ladybird beetles *(Hippodamia convergens* Guérin-Méneville), but marginal pathogenicity to milkweed bugs (*Oncopeltus fasciatus* Dallas). The factors influencing host range between isolates are complex, but

variability in the secretion of proteases and chitinolytic enzymes during the infection process has been shown to be important (Freimoser et al. 2003).

The fact that some isolates exhibit broad physiological host ranges does not necessarily imply that the ecological host range found in nature would be similarly broad. Goettel et al. (1990) cited literature indicating that entomopathogenic fungi exhibited narrower host ranges under field conditions, compared to laboratory conditions that may be more suitable for the pathogen. Careful selection of isolates for narrow ecological host ranges can reduce impacts on non-target organisms. Selected *B. bassiana* isolates have been applied to control pine caterpillars (*Dendrolimus* sp.) in areas used to rear silkworm (*Bombyx mori* Steinhaus) with no adverse effect (Butt et al. 2001). In fact, Butt et al. (1998) used honeybees to vector *M. anisopliae* for the control of the pollen beetle, *Meligethes aeneus* Fabricius, while the bumble bee, *Bombus impatiens* Cresson, has successfully vectored *B. bassiana* (Al-Mazra'awi et al. 2006). Utilizing isolates with a broad ecological host range is not without merit. Thus, a myco-insecticide, capable of infecting a wider range of arthropod pests of domestic animals (ticks, mites, flies) without the toxic side effects to humans or animals associated with chemical pesticides, could be desirable.

Origin of isolate

It is commonly assumed that an isolate is more pathogenic to the host from which it was isolated as compared to a new, unrelated host (Goettel et al. 1990). However, there are several instances where isolates derived from ticks have been found to be less pathogenic in comparison to isolates from non-tick hosts. Monteiro et al. (1998a, b) found that the *M. anisopliae* isolate (Ma319), of ant origin, was more effective than the tick isolate (Ma959) in terms of mortality to larvae and inhibition of egg hatching in *R. sanguineus*. Additionally, *I. fumosorosea*, which originated from *Rhipicephalus annulatus* (Say), was not as pathogenic to *R. annulatus* in comparison to strains of non-tick origin (Gindin et al. 2001).

Virulence

High concentrations of conidia (10^8–10^9 conidia/ml) used in laboratory bioassays produce significant mortality in cattle ticks. However, pathogenicity often declines rapidly as concentrations are reduced (Frazzon et al. 2000; Benjamin et al. 2002; Polar et al. 2005a), which implies that virulence of the isolates may not be high. Considering the small size of tick development stages, and difficulty in targeting them on the cattle surface, only few conidia are likely to attach to the target; thus there is a need to identify highly virulent isolates. In studies with *Schistocerca gregaria* (Forskål), Prior et al. (1995), using *M. anisopliae* var. *acridum*, calculated that a dose of 500–5000 conidia/insect caused 95% mortality in 6–7 days. However, minimum lethal doses for various developmental stages in ticks have not been calculated.

Although mortality is desirable, sublethal effects can contribute significantly to control efforts as they often affect reproduction and hence the future population size in the field. Reported sublethal effects due to entomopathogenic fungi on ticks generally influence reproductive parameters such as post engorgement weights, oviposition period, weight of egg mass, larval eclosion period and eclosion (Monteiro et al. 1998b; Onofre et al. 2001; Samish et al. 2001; Hornbostel et al. 2004).

Target factors

Tick species

Natural and experimental infection, by entomopathogenic fungi, has been demonstrated in a number of tick species including: *Argas persicus* Oken, *Amblyomma americanum* L., *Amblyomma cajennense* (Fabricius), *Amblyomma maculatum* Koch, *A. variegatum*, *Anacentor nitens* Neumann, *R. annulatus*, *Rhipicephalus decoloratus* Koch, *R. microplus*, *Dermacentor variablis* Say, *Dermacentor reticulatus* Fabricus, *Dermacentor marginatus* Sulzer, *Hyalomma excavatum* Kock, *Hyalomma scupense* Olenev, *I. ricinus*, *Ixodes scapularis* Say, *R. appendiculatus* and *R. sanguineus* (Table 2).

In many instances, the geographic range of tick species of economic importance overlap (Olwoch et al. 2003); hence, the ability to kill several tick species using a single isolate myco-acaricide would be ideal. There are few studies which demonstrated the pathogenicity of isolates to more than one tick species. Gindin et al. (2002) demonstrated significant variation in pathogenicity between *R. annulatus*, *R. sanguineus* and *H. excavatum* using isolates of *M. anisopliae*, *M. flavoviride* Gams & Roszypal, *B. bassiana*, *P. fumosoroseus* and *L. lecanii*. Kaaya and Hassan (2000) also demonstrated high mortality in *A. variegatum* and *R. appendiculatus* in experiments with *M. anisopliae* and *B. bassiana* isolates. Polar et al. (2005a) found that *R. sanguineus* was less susceptible to *M. anisopliae* ARSEF3297 in comparison to *R. microplus*.

Developmental stage

Not all stages of an insect's life cycle are equally susceptible to infection by entomopathogenic fungi (Butt and Goettel 2000); the same appears to be true for ticks. In several tick species, all development stages have been shown to be susceptible to entomopathogenic fungi to varying degrees. Bittencourt et al. (1994) demonstrated the inhibition of egg eclosion and larval mortality in *R. microplus* using isolates of *M. anisopliae*. Gindin et al. (2002) demonstrated that an *M. anisopliae* isolate induced mortality to varying extents, in engorged females, males, nymphs and larvae of *B. annulatus*, *R. sanguineus* and *H. sexcavatum*. It also reduced fecundity and egg viability in the same species. Samish et al. (2001) compared a few isolates of four fungal species (*B. bassiana*, *M. anisopliae*, *M. anisopliae* var. *acridum* and *I. fumosoroseus*) on *R. sanguineus* and found that unfed larvae and nymphs were more sensitive to fungal infection than engorged ones and unfed adult females were less sensitive than engorged females. The ability of fungi to kill both immature and mature stages of ticks is important as major tick-borne diseases are transmitted by the younger stages such as larvae and nymphs while engorging females cause blood loss and loss of productivity (Pegram and Oosterwijk 1990; Kettle 1995).

Finally, in insects, moulting may result in the loss of inoculum on the exuviae (Butt and Goettel 2000). This phenomenon has not been investigated in ticks where it is also likely to limit infection in a similar manner, particularly in one-host ticks with short life cycles, such as *R. microplus,* where moulting may occur before penetration of the germ tube.

Anatomy

In theory, ticks should be good hosts for fungal pathogens particularly in the engorged state when the integument is stretched (Kalsbeek et al. 1995). Bittencourt et al. (1995a, b)

demonstrated that infection of *R. microplus* by *M. anisopliae* is fairly rapid with hyphae appearing in the internal organs 3 days post-infection. Polar et al. (2005a) questioned whether ticks, relative to insect species, are indeed anatomically favourable to fungal infection considering the high concentrations of conidia which are often needed to induce mortality in vitro. The high degree of sclerotization in the integument of ixodid ticks (Evans 1992) may make fungal penetration and colonisation difficult in vivo. Additionally, water availability for the germination of conidia needs to be considered. Insects lose water through the spiracles during respiration and via faeces and saliva (Rourke and Gibbs 1999). Moore et al. (1997) suggested that, in locusts, water for germination of the conidia may be absorbed directly from the cuticle or from a boundary layer around the insect. However, the structure of the tick integument is highly impermeable, restricting water loss from the body (Evans 1992), thus water for germination of conidia on the tick surface may not be as readily available, unless a humid boundary layer occurs to supply moisture. In argasid ticks, excess water is eliminated via the coxal apparatus but no such structure exists in ixodid ticks (Kettle 1995). Little urine is secreted by the Malpighian tubules and excess fluid is eliminated by salivation passed back into the host, and hence may not be available for germination of conidia.

Life cycle

Ticks have highly variable life cycles and feeding patterns (Kettle 1995). The majority of ixodid ticks are three-host ticks where the larvae, nymphs and adults fall off the host after feeding, while in one-host ticks (e.g., subgenus *Boophilus* and *Margaropus* spp.) the developmental stages remain attached to the same individual host (Kettle 1995). As such, one-host ticks are more likely to be effectively targeted by periodic topical application, resulting in more effective control compared to three-host ticks which may spend up to 90% of their time off the cattle host.

Location on host

The preferred feeding location of the ticks is also important in a myco-acaricide application strategy. The nymphs and larvae of *R. microplus* may wander about the host while the adult females often attach on the neck, flank, brisket, inguinal region and escutcheon of the host (Kettle 1995). This suggests that *R. microplus* can be targeted easily through spraying or dipping. However, immature *Rhipicephalus evertsi* Neumann occur deep inside the ear which makes targeting more difficult, although Kaaya et al. (1996) suggested that the high humidity of the ear may assist pathogenesis while the lack of ultra violet light may favour persistence of the fungus. Targeting methods should also consider that after moulting ticks move around, thus increasing the probability of coming into contact with conidia of an applied myco-acaricide.

Host skin microenvironment

Anatomy

Cattle skin is divided into the epidermis and the dermis from which the hair follicles which produces the coat arise (Lloyd et al. 1979a; Jenkinson 1992). The hairs vary between animals in terms of length, diameter and the number per cm^2. For example the pig has a

sparse coat composed of large hairs (10–20 per cm^2) while cattle have a denser coat of finer hairs which may exceed 2000 per cm^2 in some breeds (Jenkinson 1992).

The intact skin provides a barrier against disease-causing organisms and environmental challenges (Wikel 1996). The cattle coat acts as the first line of defence by physically preventing colonisation by microbes in the environment (Jenkinson 1992). An entomopathogen applied to cattle is likely to face some aspects of the innate immune defences (i.e., non-specific factors on skin) more so than the acquired immune response as entomopathogens are not normally invasive in mammalian tissue. Additionally, conidia which land on the tick target may be affected differently by the innate immune defences compared to conidia landing on the skin surface.

Skin temperature

Temperature is a key factor influencing entomopathogen efficiency. Increasing temperature above 25–30°C generally reduces germination of conidia although some isolates are more resistant to temperature than others (Moore and Morley-Davies 1994; Morley-Davies et al. 1996). Brooks et al. (2004) demonstrated that increasing temperature from 28 to 37.5°C reduced *M. anisopliae* infection of the ectoparasitic mite *Psoroptes ovis* Hering.

Skin surface temperatures of cattle vary with environmental temperature (Wolff and Monty 1974). Monty and Garbareno (1978) found that surface temperature fluctuations of the thorax of Holstein-Friesian cattle in Arizona (USA), under normal shaded conditions, ranged from 28 to 40°C. These temperatures are higher than the optimum for germination, growth and pathogenicity of most entomopathogens (20–25°C) (Inglis et al. 2001). Polar et al. (2005b) demonstrated that different locations on cattle in Trinidad ranged from 28 to 41°C with the temperature of the udder, where *R. microplus* are most prevalent, fluctuating diurnally from 31 to 35°C.

Selection of isolates, which can survive and grow at temperatures similar to those which exist on mammalian skin, assuming the temperature in the tick and on the skin are similar, is important to the development of myco-acaricides for topical application to cattle. This may even be important for pasture application, where infected immature stages may attach to host to continue development. Tick pathogenic isolates are often selected in bioassays at standard laboratory temperatures (25–27°C) (Bittencourt et al. 1994; Gindin et al. 2001). However, these isolates are not necessarily pathogenic at the higher temperatures found on the cattle surface. Polar et al. (2005b) compared two *M. anisopliae* isolates (IMI386697 and ARSEF3297) in bioassay conditions mimicking the skin temperature of cattle (31–35°C, fluctuating in a 12 h cycle) and at a more traditional bioassay temperature (28°C, constant). At 28°C, both isolates produced similar pathogenicity to *R. microplus*, but under conditions mimicking the skin temperature, IMI386697, which had a higher optimum temperature in its growth profile, was more pathogenic. In field studies, after three weekly sprays, IMI386697 had reduced the tick population on cattle by 72%, while ARSEF3297 produced a 36% reduction, in comparison to the control. Leemon and Jonsson (2008) subsequently evaluated 31 Australian *M. anisopliae* isolates for their growth between 20 and 40°C and pathogenicity to *R. microplus* although bioassays were carried out at 25°C.

Fungal isolates which can grow at mammalian temperatures are generally avoided due to the potential risk of human infection. Burgner et al. (1998) reported disseminated infection by *M. anisopliae* in a 9-year-old boy undergoing chemotherapy for lymphoblastic leukemia. This isolate was found to exhibit limited growth at 35–37°C and can be considered temperature tolerant. Revankar et al. (1999) also suggested that *M. anisopliae* was

responsible for two cases of sinusitis in immuno-competent hosts; however, laboratory studies indicated that these isolates grew best at 25°C, but were unable to grow at 35°C. Human and animal infections by *M. anisopliae* are rare even in immuno-incompetent hosts. There is insufficient evidence to conclude whether temperature tolerant isolates are more dangerous to humans than isolates that have lower temperature tolerance. The scenarios suggest that the fungi of the above studies (Burgner et al. 1998; Revankar et al. 1999) were opportunists and although the fungi were present they were not necessarily responsible for the conditions observed. Additionally, there were no reports that the fungi were associated with exposure to myco-insecticides. Although myco-insecticides have an excellent safety record, consideration to the peculiar features of high temperature tolerant isolates will be necessary, in addition to the standard safety assessments.

Coat humidity

High ambient relative humidity is known to favour mortality as it allows for greater germination of conidia (Marcandier and Khachatourians 1987). However, it is now believed that the relative humidity of the microenvironment is more critical than the ambient relative humidity with regards to mortality (Inglis et al. 2001). The relative humidity in the cattle coat is influenced by both temperature of the hair and vapour pressure (Allen et al. 1970). The moisture content of the cattle coat can range from 5.8 to 27.5% and this can be influenced by the cattle breed and environmental factors (Allen et al. 1970). The study also indicated that in hot environments the relative humidity of the cattle coat could be up to 5.5% above the average moisture content. Additionally, a stable humidity is likely to be maintained as the cornified squames (flat cells) are hygroscopic and capable of absorbing 3–4 times their own weight in water from the atmosphere (Jenkinson 1992).

Availability of water to support germination of conidia on the tick surface has already been discussed as a potentially limiting factor above. Since the humidity within the cattle coat is not high enough for optimal germination of conidia it may possibly limit pathogenesis. However, the ability of skin and hair to retain moisture, thus providing a more stable humidity, may be more important in favouring pathogenesis. The impact of skin humidity on the pathogenesis thus remains unclear and requires further study.

Skin pH

The pH of the cattle skin varies depending on location and age (Jenkinson and Mabon 1973). The muzzle (6.4) and teats (6.13) of Ayrshire cattle had higher pH values relative to other parts on the body. The skin pH of young heifers ranged from 5.0 to 7.6, most frequently in the range 5.6–6.0, while that of adult cattle ranged from 4.5 to 7.6, but most frequently in the range of 5.0–5.5. Increasing temperature and humidity did not have an effect on skin pH.

Fungal development is generally favoured by alkaline pH and the acidic environment may affect performance of applied entomopathogens. St. Leger et al. (1999) cites literature, which indicates that *M. anisopliae* can grow over a pH range of 2.5–10.5 but specific isolates are likely to have more restricted ranges. In complex environments, such as soil, the effects of pH are not well understood, although there are a number of studies demonstrating none or minimal effects of soil pH on the distribution of entomopathogenic

fungi (Inglis et al. 2001). Skin pH is known to affect skin microflora (McBride 1993) which may have possible antagonistic or synergistic effects on the applied entomopathogen.

Skin secretions

Sebum. Sebum, a viscous/oily substance is primarily produced by a process of lipogenesis from live cells rather than holocrine glands as is the case for most mammals and its secretion is influenced by sex and season (Smith and Jenkinson 1975a, b; Jenkinson 1992). Sebum exists as an emulsion in the convex amorphous material (CAM) found on the margins of the epidermal squames in the interfollicular region of the cattle skin, and coats the hair shaft immediately above the skin, but does not flow across the skin surface (Jenkinson and Lloyd 1979). It may harden to form a sealant in the spaces between the squames and form a physical barrier to microorganisms at the surface cell margins and may regulate water flow through the corneum (Jenkinson and Lloyd 1979). The composition of lipids in sebum is complex and includes: chlosteryl esters (3%), wax diesters Type I (37.7%) and Type II (7.9%), wax triesters (29.9%), triglycerides (3.6%), 2-lyso diesters Type I (2.0%) and Type II (1.6%), lysotriesters (1.6%), free fatty alcohols (0.6%), 1-lyso diesters Type II (4.9%), cholesterol (4.0%), free fatty acids (2.3%) and an unidentified class (2.0%) (Downing and Lindholm 1982). Sebum composition in the sebaceous gland is similar to skin surface lipids, except that the sebaceous glands contain a higher proportion of phospholipid and unesterified fatty acid and a lower proportion of triglyceride and free cholesterol (Smith and Ahmed 1976; McMaster et al. 1985).

Sebum lipids impart a disinfecting activity on the skin surface and the free fatty acids of sebum are responsible for this property, with regard to bacteria (Wille and Kydonieus 2003). Smith and Ahmed (1976) reported that linoleic acid is a major constituent of the triglyceride component of the sebum on the surface (17.7%) and the glands (10.2%) and has antimicrobial properties. Additionally, myristic, palmitic and oleic acids found in bovine sebum are also known to be bacteriostatic and even bacteriocidal. Palmitoleic acid from human sebum, which is also present in bovine sebum, was found to be bactericidal to gram positive bacteria (*Staphylococcus aureus* Rosenbach, *Staphylococcus pyogenes* Rosenbach and *Corynebacterium* sp.) but not to *Candida albicans* (C. P. Robin) Berkhout (yeast) and gram negative bacteria such as *Escherichia coli* (Migula) Castellani & Chalmers, *Enterobacter saerogenes* Hormaeche & Edwards, *Klebsiella pneumoniae* (Schroeter) Trevisan and *Propiobacterium acne* (Gilchrist) Douglas & Gunter (Wille and Kydonieus 2003). Palmitoleic acid was also found to inhibit the adhesion of *C. albicans* to the stratum corneum (Wille and Kydonieus 2003).

Sebum potentially affects fungal germination. Barnes and Moore (1997) demonstrated that caprilic (C-8) and capric (C-10) fatty acids are inhibitory to germination of *M. anisopliae* while stearic acid overcame the inhibition. Sebum from cattle skin washing was found to have 14.3% stearic acid (C-18) (McMaster et al. 1985). However, it is unclear if capric or caprilic acid is present. Downing and Lindholm (1982) indicated that the majority of aliphatic components in cattle sebum are above C12, however, a C10 fatty acid component was reported. Both capric acid (0.3%) and stearic acid (0.2%) are present in wool wax of sheep (Weitkamp 1945), but it is not known if they are in cattle sebum.

Sweat. Cattle sweat also has the potential to influence germination of fungal conidia. Cattle sweat contains a range of ions (e.g., sodium, potassium, magnesium, calcium, chloride and phosphorus), lactate, 3-methoxy-4-mandelic acid, proteins and corticosterols (Mabon and Jenkinson 1971; Jenkinson et al. 1974a, b; Jenkinson and Mabon 1975; Lloyd et al. 1977).

Increasing temperature also increased the nitrogen, sodium and potassium content of sweat (Singh and Newton 1978; Jenkinson and Mabon 1973; Jenkinson et al. 1974b).

Soluble proteins in cattle sweat, particularly immunoglobin A and transferrin are known to play a role in the immune response against microorganisms (Jenkinson et al. 1979). Jenkinson et al. (1974b) suggested that the increased nitrogen from sweat could increase bacterial growth on the skin surface. A similar effect may occur with fungi, as Li and Holdom (1995) demonstrated that increased nitrogen could increase fungal growth in vitro. The skin microflora is known to coincide with the distribution of surface sebum and sweat emulsion which is a likely nutrient source (Lloyd et al. 1979b).

Skin microflora

The microbial population found on the cattle skin is present in the outer layers of the stratum corneum and in the hair follicle infundibulum (Lloyd et al. 1979b). This population consists mainly of mixed microcolonies of coccoid and rod shaped bacteria and, occasionally, yeast and filamentous fungi are also observed (Lloyd et al. 1979b). The skin microbial population is highly specialised and only a limited number of inhabitants are capable of continued growth and development (Jenkinson 1992). Non-resident pathogenic bacteria face not only the skin's defence mechanism, but intense biological competition (Jenkinson 1992). This may also influence the survival of an applied entomopathogen.

Ticks which are reported to have shown natural infection by fungi have been collected from soil or vegetation (Kalsbeek et al. 1995; Samsinakova et al. 1974; da Costa et al. 2002) and directly from animals (Polar 2007; Kalsbeek et al. 1995). As ticks are intermittent ectoparasites with at least one developmental stage in the natural environment, it remains unclear if ticks are infected by fungi on the cattle surface, either as part of the natural flora or as contamination, or if their immature stages pick up pathogens solely, or mostly, from the natural environment. The latter is probably more likely, considering the relative rarity with which entomopathogenic fungi have been recorded from cattle skin. In skin scrapings of ruminants, the fungal dermatophytes *Trichophyton mentagrophytes* (Robin) Blanchard, *Trichophyton rubrum* (Castell.) Sabouraud and *Microsporum gypseum* (Bodin) Guiart & Grigorakis have been isolated (Mitra et al. 1998), but none of these organisms have been isolated from ticks. However, non-dermatophyte fungi including *Alternaria* spp., *Aspergillus* spp., *B. bassiana*, *Curvularia* spp. and *Penicillium* spp. have been isolated from the skin of cattle, goats and sheep (Mitra et al. 1998; Aquino de Muro et al. 2003) and similar fungi have been isolated from ticks (Samsinakova et al. 1974; da Costa et al. 2002; Monteiro et al. 2004).

It is of interest that, from the literature reviewed, there are no entomopathogenic fungi isolated from permanent ectoparasites, (i.e., those with no developmental stage in the natural environment) such as lice (Phthiraptera) and mites of the Sarcoptidae, Psoroptoidae and Analogoidea, of warm blooded animals. All the fungal infected mites reported in Chandler et al. (2000) and Van der Geest et al. (2000) were phytophagous or soil associates and may have picked up the fungi from the wider environment. The one possible exception cited by Van der Geest et al. (2000), *Hirstionyssus* sp., was infected by an unknown Laboulbeniales (Ascomycota) which may have been a rodent ectoparasite such as *Hirstionyssus isabellinus* Oudemans (Baker et al. 1956). This suggests that skin microflora or contaminants may not be contributing significantly to infection of permanent ectoparasites possibly because the skin microenvironment may be hostile for infection. It should be considered that entomopathogenic fungi capable of surviving on the cattle surface may be highly effective because the target organisms may not have developed natural immunity.

Routes of infection

Mortality of a target depends on the organism picking up a lethal dose of the entomopathogen. In field studies with grasshoppers and locusts, three distinct routes of fungal infection were identified: (a) direct impaction of the target with spray droplets, (b) secondary pick-up by the target (residual infection) of spray residues from vegetation and soil, and (c) secondary cycling of the pathogen from individuals infected from the first two modes (Bateman 1997; Bateman and Chapple 2001). The extent to which the three routes contribute overall tick mortality from an applied pathogen on cattle is likely to vary due to the peculiarities of the cattle skin microenvironment.

Direct impaction

The hair density and length in the cattle coat varies between cattle breeds, season and other environmental effects (Berman and Volcani 1961; Steelman et al. 1997). The nature of the cattle coat is likely to limit the penetration of applied conidia thus limiting contact with ticks on the skin surface. Formulation and application techniques are likely to strongly influence the contribution of direct impaction to overall mortality.

Secondary pick-up

Residual infection can also make a significant contribution to overall mortality. Thomas et al. (1997) stated that in situations where direct impaction of the target with entomopathogen is limited, secondary pick-up is essential for effective control. In field experiments, 40–50% of the total infection of the grasshopper *Hieroglyphus daganensis* Krauss resulted from residual infection.

Residual infection is influenced by initial infectivity, persistence (Thomas et al. 1997) and availability of conidia. Polar et al. (2005c) demonstrated that emulsifiable adjuvant oils (e.g., Newman's Cropspray 11-E, Codacide oil) increased pathogenicity in laboratory bioassays when directly applied to ticks compared to Tween 80 formulations. However, it should be considered that emulsifiable adjuvant oils may cause conidia to be too strongly bound to hair, limiting availability to transfer to the target. Alternatively, conidia too loosely bound may become easily dislodged by movement of animals or rainfall.

Pre-soaking of conidia is one method of improving initial infectivity. Dillon and Charnley (1985) demonstrated that pre-soaking can reduce the time to germination of conidia. Pre-soaking has been shown to increase pathogenicity of *M. anisopliae* to *Manduca sexta* L. (Hassan et al. 1989) but not to *S. gregaria* (Moore et al. 1997). Further study is required to determine if pre-soaking can improve pathogenicity in ticks.

Prolonging field persistence of the conidia may improve the performance of the fungus in the field as there is a higher probability of the target encountering the entomopathogen (Inglis et al. 2001) as well as increasing the chances of circumstances more favourable for infection. There are few studies that have attempted to measure persistence of applied entomopathogens on cattle. Kaaya et al. (1996) recovered Colony Forming Units (CFUs) from inside the ears of cattle up to 3 weeks and 1 week with *M. anisopliae* and *B. bassiana* applications, respectively. Polar (2007) recovered CFUs of *M. anisopliae* from the escutcheon of treated cattle using Sabouraud Dextrose Agar-Yeast plates with 100 μg/l dodine (Lui et al. 1993). The number of CFUs declined rapidly between 24 and 48 h and

there was limited recovery 72 h after application. This suggests that time which conidia can persist on cattle may be relatively short and may limit residual infection.

Several factors which either encourage death or germination of conidia may influence persistence of conidia. The UVB portion (285–315 nm) of solar radiation has been shown to be detrimental to conidial persistence (Moore and Morley-Davies 1994; Hedimbi et al. 2008, this issue). Moore et al. (1996) cited literature indicating that *M. anisopliae* has a half life of 110–360 min when exposed to UV radiation (simulated sunlight) and demonstrated the detrimental effects of UV radiation on conidial persistence increased with increasing temperature. These laboratory results were not replicated in the field where persistence was much greater, presumably because many conidia were shielded from direct sunlight, perhaps by their location on the vegetation. Little is known about the tolerance of an entomopathogen to sunlight on the insect body, as it is assumed that penetration occurs within 24 h in most insects (Inglis et al. 2001), but again it is likely that avoidance to the damaging UV rays is important for persistence. The cattle coat is likely to reduce UV damage, however, no studies have been conducted to measure the UV levels within the cattle coat. The effect of other factors described above (e.g., skin secretions, microflora) are likely to influence persistence but knowledge in this area is limited.

Secondary cycling

Secondary cycling is unlikely to contribute to overall infection on the cattle surface as infected ticks are likely to detach from the cattle host and fall off the animal. However, increasing the amount of fungal inoculum in the natural environment through secondary cycling, akin to pasture application, is likely to increase the levels of infection in the tick population.

Conclusion and recommendations

Based on the constraints identified, detailed recommendations for research are listed in Table 3. Myco-acaricides are likely to become a necessary tool considering the rate at which resistance is developing to existing products, the high cost of developing new chemical acaricides and the projected expansion of the geographic range of African tick species.

This paper reviews the current status of control of cattle ticks by topical application of myco-acaricides, but in general, lays the foundation for the development of myco-insecticides for application to animal systems to control ectoparasites. There are numerous studies which demonstrate that entomopathogenic fungi are pathogenic to ticks but few which are useful for the development of an effective system for control based on myco-acaricides. This is similar to the position with the control of crop pests less than 20 years ago hence lessons can be drawn from recent studies which recognise that improvements in a succession of components are required to move successfully from isolating a fungus, to the development of a viable myco-insecticide.

There is considerable potential for a myco-acaricide developed for pasture or topical application to cattle for the control of ticks. Experiments with pasture application have had excellent results while trials with topical application to cattle have been variable. The animal skin is very complex, and temperature, moisture, pH and skin secretions are singly or more likely in combination, important factors to be considered if effective myco-

Table 3 Research priorities for the development of a myco-acaricide for the control of ticks

Influencing factors	Key findings/possible impacts	Recommendations for research
Pathogen factors		
Fungal species	*Metarhizium* and *Beauveria* spp. are the key pathogens of ticks and have very good safety characteristics for development as myco-acaricides.	Focus on evaluating isolates of *Metarhizium* and *Beauveria* for tick pathogenicity. Determine factors in these species which are responsible for the strong pathogenicity to ticks.
Host specificity of isolate	An isolate with a broad physiological host range does not necessarily mean the ecological host range will be similarly wide.	Although narrow ecological host range isolates may have limited impacts on non-targets, a broad host range isolate may be used to target a wider range of ectoparasites. Determination of the ecological host range of isolates should only be a priority at later stages of research.
Origin of isolate	Contrary to a common belief, isolates from tick species have not proven to be more pathogenic to ticks than non-tick isolates.	Limit focus on bioprospecting for isolates from ticks and screen isolates from international collections with good production characteristics for pathogenicity to ticks.
Virulence	In bioassays, high concentrations of conidia are generally required to produce mortality in ticks which may indicate that highly virulent isolates are not known. Sublethal effects can affect reproduction in ticks and could be used in control strategies.	Identify highly virulent tick pathogenic isolates and calculate minimum lethal doses for all tick stages. Determine contribution of sublethal effects to tick control.
Host factors		
Tick species	A range of tick species are susceptible to entomopathogenic fungi. Single isolates may vary in pathogenicity between tick species.	Identify a suite of isolates which are pathogenic to a wider range of species—these studies will need to take a geographic focus depending on which ticks are important in particular areas or if formulation can improve range.
Development stage	All developmental stages are susceptible to entomopathogenic fungi but single isolates may vary in pathogenicity. Several tick developmental stages may be present on animal at any given time.	Identify a suite of isolates which are pathogenic to mature and immature stages and test if formulation can improve range.
Anatomy	Ticks, particularly non-engorged stages, may provide a greater challenge to fungal colonisation than insects due to the nature of the tick body and other anatomical features.	Determine if the anatomy of ticks makes the use of myco-acaricides impractical for the control of certain tick species.

Table 3 continued

Influencing factors	Key findings/possible impacts	Recommendations for research
Life cycle	One-host ticks spend most of their life cycle attached to a single host and are easy to target using topical applications of myco-acaricide. The development stages of three-host ticks spend the majority of their time off host, making topical applications of myco-acaricide less effective.	Determine how long various tick species are on cattle and determine appropriate application strategy for myco-acaricides.
Location	Tick species have specialised habitats on animal host. Microenvironmental factors may vary in parts of the animal host which may affect fungal efficacy.	Determine where tick species reside on animals and implications for myco-acaricide application.
Host skin microenvironment		
Skin temperature	Efficiency of entomopathogenic fungi is generally reduced at mammalian skin temperatures. Temperature may be a major limiting factor to the performance of a topically applied myco-acaricide. High temperature tolerant isolates show promise.	Identify high temperature tolerant isolates, either those which can grow or merely survive without detriment, for use in future studies. Evaluate safety of high temperature tolerant isolates to mammals, at suitable stage in research.
Coat humidity	Humidity of the skin surface is relatively low and may not provide moisture necessary for rapid germination of conidia. Conversely, it may provide a stable humidity allowing for fungal growth.	Conduct further studies on humidity in the cattle coat and its effect on germination of conidia.
Skin pH	Skin pH is acidic, ranging from 4.5 to 7.6, generally favouring bacteria rather than fungi. It is unclear if pH of the relevant range has major impacts on fungal performance.	Assess the impact of pH (4–8) on germination and persistence of conidia of promising isolates Identify lower pH tolerant strains of entomopathogenic fungi. Assess formulation using buffers.
Secretions/excretions	Secretions found on the cattle surface are complex and consist of a range of fatty acids, ions, proteins and other compounds. Skin secretion may be a major limiting factor for a topically applied myco-acaricide.	Evaluate the effect of skin secretions on fungal performance Conduct studies on using microencapsulation to minimize any negative effect of skin secretions.

Table 3 continued

Influencing factors	Key findings/possible impacts	Recommendations for research
Skin microflora	Microflora of the skin is mainly composed of specialized bacteria. Dermatophytes isolated from the skin of cattle have not been recovered from ticks; however, non-dermatophyes such as *Alternaria* spp., *Aspergillus* spp., *Beauveria bassiana*, *Curvularia* sp., and *Penicillium* sp. have been isolated from ticks. It is unclear if infected ticks obtain their fungi while on the host or from the wider environment when juvenile.	Studies the effects of skin microflora, incl. microbial secretions, on germination and growth of entomopathogenic fungi. Determine if fungal infection in ticks originate from the skin microflora or the wider environment. Determine if an entomopathogenic fungus can be integrated into the skin microflora or if entomopathogens in the skin microflora, if they exist, can be enhanced.
Mode of action		
Direct impaction	The cattle coat, which varies with breed and season, is likely to reduce direct impaction of conidia onto tick targets hidden deep within the hair.	Focus on quantifying levels of direct impaction using various formulations.
Residual infection	Residual infection is likely to be influenced by availability, initial infectivity and persistence. Emulsifiable adjuvant oil formulation which enhanced pathogenicity to ticks in bioassays may be detrimental to residual infection due to greater adherence to hair. Pre-soaking in tap water improved pathogenicity to *R. microplus*. Persistence of conidia on the cattle surface is low.	Focus on maximizing residual infection through addressing availability, initial infectivity and persistence. Conduct formulation studies to balance reduction of loss of conidia from cattle coat after spraying with ease of being picked up by ticks. Conduct further studies on pre-soaking including the addition of nutrients to synchronize germination. Conduct studies on using microencapsulation to maximize persistence.
Secondary cycling	Increasing inoculum through secondary cycling is likely to reduce tick populations similar to pasture application.	Determine the contribution of secondary cycling to tick control.

acaricides for topical application are to be developed. Therefore, fundamental research is required to further understand how entomopathogenic fungi interact with the physical, chemical and biological parameters of the cattle surface. These studies will then influence the selection of isolates, and formulation and application technologies, which can lead to the development of effective control strategies for specific tick species.

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Protection of *Metarhizium anisopliae* conidia from ultra-violet radiation and their pathogenicity to *Rhipicephalus evertsi evertsi* ticks

M. Hedimbi · G. P. Kaaya · S. Singh · P. M. Chimwamurombe · G. Gindin · I. Glazer · M. Samish

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Abstract *Metarhizium anisopliae* conidia were formulated in water or in olive oil containing 3% commercial sunscreens (Everysun® or E45 Sun Block 50®) and exposed to an artificial UV source for up to 5 hours. Survival of conidia after 5 h of exposure to UV in oil formulation was 29% when protected with Everysun, 40% when protected with E45, and 4% in control. In comparison, survival of conidia formulated in water was 13% when protected with Everysun, 24% when protected with E45, and 0% in control. Furthermore, the influence of sunscreens on conidia viability and virulence to *Rhipicephalus evertsi evertsi* larvae and unfed adult ticks was evaluated. Adding these compounds to the conidial formulations did not reduce the viability of the conidia. Larval mortality was 95 and 100%, while unfed adult mortality was 90 and 97% after being exposed to unprotected conidia formulated in water or in oil, respectively. Conidia protected by Everysun or E45 formulated in water, induced 88 and 83% mortality in larvae, and 92 and 90% mortality in unfed adults, respectively. Conidia suspended in oil and protected by Everysun or E45 induced 94 and 91% mortality in larvae, and 83 and 81% in unfed adults, respectively. These observations indicate that olive oil and the two sunscreens confer protection to conidia against damages by UV radiation without interfering with their pathogenicity to ticks.

Keywords *Metarhizium anisopliae* · Ultra-violet radiation · Sunscreens · *Rhipicephalus evertsi evertsi* · Conidial germination

M. Hedimbi · G. P. Kaaya (✉) · P. M. Chimwamurombe
Department of Biological Sciences, University of Namibia, Private Bag 13301 Windhoek, Namibia
e-mail: gkaaya@unam.na

S. Singh
Department of Physics, University of Namibia, Private Bag 13301 Windhoek, Namibia

G. Gindin · I. Glazer
ARO, The Volcani Centre, P.O. Box 6, Bet Dagan 50250, Israel

M. Samish
Department of Parasitology, Kimron Veterinary Institute, P.O. Box 12, Bet Dagan 50250, Israel

Introduction

Promising results have been reported for the potential of the entomopathogenic fungus *Metarhizium anisopliae* to serve as a bio-control agent of ticks (Kaaya et al. 1996; Kaaya 2000, 2003; Kaaya and Hassan 2000). The formulation in which the conidia are suspended is known to influence the efficacy of the fungus (Burges 1998; Kaaya and Hassan 2000). Conidia formulated in oil have been reported to induce higher mortalities than those formulated in water alone (Kaaya 2000; Kaaya and Hassan 2000; Maranga et al. 2005). Although the reason for higher mortality induced by oil formulations is not known, it is believed to be due to the fact that oil blends better with insect's lipophilic cuticle than water, and that oil spreads rapidly, presumably carrying fungal conidia to areas of cuticle that are normally protected from the unfavorable environmental conditions (Wraight et al. 2001). Furthermore, in the field, tiny oil droplets do not evaporate as quickly as those of water, thus providing moisture to conidia for longer periods than water alone (Wraight et al. 2001; Maranga et al. 2005).

A major obstacle in using entomopathogenic fungi under field conditions is the rapid inactivation of the conidia caused by ultra-violet (UV) radiation, humidity and extreme temperatures (Fargues et al. 1996). Fungal conidia are very susceptible to solar radiation and investigators have for many years tried to find ways of protecting conidia against damage caused by UV radiation (Shah et al. 1998). However, such efforts have not been very successful. In addition to inactivating conidia, UV radiation has been shown to cause delay in the germination process of the surviving conidia (Moore et al. 1993). All these reduce the efficiency of fungi as biocontrol agents under field conditions where there is a strong solar irradiation (Moore et al. 1993).

Rhipicephalus evertsi evertsi is a two-host tick, an economically important pest of livestock throughout most of Africa. It transmits *Babesia equi* to horses, *Anaplasma marginale* to cattle, and its saliva contains toxins that cause paralysis in lambs, adult sheep and calves (Walker et al. 2003).

In this study, the potential of two commercial sunscreens as improving compound for a tick biopesticide was studied. The sunscreens protection of *M. anisopliae* conidia exposed to UV radiation was measured by the ability of conidia to germinate and to form colonies. The direct influence of the sunscreens on the ability of the conidia to induce mortalities in unfed larvae and adult *R. e. evertsi* ticks are also reported.

Materials and methods

Sunscreens

Two commercial sunscreens sold for application on the skins of people were used and their chemical compositions are provided in Table 1. These types of sunscreens were chosen because they are readily available and they are relatively cheap compared to pure chemical sunscreens like benzylcinnamate, and hence likely to be available even to peasant farmers. Sunscreens developed for humans are likely to be safer to animals and probably to the environment than pure chemicals, hence their use in this study. Furthermore, the SPF values of the sunscreens used (30 and 50) are likely to confer better protection from UV radiation than sunscreens with lower SPF values, especially in harsh environmental conditions with high solar radiation.

Table 1 UV protectants used in the study and their chemical compositions obtained from the bottle labels

UV protectants	Sun protection factor (SPF)	Chemical compositions	Manufacturer
Everysun® (Everysun)	30	Aqua, ethylhexyl methoxycinnamate, benzophenone-3, c12–15 alkyl benzoate cinnamate, glycerine, ethylhexyl salicylate, cyclomethicone, 4-methyl benzylidene camphor, steareth-100, steareth-2, glyceryl stearate citrate, sucrose, mannan, xanthan gum, phenoxy ethanol, alkyl stearate, methyl thiazolinone	Permark International (South Africa)
E45 Sun Block 50® (E45)	50	Aqua, zinc oxide, octyl stearate, titanium dioxide, isopropyl myristate, isohexadecane butylenes glycol, polyglyceryl-3 oleate, cetyl dimethionine copolyol, magnesium sulphate, sodium chloride, phenoxyethanol, aluminum stearate, alumina, lecithin, isopropyl palmitate, methlyparaben, propylparaben 2-bromo-2-nitropropane-1,3-diol	Healthcare (South Africa)

Tick culture

Engorged *R. e. evertsi* adult ticks were collected from zebu cattle in Northern Namibia. All off-host stages were maintained in the laboratory at 25°C and 100% relative humidity (RH) and the on-host stages were fed on rabbits (Kaaya et al. 1996). Pathogenicity of conidia was assessed on unfed stages of larvae and adult *R. e. evertsi.*

Fungal cultures

Metarhizium anisopliae RS2 (originally isolated from *Amblyomma variegatum*) was cultured in Petri dishes for 3 weeks at 25°C on Sabourauds Dextrose Agar (SDA) (Kaaya et al. 1996). Conidia were harvested by rinsing agar with sterile, distilled water containing 0.05% (v/v) Triton X-100. Conidia were then washed twice in sterile distilled water by centrifugation at 5,000 rpm for 5 min. A hemocytometer was used to determine the concentration of conidia in the initial suspension. Serial dilutions were then made to get the desired concentration of conidia.

Preparation of conidial formulations

Chemical sunscreens Everysun and E45 were tested for their efficacy in protecting fungal conidia from UV radiation in both water (including 0.05% Triton X-100), and oil formulations (20% olive oil + 0.05% Triton X-100 in water). A solution of 3% (v/v) of the commercial sunscreen was prepared in the oil or water formulations. In the control groups, conidia were suspended in the same solutions without the sunscreens. All conidial components used in the tests started with a pre-incubation period of 30 min at standard room temperature to get all conidia in the same temperature range and humidity.

UV source

An artificial UV source was used at a wavelength of 200–400 nm (AVANTES: AvaLight-DHS, Serial No. LS-0501001; compact and closed system). The wavelength of light emitted by the UV source was detected using an AvaSpec-2048 monitor and was analyzed and adjusted using AvaSoft 6.2 software for the AvaSpec Spectrometer. Samples were exposed in a closed chamber at ca. 1 m from the UV source. Temperature in the chamber was maintained at 25 ± 2°C during exposure.

Effects of UV protectants on conidia viability

One ml of UV-exposed conidial suspension (200 conidia/ml) samples in water or in oil formulations with and without (3%) sunscreens were spread on SDA plates using glass beads and incubated at 25°C and 100% RH in the dark. Germinating conidia were counted after 48 h of incubation under a dissecting microscope and recorded as percentage germination. Conidia were considered to have germinated if the germ tube was longer than half the diameter of the germinating spore.

Protection of conidia from UV damage using sunscreens

One-ml samples (200 conidia/ml) of each formulation containing sunscreen were placed in macro disposable cuvettes (10 × 10 × 45 mm, <1 mm thick) during exposure to UV radiation. Samples were exposed to UV radiation in a compact and closed environment for 0, 1, 2, 3, 4, and 5 h, respectively. A 0.1 ml (~20 conidia/dish) of exposed samples were then cultured on SDA and spread using glass beads and incubated at 25°C and 100% RH in the dark. The colonies developing from each sample were counted daily for 3 days under a dissecting microscope. This provided an estimate of the ability to form colonies (colony forming units, CFUs). The mean ability to form colonies (%) is represented as $(S_t/S_c) \times 100$, where S_t is the mean CFUs of the nine replicates at exposure time t, and S_c is the mean CFUs of the water control (no exposure) after 3 days germination (Lee et al. 2006).

Influence of protectants on the virulence of conidia to *Rhipicephalus evertsi evertsi*

Unfed *R. e. evertsi* larvae were infected with *M. anisopliae* conidia by placing 20–30 larvae on filter paper in disposable Petri dishes (65 mm diameter) previously wetted with 1 ml of *M. anisopliae* suspension (1×10^8 conidia/ml) with or without 3% of the sunscreens E45 or Everysun in 20% oil or water formulations. Similarly, unfed adult ticks were infected by dipping them in conidial suspension and placing them on filter paper in Petri dishes. The dishes were then incubated at 25°C and 100% RH in the dark. Mortalities were recorded daily until 21 days post-infection.

Data analysis

Normality was tested using the Kolomogorov–Smirnov test and normally distributed data were analyzed by ANOVA and means were compared using a post-hoc Scheffé test, using SPPS for Windows®. Each test was based on three replicates per sample and each experiment was repeated three times, except the tick virulence test of conidia which was done only once.

Results

Effects of UV protectants on conidial viability

Addition of sunscreens did not affect the viability of *M. anisopliae* conidia in water or in oil formulations. Germination rate was 99% in control (water), and 99 and 96% among conidia protected with Everysun or E45 formulated in water, respectively ($P > 0.05$). Germination rate was 95% in control (oil), and 92 and 87% in conidia protected with Everysun or E45 formulated in oil, respectively ($P > 0.05$) (Fig. 1). There was no statistical difference ($P > 0.05$) in germination of conidia in any of the formulations with or without UV protectants.

Protection of conidia from UV damage using sunscreens

Survival of conidia (ability to form colonies) was observed to vary when sunscreens were included in formulations and exposed to UV radiation for varying periods of time. The ability of conidia to germinate and form colonies decreased faster in unprotected conidia in the two formulations as the exposure to UV radiation increased, compared to those protected with sunscreens. After 5 h of exposure to UV radiation, none of the conidia formulated in water were able to colonize, while 13% of the conidia protected with Everysun, and 24% of the conidia protected with E45 germinated and formed colonies ($P < 0.01$). The ability to form colonies after 5 h of exposure to UV radiation in conidia formulated in oil was 4% in unprotected conidia, 29% in conidia protected with Everysun, and 40% in conidia protected with E45 ($P < 0.001$) (Fig. 1).

Influence of protectants on the virulence of conidia to *Rhipicephalus evertsi evertsi*

Larval mortality was found not to vary significantly in most of the treatments and controls tested, whereas mortality of unfed adults was found not to vary significantly between controls and treatments in the same formulation, but varied between formulations. Conidia (1×10^8 conidia/ml on filter paper) suspended only in water with Triton X-100 (unprotected) caused 95% mortality to unfed larvae and 83% to unfed adult *R. e. evertsi* ticks. Conidia protected with Everysun or E45 in water formulation caused mortalities of 88 and 83% to larvae ($P > 0.05$), and 83 and 81% to unfed adults ($P > 0.05$). Conidia formulated in oil (unprotected) caused 100% mortality to larvae and 91% to unfed adults, whereas those protected with Everysun or E45 in oil formulation caused larval mortality of 94 and 91% ($P > 0.05$), respectively, and unfed adult mortality of 92 and 90% ($P > 0.05$) (Table 2).

Discussion

Unfed larvae and adult *R. e. evertsi* ticks were found to be highly susceptible to the entomopathogenic fungus *M. anisopliae* (Table 2). The high susceptibility of unfed larvae and adults from several tick species to *M. anisopliae* was reported earlier (Kaaya and Hassan 2000). The viability and virulence of the conidia was not influenced by any of the formulations including the sunscreens tested. This implies that olive oil and chemical sunscreens (Everysun and E45) can probably be incorporated in fungal formulations without affecting the conidial ability to germinate on tick cuticle. Conidia of *M. anisopliae* and *B. bassiana* in oil formulation induced higher mortalities in *Rhipicephalus appendiculatus* and *A. variegatum*

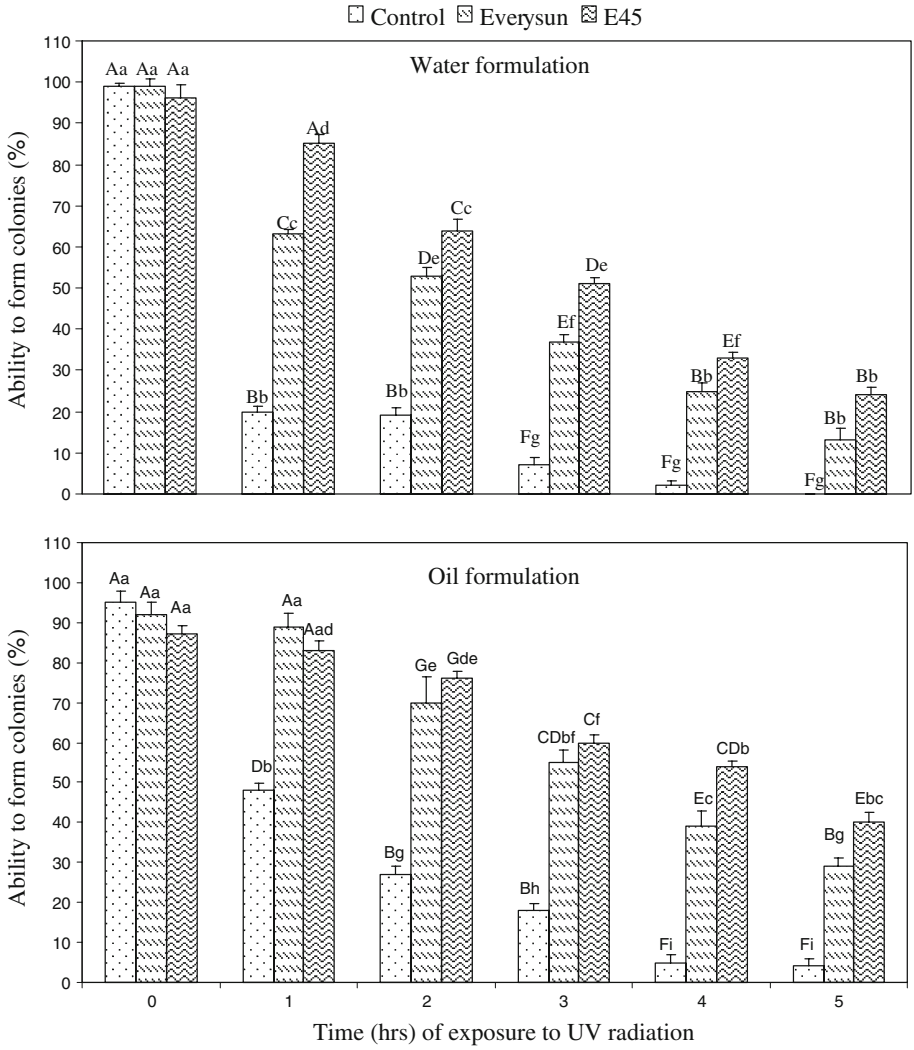

Fig. 1 The ability of *Metarhizium anisopliae* conidia protected with E45 and Everysun to form colonies after exposure to UV radiation for periods of 0–5 h in water and oil formulations. Controls contained no sunscreens. Means (±SE) of nine replicates are presented. Means with the same lower case letter are not significantly different from each other among treatments in the same formulation, whereas means with the same upper case letter are not significantly different among treatments in different formulations (Scheffé's post-hoc test, $P > 0.05$)

ticks than conidia formulated in water, under laboratory and field conditions (Kaaya and Hassan 2000; Maranga et al. 2005, 2006). The better performance by oil formulation may be due to the fact that oil blends better with tick cuticle since the cuticle is lipophilic and hydrophobic (Bateman et al. 1993; Maranga et al. 2005).

Oil formulation (without sunscreens) was found to improve survival rates of conidia following UV exposure for different durations, as compared to water formulation (Fig. 1). The ability of the conidia to form colonies was consistently higher in oil than in water formulation at all durations of UV exposure. Improved protection by oil may be due to UV absorption

Table 2 Ability of *Metarhizium anisopliae* conidia formulated in water and oil and protected with Everysun and E45 to induce mortality in *R. e. evertsi* larvae and unfed adults

Protectants	Larvae		Unfed adults	
	Water	Oil	Water	Oil
Control	95 ± 5.0 ab	100 ± 0.0 a	64 ± 1.8 c	72 ± 1.5 d
Everysun	88 ± 2.0 ab	94 ± 1.7 ab	60 ± 1.5 c	69 ± 2.1 d
E45	83 ± 1.5 b	91 ± 1.2 ab	58 ± 2.1 c	66 ± 2.1 d

Controls contained no sunscreen. Means (±SE) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé's post-hoc test, $P > 0.05$)

properties of oil (Moore et al. 1993). Since even the hydrophobic conidia in aqueous suspensions have high moisture content and are metabolically active, they are more likely to suffer DNA damage than conidia in oil formulation (Moore et al. 1996).

The addition of sunscreens significantly improved the ability of conidia to form colonies in each formulation after UV treatment compared to the control groups without sunscreen (Fig. 1). This indicates that the efficacy of a formulation in protecting conidia from UV damage increases several fold with addition of a sunscreen. Moore et al. (1996) reported a significant difference in the ability of *Metarhizium flavoviride* to germinate after addition of different types of chemical sunscreens. However, their investigations used different irradiation energy levels rather than exposure times.

Sunscreens may extend the survival of spores, especially those in direct sunlight (Moore et al. 1993). Furthermore, fungal isolates may vary in their susceptibility to UV radiation and selection or genetic engineering may result in increased UV resistance of a mycopesticide (Ignoffo and Garcia 1992). Results of this study show that olive oil and chemical sunscreens can increase the ability of UV radiated conidia to colonize. However, the overall efficacy of UV protectant under field conditions will depend also on additional factors, such as target arthropods, season, geographical location, and time of application (Moore et al. 1993, 1996; Shah et al. 1998; Lee et al. 2006).

This study has also shown that the sunscreens Everysun or E45 do not affect the ability of the conidia to germinate nor their pathogenicity to larvae and adults of *R. e. evertsi*. Similarly, Shah et al. (1998) obtained 98 and 96% mortality in *Kraussella amabile* (Orthoptera), under field conditions, after spraying conidia of *M. flavoviride* in formulations without and with the sunscreen oxybenzone (2%), respectively, demonstrating that protecting conidia with sunscreen does not affect pathogenicity. Their results, as ours, suggest that the sunscreens can be incorporated into conidial formulations to improve their survival in the field, without reducing or interfering with their pathogenicity to target arthropods.

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Evaluation of *Metarhizium anisopliae* (Deuteromycota: Hyphomycetes) for control of broad mite *Polyphagotarsonemus latus* (Acari: Tarsonemidae) in mulberry

Monchan Maketon · Patricia Orosz-Coghlan · Jittranon Sinprasert

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Abstract A study on 12 entomopathogenic fungi for controlling broad mite (*Polyphagotarsonemus latus* (Banks)) in mulberry found that *Metarhizium anisopliae* CKM-048 was the most virulent strain in controlling both larvae and adult broad mites at the concentration of 2×10^8 conidia/ml. There was no ovicidal effect when tested with broad mite eggs. Median lethal concentrations (LC_{50}) of *M. anisopliae* in killing larvae and adults were 8.7×10^6 and 1.3×10^7 conidia/ml, respectively. Median lethal times (LT_{50}) of larvae and adults were 2.4 and 3.8 days, respectively, at the concentration of 2×10^8 conidia/ml. The fungus was found to produce protease and chitinase. Scanning electron microscope (SEM) studies were done to monitor the infection steps of the fungus on broad mites. A greenhouse test on mulberry trees revealed that *M. anisopliae* could reduce the broad mite population within 4 days after treatment. However, after 7 days, its efficacy was decreased significantly.

Keywords Broad mite · *Polyphagotarsonemus latus* · *Metarhizium anisopliae* · Entomopathogenic fungi · Mulberry

Introduction

Broad mite (*Polyphagotarsonemus latus* (Banks)) has a world-wide distribution and is known by a number of common names. In Thailand it is called the yellow mite, and it is a serious problem in areas where chili (*Capsicum annum*) is cultivated (Kemsawasd 1976). Besides chili, a wide variety of agricultural crops, ornamentals, and wild plants have been recorded as hosts (Jeppson et al. 1975; Yang and Chen 1982; Ibrahim and Low 1998; Pena

M. Maketon (✉) · J. Sinprasert
Zoology Department, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
e-mail: fscimcm@ku.ac.th

P. Orosz-Coghlan
Soil, Water and Environmental Sciences Department, University of Arizona, Tucson, AZ, USA

and Campbell 2005). In the Philippines, this mite is a pest of young plants including tomato, potato, tobacco, and ornamental plants.

White mulberry (*Morus alba*) is a short-lived, fast-growing, about 15–20 m tall tree. It is native to eastern Asia and its natural fruit color is deep purple. The leaves are the preferred feedstock for silkworms (*Bombyx mori*). White mulberry is extensively planted throughout the warm temperate Northern Hemisphere, mainly for the silk industry. Recently tea made of mulberry leave has become popular in some countries. Besides thrips and whitefly, broad mite is one of the serious sucking pests on mulberry leaf. Some chemical pesticides are being used, such as sulfur and amitraz, but they caused some phytotoxic effect, especially during the summer season, with ambient temperature as high as 40°C. Therefore, an integrated pest management program has initiated, employing predators and parasites, but also microbial miticides. Nugroho and Ibrahim (2004) have reported on a laboratory bioassay of three entomopathogenic fungi against broad mites on chili. They found that the most virulent strain was *Paecilomyces fumosoroseus* followed by *Beauveria bassiana* and *Metarhizium anisopliae*.

In this study, several entomopathogenic fungi were tested for their ability to control broad mite adults, larvae, and egg stages on mulberry leaves in both laboratory and greenhouse conditions. The lethal concentration (LC_{50}) and time (LT_{50}) were evaluated for the most virulent fungus. Enzyme production from the fungus was studied. Microscope and scanning electron microscope (SEM) studies for the fungal infection steps were performed.

Materials and methods

Preparation of conidial suspension

Eighty-eight soil samples and 75 homopteran, 220 isopteran, and 86 hemipteran cadavers were sampled from northeastern and central parts of Thailand. Twelve entomopathogenic fungi were isolated by the method of Meikle et al. (2005) and sent to the Thailand Institute of Scientific and Technological Research (TISTR, Bangkok, Thailand) for identification.

All screen cultures were grown on potato dextrose agar (PDA) (Difco, Becton-Dickson, Sparks, MD, USA) at 27 ± 1°C in the dark for 14 days. Conidial suspensions were made by lightly scraping the fungal culture surface with a sterile cell spreader into a 100-ml plastic container. The conidial clumps were suspended in distilled water with 0.01% Tween 80 (ICI Americas, Norwich, NY, USA). The suspensions were vortexed for 5 min to dissociate clumps and then filtered through one layer of cheesecloth to remove conidial clumps and mycelial debris. Concentration of each suspension was diluted to 2×10^8 conidia/ml determined by a Neubauer hemocytometer under a phase-contrast microscope. The suspensions were used on the same day or the day after preparation and shaken before use. The pure fungal culture of the most virulent strain was deposited at TISTR.

Preparing mulberry trees for rearing broad mite

A mulberry stalk of 1 cm diameter and 30 cm length was transferred into a 20-cm diameter pot. The pots were placed in a cage (60 × 60 × 200 cm) and covered with a nylon sheet to prevent infestation with pests. For the laboratory and greenhouse tests, 20 and 40 pots were prepared, respectively. After new leaf growth emerged to about 10 leaves, 20 male and 20 female adult broad mites per pot were randomly introduced on the leave surface. Broad mites were obtained from the Thai Department of Agriculture, where they had been reared

on mulberry. Broad mites were transferred by a single-hair brush pen. All work was done under a stereo microscope.

Screening for the most virulent fungus against broad mite adults, larvae and eggs

Fourteen treatments consisting of 12 entomopathogenic fungi with an untreated control and a water treated control were performed in three replicates. A mulberry leaf without broad mites was cut into a 5-cm diameter circle and placed on moist cotton wool in an autoclaved 9-cm Petri dish. Twenty broad mite adults or larvae were transferred into each dish using a single-hair brush pen. For the assays with eggs, 20 adult female mites were placed on a clean mulberry leaf disc for 6 h at room temperature. Then females were removed and the number of eggs laid was reduced to 20 per leaf disc by puncturing the excess eggs with a needle.

Aliqouts of 1 ml of a fungal suspension were sprayed into a dish using a thin-layer chromatography (TLC) sprayer (Merck, Germany), resulting in $\sim 1 \times 10^7$ conidia/cm^2 leaf surface (Cuthbertson and Walters 2002). Observations were made every 12 h to check for broad mites falling off the leaf margin. Every day for 5 days broad mite adult and larva mortality (%) was recorded, or the percentage of unhatched eggs. All observations were done under a stereomicroscope.

For confirmation of fungus infestation of the dead broad mites, cadavers were dipped in a 10% sodium hypochlorite (NaOCl) solution (Sigma-Aldrich, MO, USA) for 5 min, allowed to dry, placed on PDA, and monitored for possible mycelium germination.

Median lethal concentration (LC_{50}) and time (LT_{50}) assessment

The most virulent fungus was further studied for its median lethal concentration (LC_{50}). Suspensions were prepared at 2×10^6, 2×10^7 and 2×10^8 conidia/ml and tested on larvae and adults separately (seven replications per treatment). Mortality was checked daily for 3 days. For the median lethal time (LT_{50}), a suspension was prepared at 2×10^8 conidia/ml and tested on larvae and adults. Mortality (%) of broad mites was recorded daily for 5 days.

Analysis of enzymes produced by the most virulent fungus

Qualitative study

The most virulent fungus was cultured on PDA for 14 days at room temperature (27 ± 1°C) in the dark. A 1-cm diameter piece of agar covered with mycelium was cut using a cork borer, placed on skimmed milk agar (Difco, Becton-Dickson, Sparks, MD, USA) and incubated in the dark at room temperature for 7 days. The occurrence of clear zones depicts protein-digesting enzymes produced by the fungus. The efficacies of enzyme production can be determined in terms of the ratio between the clear zone diameter and the colony's diameter (Chongcharoen and Vatanyoopaisan 2005). To investigate chitin-digesting enzymes, the fungus was cultured on chitin agar (chitin from crab shells from Sigma-Aldrich, MO, USA) (Chatdumrong 1996).

Quantitative study

Methods of Khan et al. (2003) and Dackman et al. (1989) were employed. For protease production, 0.2% (W/V) skimmed milk (Sigma-Aldrich) was added to minimal-medium

(Bonants et al. 1995) cultures in 1-l conical flasks containing 250 ml culture medium and inoculated with the most virulent fungus at 1.0×10^6 conidia/ml. Cultures were incubated on a shaker at 125 rpm for 7 days at 27°C. Samples were drawn daily and diluted in a carbonate-bicarbonate buffer pH 10.2 (Dawson et al. 1969). One milliliter of diluted sample was incubated with 1 ml of 1% azocasein (Sigma-Aldrich) at 50°C for 30 min. After that, the reaction was terminated by the addition of 1.5 ml 5% trichloroacetic acid (Sigma-Aldrich). Non-digested azocasein was separated by centrifuging at 600*g* for 20 min and culture supernatants filtered through a 0.2-µm membrane. Protease activity was measured at 345 nm and expressed as mg dry weight per ml azocasein solubilized, based on the formula $A_{345} \times 0.566 \times$ dilution factor (Lovrien et al. 1985). Protein content of the filtrate was determined by the method of Lowry et al. (1951).

For chitinase production, minimal medium was supplemented with 0.5% (W/V) chitin (Sigma-Aldrich). One-liter conical flasks containing 250 ml medium were inoculated with 1.0×10^6 conidia/ml of the most virulence fungus and incubated at 125 rpm for 7 days at 27°C. Daily, 1.5 ml of sample was drawn and incubated with 1 ml of 1% colloidal chitin at 37°C for 2 h (Elad et al. 1982). Precipitate was separated and the supernatant was measured at 285 nm. Chitinase activity was indicated by the increase in absorbance of *N*-acetyl-L-glucosamine.

Infection characteristics of the most virulent fungus against broad mite larvae

Twenty larvae were transferred onto a clean mulberry leaf lined with moist cotton wool and placed in a Petri dish ($n = 5$). A suspension of 2×10^8 conidia/ml was sprayed on the leaf. For the light (stereo) microscope study, cadavers were collected after 5 days, rinsed with distilled water and dipped into Nesbitt solution (40 g chloralhydrate crystals, 10 ml HCl, 40 ml water; Sigma-Aldrich), for 10 min until the body was cleared. Infection stages were photographed.

For the SEM study, the leaves filled with larvae were collected after 24, 48, 72, 96, and 120 h after the start of infection. Due to the tiny size of the broad mite, the leaf was cut into 1-cm^2 portions, placed into a micro well plate. The plate was kept in a 2.5% glutaraldehyde solution (Sigma-Aldrich) adjusted with 0.2 M cacodylate buffer (Sigma-Aldrich) to pH 7.2 for 12 h at 4°C. The sample was then rinsed three times with 0.1 M cacodylate buffer pH 7.2 for 10–15 min each. The rinsed sample was placed in 1% osmium tetroxide solution (Sigma-Aldrich) at pH 7.2 for 1 h at 4°C. The rinse with 0.1 M cacodylate buffer was repeated three more times and the final sample was washed with an increasing series of 30%, 50%, 70%, 90% and 100% acetone solutions (Merck, NY, USA). Using the critical point drying technique (Denton Vacuum, Cherry Hill, NJ, USA) with CO_2, the samples were dried and fumed with carbon and gold vapors, respectively (Wagner and Lewis 2000). The samples were observed under a SEM (Hitachi S-2500).

Greenhouse study of the most virulent fungus against broad mite

Forty broad mites on a mulberry leaf were prepared as described earlier. Two suspensions of 2×10^7 and 2×10^8 conidia/ml, resulting in $\sim 1 \times 10^6$ and 1×10^7 conidia/cm^2, respectively, were applied as described earlier. Amitraz 20% EC (Mitac™, AgrEvo) at 60 ml per 20 l water, resulting in $\sim$0.01 mg a.i./cm^2, was applied for comparison, as were an untreated and a water-treated control. All five treatments were conducted in a completely randomized design ($n = 8$). Average temperature ranged between 26 and 34°C and relative humidity was $80 \pm 5\%$.

One day before treatment, juvenile plus adult broad mites were counted on five young leaves at the top of the 120-cm-tall plants. A TLC sprayer was used to treat the whole plant, especially upper and lower leaves. Broad mites were counted at 1, 4, and 7 days on leaves on the plants.

Statistical analysis

In the assays screening for virulence, treatment effects were corrected for control mortality using Abbott's (1925) formula. Data from each trial were analyzed using analysis of variance (ANOVA) and means were compared by Duncan's new multiple range test (DMRT). Toxicological data were analyzed by Probit analysis (Finney 1971) with 95% confidence intervals, using SAS version 9.1.3. The greenhouse data were analyzed by ANOVA, because no significant difference was observed before spraying.

Results

Screening for the most virulent fungus against broad mite adults, larvae and eggs

Twelve entomopathogenic fungi were clarified (Fig. 1). After 5 days at room temperature (27 ± 1°C) and 72 ± 2% r.h., *M. anisopliae* CKM-048 yielded the highest mortality (mean ± SE, 71.7 ± 6.0%) of broad mite adults. Larval mortality was highest by *M. anisopliae* CKM-048 (100%) and *B. bassiana* CKB-048 (95 ± 5%) (Fig. 2). The 10 remaining isolates caused significantly lower larval mortality than these two isolates. Dipping of the cadavers in NaOCl solution and incubating them on PDA, confirmed that all new mycelia emerged from the cadavers were related to the original isolates. None of the 12 isolates had ovicidal activity: eggs in all treatments hatched within 1–2 days, so no fungal isolate in this experiment was effective in controlling broad mite eggs.

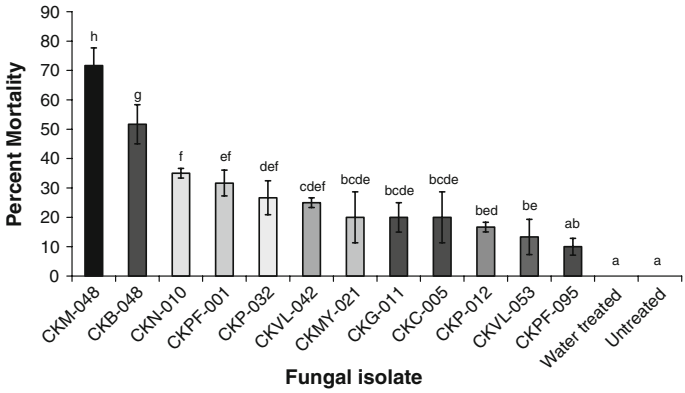

Fig. 1 Mortality (mean %) of broad mite adults on mulberry leaf discs, 5 days after being sprayed with suspensions from 12 entomopathogenic fungi. Bars with different letters are significantly different ($P < 0.05$, DMRT). The fungi are: *Beauveria bassiana* CKB-048, *Paecilomyces lilacinus* CKP-012, *P. lilacinus* CKP 032, *P. fumosoroseus* CKPF-001, *P. fumosoroseus* CKPF-095 (reclassified in part as *Isaria fumosorosea*; Luangsa-Ard et al. 2005), *Gliocladium virens* CKG-011, *Verticillium lecanii* CKVL-042, *V. lecanii* CKVL-053 (reclassified in part as *Lecanicillium muscarium* (Petch); Zare and Gams 2001), *Nomuraea rileyi* CKN-010, *Cordyceps brongniartii* CKC-005, and *Myrothecium verrucaria* CKMY-021

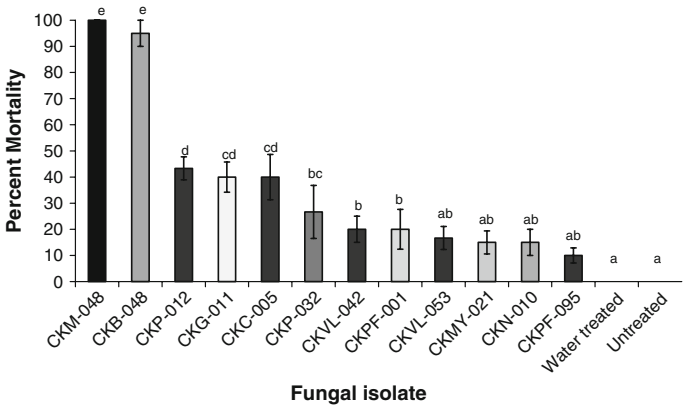

Fig. 2 Mortality (mean %) of broad mite larvae on mulberry leaf discs, 5 days after being sprayed with suspensions from 12 entomopathogenic fungi. Bars with different letters are significantly different ($P < 0.05$, DMRT). Fungi: see Fig. 1

Based on these results, *M. anisopliae* CKM-048 was coined the most virulent fungus and selected for further experiments.

Median lethal concentration (LC_{50}) and time (LT_{50})

The *M. anisopliae* CKM-048 suspension causing 50% mortality in broad mite larvae was found to lay between 2×10^6 and 2×10^7 conidia/ml. Therefore, two additional concentrations, 4×10^6 and 1×10^7, were prepared and tested. Probit analysis showed that the calculated LC_{50} for broad mite larvae was 8.71×10^6 conidia/ml. A similar procedure was done with adults, resulting in an LC_{50} of 1.32×10^7 conidia/ml (Fig. 3a).

At a concentration of 2×10^8 *M. anisopliae* CKM-048 conidia/ml, the LT_{50} for larvae was 2.36 days, and for adults 3.82 days (Fig. 3b).

Enzymes produced by the most virulent fungus

After 7 days incubation, *M. anisopliae* CKM-048 produced a protein- and a chitin-digesting enzyme. The ratio between clear zone and colony diameter that occurred on skimmed milk agar was 1.36 and on chitin agar it was 1.09. The quantitative study confirmed that the fungus was capable of producing protease and chitinase activity (Table 1).

Microscopic study of infection characteristics

After dipping a broad mite cadaver infected with *M. anisopliae* CKM-048 in Nesbitt solution, the cadaver became transparent and mycelium was seen inside and outside the body, whereas no mycelium was seen in the uninfected broad mite (Fig. 4).

A series of SEM pictures illustrates the infection process. At 1–2 h after contact, the conidia of *M. anisopliae* CKM-048 adhered to the cuticle of a broad mite larva (Fig. 5a). After 48–60 h, the fungus started to germinate and penetrate into the broad mite's body (Fig. 5b, c). During 72–96 h, fungal mycelia extrude from the broad mite and cover most of its body (Fig. 5d, e). Finally, after 120 h, the fungus started conidiogenesis and new conidia were formed (Fig. 5f).

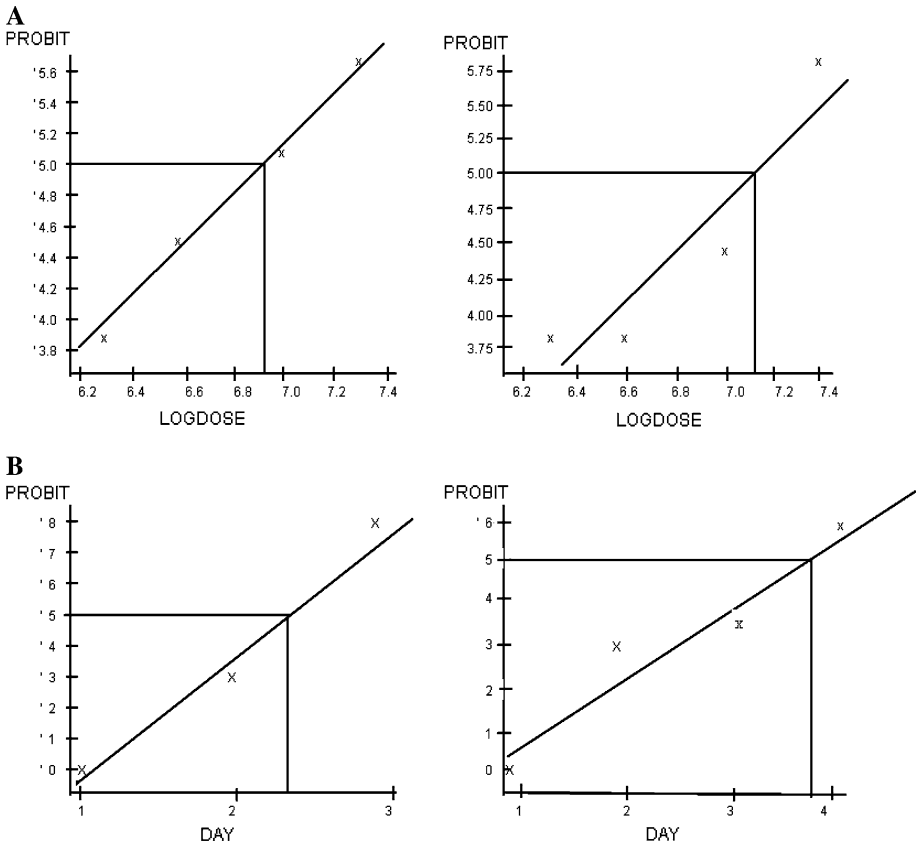

Fig. 3 Median lethal concentration (LC_{50}) of *Metarhizium anisopliae* CKM-048 suspensions and median lethal time (LT_{50}) when treated with *M. anisopliae* CKM-048 at 2×10^8 conidia/ml. (**A**) LC_{50}, and (**B**) LT_{50}, of broad mite larvae (left) and broad mite adults (right)

Table 1 Enzyme production by *Metarhizium anisopliae* CKM-048 on agar

Day	Protein (mg/ml)	Protease activity	*N*-acetyl-L-glucosamine	Chitinase activity
1	0.911	0.0000	0.122	0.0819
2	0.567	0.0137	0.143	0.0837
3	0.472	0.0176	0.222	0.0902
4	0.480	0.0279	0.222	0.0938
5	0.588	0.0245	0.268	0.0992
6	0.574	0.0339	0.296	0.1016
7	0.567	0.0475	0.323	0.1056
Control	0.790	0.0000	0.163	0.0000

Greenhouse study of the most virulent fungus against broad mite

Before application, the average number of broad mites found in all treatments did not differ significantly, hence the data could be analyzed by ANOVA. One day after spraying, amit-

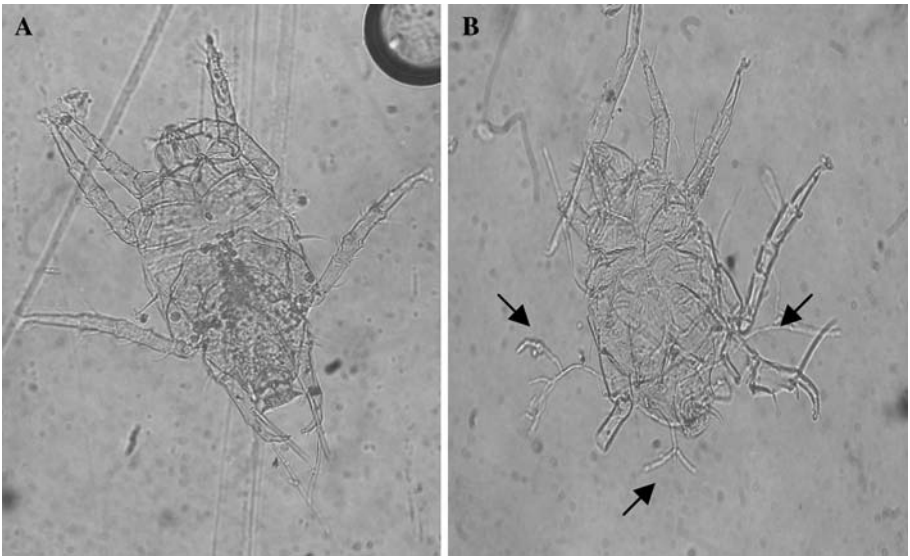

Fig. 4 Broad mite cadavers under the light (stereo) microscope: (**A**) Uninfected mite, no fungal mycelium inside the cadaver (control). (**B**) Mycelium of *Metarhizium anisopliae* CKM-048 extruded out of the infected cadaver (indicated by *arrows*)

raz had immobilized almost all broad mites, whereas the numbers of moving broad mites in the other four treatments were all about the same as before treatment (Table 2).

After 4 days, amitraz had killed all mites. The highest concentration of *M. anisopliae* CKM-048 suspension had killed significantly more mites than the lower fungus concentration or the water-treated control. At the untreated control the most mites were alive (Table 2).

Surprisingly, after 7 days the number of broad mites in the treatment with the higher rate of fungus suspension had increased tremendously, and was now not different from the number of live mites treated with the lower fungus rate. Still, the numbers of mites alive after 7 days were significantly lower in the fungus treatments than in the two control treatments.

Discussion

Metarhizium anisopliae CKM-048 was the most virulent fungal strain, hence the most promising candidate for controlling broad mite larvae and adults. Also *B. bassiana* CKB-048 showed good efficacy against larvae (95% mortality) and it was the second best against adults (ca. 50% mortality), so this may be considered another effective microorganism against broad mites, worthy of further investigations.

The concentrations of *M. anisopliae* CKM-048 needed for killing 50% of broad mite larvae and adults (8.71×10^6 and 1.32×10^7 conidia/ml, respectively), were not far apart. Yet, the time needed for killing 50% (when treated with 2×10^8 conidia/ml) of adults (3.4 days) was clearly longer than the time needed for killing half of the larvae (2.4 days). This might point at a higher tolerance of adults compared to larvae. Mite eggs were not found to be infected by any of the fungal isolates—perhaps the duration of the egg stage (1–2 days) is simply too short for the fungal mycelium to cause harm.

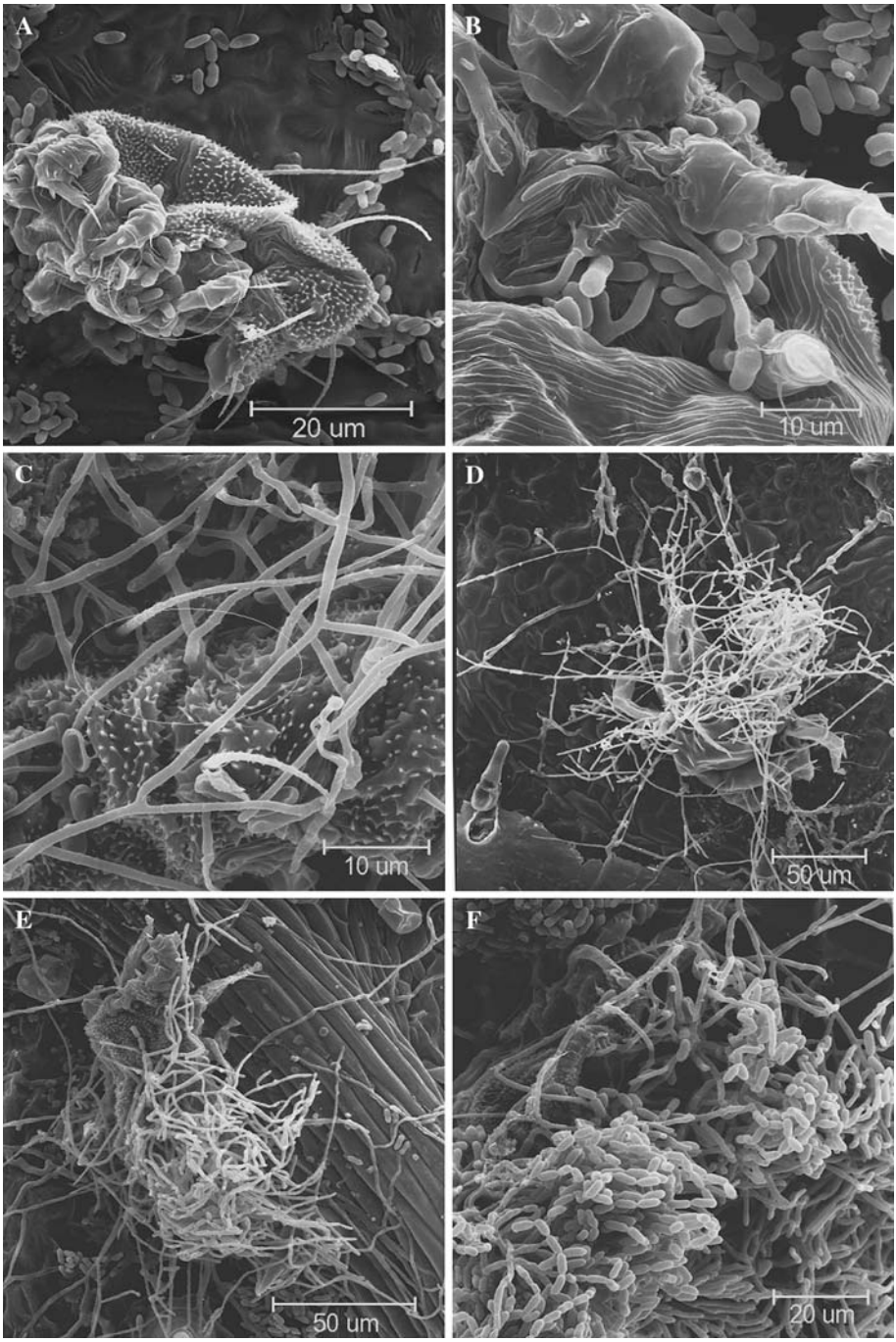

Fig. 5 A series of SEM pictures showing infection steps of *Metarhizium anisopliae* CKM-048 on the broad mite. (**A**) Conidia adhere on the broad mite's cuticle at 1-2 h after contact. (**B**) Germination from conidia at about 48 h after contact. (**C**) Penetration into the broad mite's body about 60 h after contact. (**D**) Extruding of fungus from the broad mite's body about 72 h after contact. (**E**) Colonization over the broad mite's body about 96 h after contact. (**F**) After 120 h, fungus started its conidiogenesis stage

Table 2 Mean number of broad mites (±SE) surviving per mulberry leaf up to 7 days after treatment

Treatment	Number of moving broad mites per leaf*			
	Before spray	1 day	4 days	7 days
Amitraz 20% w/v	26.40 ± 0.75^{ns}	0.05 ± 0.03^{b}	0^{d}	0^{d}
M. anisopliae CKM-048 2×10^8 conidia/ml	25.73 ± 0.92	23.67 ± 0.36^{a}	19.13 ± 2.76^{c}	30.97 ± 1.95^{c}
M. anisopliae CKM-048 2×10^7 conidia/ml	25.83 ± 0.78	23.65 ± 0.81^{a}	32.43 ± 1.51^{b}	33.80 ± 0.49^{c}
Control (treated)	26.73 ± 0.31	24.00 ± 0.43^{a}	37.18 ± 1.28^{b}	38.80 ± 1.08^{b}
Control (untreated)	25.80 ± 0.74	23.99 ± 1.03^{a}	47.82 ± 1.52^{a}	52.28 ± 2.38^{a}
% Coefficient of variation	7.29	9.48	17.21	13.39

* Means followed by the same letter within a column did not differ significantly ($P > 0.05$, DMRT)

The finding of enzyme activity confirmed the fungus' modes of action, because both protein- and chitin-digesting enzymes would be needed for lysis of the mite's cuticle, prior to mycelia penetration. Furthermore, the microscopic evidence underpinned the fungal infection of the mite.

The greenhouse assay suggested that *M. anisopliae* CKM-048 has a fairly short persistence on mulberry leave. Therefore, re-application of *M. anisopliae* CKM-048 within 3–4 days will be necessary. The application of microbial pesticides needs to be repeated especially for short-life-cycle pests, such as broad mites. Broad mites not only have a short life cycle, they are also parthenogenetical (Gerson 1992), and they are known to use insect hosts, specifically some whiteflies species, to move phoretically from plant to plant (Palevsky et al. 2001)—both factors will favour their potential rapid re-emergence.

Broad mite has not yet developed resistance to amitraz. However, *M. anisopliae* CKM-048 could be considered as an alternative tool for broad mite control in an integrated pest management program.

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Enabling mycelial application of *Hirsutella thompsonii* for managing the coconut mite

P. Sreerama Kumar · Leena Singh

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Abstract Laboratory and field studies were conducted to examine the prospect of mycelial application of *Hirsutella thompsonii* as an alternative to the use of mycelial–conidial formulations of the fungus in the suppression of the coconut mite, *Aceria guerreronis*. In a series of laboratory experiments, glycerol, yeast extract powder and dehydrated malt extract broth were found to be the best among nine substances investigated as possible adjuvants for use on coconut palms in the field along with *H. thompsonii* mycelia. *H. thompsonii* biomass in the presence of adjuvants not only produced more colonies but also yielded more conidia per pellet. In terms of the density of conidia generated on a mycelial mat the treatments varied highly significantly in two methods, with glycerol showing an average of 106% increase over control. Though irradiance with simulated sunlight resulted in reduced conidiogenesis, in general, adjuvant-treated pellets, both exposed and unexposed to simulated sunlight, produced substantial conidiation compared with control, irrespective of the two incubation conditions. Better conidiation was observed under alternating light–dark regime than under total darkness in all the treatments. Glycerol boosted the pathogenicity of *H. thompsonii* by 16.5% over control. In the field, a newly developed mycelial formulation of *H. thompsonii* applied after tank-mixing separately with the three selected adjuvants brought down the post-treatment population of the coconut mite by 85.6–97.1%. Application of the fungus in combination with glycerol resulted in a tolerable mean nut damage grade of 2.0 during the pre-harvest stage, compared with an acute score of 4.0 in control palms.

Keywords *Aceria guerreronis* · Adjuvants · Coconut eriophyid mite · *Hirsutella thompsonii* · Mycelial application

Introduction

Aceria guerreronis Keifer (Acari: Eriophyidae), one of the most destructive pests of the coconut palm (*Cocos nucifera* L.), is present in 22 countries spread over the Americas,

P. Sreerama Kumar (✉) · L. Singh
Project Directorate of Biological Control, P. O. Box 2491, H.A. Farm Post, Hebbal,
Bellary Road, Bangalore 560 024, India
e-mail: psreeramakumar@yahoo.co.in

Africa and the Indian sub-continent (Navia et al. 2005). Its occurrence in the Sultanate of Oman is also now confirmed (Sreerama Kumar, personal observation, 2007). India, which ranks third behind Indonesia and the Philippines in terms of coconut production, is the only one out of these three countries to be afflicted with this nut-inhabiting mite. Both in India and Sri Lanka—the other major coconut-producing country suffering from this pest since about the same time—the acarofungal pathogen, *Hirsutella thompsonii* Fisher (mitosporic fungi: Hyphomycetes), is perceived as one of the most potent biological control agents for this pest (Sreerama Kumar and Singh 2000; Sreerama Kumar 2002; Fernando et al. 2007) especially because a majority of coconut growers desist using chemical pesticides.

Because of the huge demand, a powder formulation (Mycohit) of *H. thompsonii* was developed during 2000 in India to enable its registration and commercialisation (Sreerama Kumar and Singh 2000; Sreerama Kumar 2002). Later, two liquid variants of this product, viz. Mycohit-LG20 and Mycohit-OS, have also been formulated, extensively field-tested and found to be equally effective (Sreerama Kumar 2006). Unlike most of the commercial mycopesticides, which contain only spores or conidia (Jenkins et al. 1998), these bio-products include both mycelia and conidia: mycelia for secondary cycling and disseminating the fungus, and conidia to target stray mites and to passively enter the perianth along with the spray fluid.

Production of high-concentration, mycelial–conidial formulations of *H. thompsonii* with an acceptable shelf-life usually involves a long biomass production cycle consisting of the submerged fermentation phase to generate mycelial biomass followed by the aerial conidiation or sporulation phase to obtain enough conidia. Therefore, our simultaneous research focussed on reducing the production cycle (i.e., eliminating the sporulation phase) and the prospect of using mycelia alone as an effective alternative to control the coconut mite. Since the shorter shelf-life of formulated *H. thompsonii* could be a deterrent to commercialisation to some extent (McCoy 1981), mycelia-based products could be used to meet the niche, 'no storage' or 'immediate use' market through advance orders from consumers.

There are several advantages of using *H. thompsonii* mycelial fragments in the field (McCoy et al. 1971). For a cryptic pest like the coconut mite, the amount of inoculum coming in direct contact with the pest immediately after application is negligible. The triggering of microepizootics within the niche of the perianth depends mostly on the sporulation of mycelia on the plant surface proximate to the infested nuts. The repeated cycles of sporulation on the plant surface determine the rate of spread of the disease in the mite population. Therefore, once applied, growth and sporulation of *H. thompsonii* mycelium on the plant surface is essential.

Availability of enough nutrition should be ensured for the initial establishment and saprophytic growth of the fungus through inclusion of nutrients in the spray (McCoy and Couch 1982). Furthermore, since several environmental factors either directly or indirectly influence the survival and persistence of fungal propagules (Roberts and Campbell 1977; Fuxa 1987), specifically the delicate mycelial fragments, protection against these fluctuating factors needs to be taken care of. Natural sunlight, in particular, is considered to be the harshest of factors as it is capable of killing fungi within hours of exposure (Moore et al. 1993; Inglis et al. 1995; Fargues et al. 1996). Moisture is another critical factor for the fungus to survive and propagate (Inglis et al. 2001). Several additives have multifarious qualities and are known to simultaneously act as a nutrient, humectant, sunscreen, etc. (Burges 1998).

Hence, the idea behind the work reported in this paper was to find out whether *H. thompsonii* mycelia alone could be used as a viable alternative to the mycelial–conidial versions of the fungus. The investigations commenced with laboratory selection of suitable

adjuvants and concluded with a field trial that proved the ability of mycelia alone to bring down the population of *A. guerreronis* with appreciable decline in concomitant nut damage.

Materials and methods

Fungal culture

An Indian isolate [MF(Ag)66] of *H. thompsonii* originating from infected *A. guerreronis* collected in Kerala was used throughout the studies. A monosporal sub-culture of the isolate was propagated on homemade potato dextrose agar (PDA) in Petri dishes (90 mm diameter) at 25°C for 30 days for use as inoculum. Stock cultures were stored at 4–5°C on slants of the same medium.

Mycelial beads or pellets (ca. 2.5 mm diameter) of *H. thompsonii* were produced in an Erlenmeyer flask (500 ml capacity) containing 200 ml of Sabouraud dextrose broth (SDB; HiMedia) through shake-flask fermentation with an orbital shaker (200 rpm, 5 days) ['Orbitek-L', Scigenics Biotech (Private) Limited, Chennai, India].

Effect of different adjuvants on the growth characteristics of *Hirsutella thompsonii*

Nine substances (Table 1) were tested for their suitability as adjuvants at 0.5% concentration (w/v or v/v) in three laboratory experiments consisting of treatments each of which was replicated thrice. In all the experiments, incubations were done under a 12-h photoperiod at room temperature (28 ± 2°C).

Table 1 Details of substances used as adjuvants

Substance	Manufacturer	Code	Known properties
Gelatine	HiMedia Laboratories, Mumbai, India	RM019	Humectant, nutrient
Glycerol	S.D. Fine-Chem, Mumbai, India	38454 L 05	Humectant, nutrient
Malt extract broth (dehydrated culture medium)	Laboratorios Conda, Madrid, Spain (Supplier: Colloids Impex, Bangalore, India)	1245	Nutrient, humectant
Nutrient broth (dehydrated culture medium)	HiMedia	M002	Nutrient, humectant
Potato dextrose broth (dehydrated culture medium)	HiMedia	M403	Nutrient, humectant
Potato flour	HiMedia	RM848	Nutrient, binder, humectant
Sabouraud dextrose broth (dehydrated culture medium)	HiMedia	M033	Nutrient, humectant
Skimmed milk powder (partly skimmed milk & sucrose)	'Amulya Dairy Whitener', Sabarkantha District Co-operative Milk Producers' Union, Himatnagar, India	–	Nutrient, humectant
Yeast extract powder	S.D. Fine-Chem	49117 K 05	Nutrient, humectant

In experiment 1, test adjuvants were added separately to 10-ml aliquots of the *H. thompsonii* biomass suspension in spent medium. Quantities of 0.1 ml were pipetted out onto a sterile filter paper disc placed inside a Petri dish and the resultant colonies were counted after 5 days of incubation. Biomass suspension without any added adjuvant served as control.

In experiment 2, the adjuvants were added separately to sterile deionised water and nine pellets were dunked in each test liquid (9 ml) separately for 30 min. Pellets dunked in sterile deionised water served as control. Sterile insect-mounting pins (38 mm long) were used to pierce through the treated pellets at the rate of three beads per pin. Three such loaded pins were kept in 15-ml sterile glass vials at the rate of one pin per vial for each treatment and incubated for 48 h. At the end of the incubation period, pellets from each pin were transferred to 1 ml of sterile deionised water containing 0.2% Tween 80, vortexed for 5 min, and the conidia were counted per pellet with a haemocytometer ('Bright-Line', Hausser Scientific, Horsham, PA, USA).

In experiment 3, the ability of the fungal pellets to form a sporulating mycelial mat in the continuous presence of the adjuvants was assessed through two ways of pellet treatment. In the first method, 5 ml of the biomass was transferred along with the spent medium to 15-ml glass vials and each adjuvant was added separately, swirled and incubated for 30 days by which time a sporulating mycelial mat (ca. 20 mm diameter) was formed on the surface of the undisturbed medium. Biomass without any additive served as control. In the second method, the adjuvant solutions were prepared separately, and the pellets obtained from 10 ml of shake-flask culture of *H. thompsonii* were added to 5 ml each of the adjuvant liquid taken in 15-ml glass vials. Pellets added to sterile deionised water were used as control. In both methods, at the end of the incubation period, the 20-mm-diameter mycelial mat was transferred to 10 ml of 0.2% aqueous Tween 80, vortexed for 5 min, and the conidial productivity was estimated with a haemocytometer.

Growth and conidiation of mycelial pellets on excised parts of the coconut palm

The following parts of the coconut palm (Purseglove 1972) were tested for their suitability as substrates for germination and conidiation of *H. thompsonii* mycelial pellets treated separately with the three selected adjuvants [glycerol, yeast extract powder (YEP) and malt extract broth (MEB)]: tepals or perianth lobes (Nos. 1, 2 & 3) (outer surface); tepals or perianth lobes (Nos. 4, 5 & 6) (inner surface); nut surface or exocarp (green portion of tender nut); healthy meristematic portion of tender nut; main axis or peduncle; sub-axis or short peduncle; leaflet (adaxial and abaxial surfaces); exposed root; trunk bark and suberised or necrosed tissue of mite-infested tender nut. These plant parts were excised into small pieces (2×2 cm^2 for flat parts, or 5 cm long for cylindrical parts) or used as such (only tepals) with each piece serving as a replicate. Fungal pellets treated for 30 min with the adjuvants (0.5%) were placed individually on the plant parts (5 pellets/part) kept in Petri dishes (3 replicates/part) lined with a thin layer of damp cotton and incubated at room temperature with a 12-h photoperiod. Observations were recorded for growth and conidiation of mycelial pellets frequently (at least three times in a 24-h period) for up to 96 h. Comparisons were made with pellets treated with sterile deionised water.

Effect of simulated sunlight on the conidiation of *Hirsutella thompsonii*

Mycelial beads of *H. thompsonii* treated with the three adjuvants in the same fashion as in the previous experiment were placed equidistant from each other in a Petri dish (90 mm diameter) (10 pellets/dish) lined with a circle of moistened filter paper. Pellets treated with

only sterile deionised water served as control. For each adjuvant and control, a set of six Petri dishes (without lids) containing the treated pellets was exposed to an irradiance of 500 W/m^2 for 1 h at 35°C in a sunlight simulator ('Suntest CPS+', Atlas Material Testing Technology, Linsengericht, Germany). A 1100-W air-cooled xenon arc lamp gave an output spectrum closely resembling sunlight in a total exposure area of 560 cm^2 inside the simulator chamber.

After sunlight treatment, the lids were replaced and two sub-sets of three Petri dishes each for the adjuvants and control were further incubated at alternating light–dark regime (12:12 h) and total darkness, respectively, for 48 h at room temperature. For non-irradiated control, a similar protocol was followed with Petri dishes enclosed in black paper while inside the simulator, but other incubation conditions remained the same. At the end of the incubation period, all pellets from each Petri dish were transferred to 5 ml of sterile deionised water containing 0.2% Tween 80, vortexed for 5 min, and the conidia per pellet were counted with a haemocytometer.

Pathogenicity of adjuvant-treated pellets

Before the field trial, the three best adjuvants were tested for their effect on the pathogenicity of *H. thompsonii* towards the coconut mite through a mortality-based assessment. Chips (20 mm diameter) were sliced from beneath the perianth of young, freshly harvested nutlets showing very high mite infestation (>20 live adult mites/mm^2) after carefully removing the bracts. The pellets treated as in the plant parts study were first allowed to germinate for 24 h and then transferred to the surface of the chip contained in the centre of a clean 200-mm glass Petri dish, at a rate of five pellets per chip. The Petri dishes arranged in this manner were then closed and kept at room temperature with a 12-h photoperiod. There were five replicate Petri dishes for each adjuvant and control. After 96 h, the rate of infection by *H. thompsonii* was determined by assessing the percentage of mites infected (those showing exiting hyphal strands) in a 4-mm-diameter area at three randomly selected places on the Petri dish surface (Sreerama Kumar 2007); they were treated in Nesbitt's clearing reagent, mounted in Hoyer's fluid, and examined with a phase-contrast microscope (McCoy et al. 1971).

Development of a mycelial formulation of *H. thompsonii*

For field evaluation, a mycelia-alone variant (Mycohit-M) of the already available powder formulation (Mycohit) was developed during 2006–2007. The isolate MF(Ag)66 constituted the active ingredient of the new product with a concentration of 2.5×10^6 colony-forming units (cfu) per gram. The formulation process and ingredients, including the carrier and the additives (or formulants) incorporated into the final product were the same as the original product. However, the finer details of the formulation are not disclosed in this paper.

Field trial

A field trial was conducted between September 2006 and June 2007 at a heavily infested coconut plantation in Huskuru village, Bangalore Rural district, Karnataka, India, for evaluating *H. thompsonii* mycelial formulation in combination with the three selected adjuvants against the coconut mite. Random pre-treatment grading of mature nuts in the

field indicated a high level of mite incidence as evidenced by a damage score of 4.0. A block of 84 palms (7 rows × 12 palms) at the centre of the grove was selected, out of which the first three rows were used as a set for the fungal treatment and the last two as a set for the chemical and control treatments, with a buffer of two untreated rows in between these sub-blocks. The individual treatments were randomised 12 times each within their respective sets.

The treatments were as follows: (1) *H. thompsonii* formulation (10 g l^{-1}) + glycerol (5 ml l^{-1}), (2) *H. thompsonii* formulation (10 g l^{-1}) + YEP (5 g l^{-1}), (3) *H. thompsonii* formulation (10 g l^{-1}) + MEB (5 g l^{-1}), (4) chemical acaricide (Triazophos 40% EC; Trifos 40®, Cheminova India Limited, Panoli, India) (5 ml l^{-1}), and (5) control (plain water).

After harvesting the mature coconuts from each experimental palm, the bunches were numbered by considering the fully open inflorescence as the first bunch and the preceding older bunches sequentially as second, third, etc. The second and third bunches were tagged by tying insulated electric wire of the best-visible colours, viz. black and red, respectively, at the base of the main axis or peduncle. For obtaining pre-treatment population data, the third nutlet from the bottom of the bunch was sampled from the fourth and fifth bunches. The population of live mites (all motile stages, i.e. larvae, nymphs and adults) was estimated at three places on the nut surface with the aid of a stereozoom microscope (63×) in the laboratory and expressed as mean number of live mites per mm^2. Following the pre-treatment sampling, all the bunches were treated with the specific spray fluid (2 l/palm) using a portable, lightweight, hand-compression sprayer (3.5 l capacity) ('Marut' MT-36, Aspee-American Spring & Pressing Works, Mumbai, India). All the spray fluids were prepared in plain water and applied thrice as sprays at fortnightly intervals during early mornings.

The post-treatment population count of the mite was recorded in all the palms 6 weeks after the first round of treatment. Population counts were made on two nuts, one each from both the tagged bunches, in the same way as pre-treatment analysis. Finally, during the pre-harvest stage, both the tagged bunches were cut off entirely from the palm and brought down for grading. The nuts were separated from the short peduncles from each bunch separately and were graded individually based on the damage caused by the mite. The grading system was as follows: 1: no damage, 2: 1–10%, 3: 11–25%, 4: 26–50%, and 5: >50% of damage with reduction in size and great distortion.

Data analysis

All laboratory experiments were performed twice and the field trial once. For the laboratory experiments, the results from only one trial are presented because a similar trend was observed between the trials with homogeneity of variances determined with Bartlett's test. A completely randomised design was used for all laboratory experiments. Data from all experiments were analysed by one-way analysis of variance (ANOVA), except the data from the sunlight study, for which two-way ANOVA was followed. Prior to analysis, the data from conidial counts were subjected to $\log(x)$-transformation to improve homogeneity of variances. Data on colony counts on the filter paper and pathogenicity were square-root-transformed ($\sqrt{x}$). The pre-treatment ($\sqrt{x}$) and post-treatment $[\sqrt{(x+0.5)}]$ data from the field trial were also subjected to square-root transformation. Treatment means were separated by Tukey's honestly significant difference (HSD) test or Bonferroni's test (only for sunlight study) at a significance level of $\alpha = 0.05$.

Statistical analysis was done using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com).

Results

Effect of adjuvants on the growth characteristics of *Hirsutella thompsonii*

The number of fungal colonies formed on the filter paper by *H. thompsonii* biomass in the presence of adjuvants varied significantly ($F_{9,20} = 35.38$, $P < 0.0001$). The most colonies (19.3) emerged from the biomass treated with glycerol (Table 2), which was followed by MEB and YEP treatments. The lowest number of colonies (2.3) was formed by gelatine- and nutrient broth-treated pellets. Hyphal development and extension occurred in less than 24 h only in glycerol treatment. In other treatments, it took anywhere between 24 and 48 h, except in the case of gelatine and nutrient broth, both of which took longer.

Several test adjuvants were able to take sporulation levels much higher than the untreated control ($F_{9,20} = 15.06$, $P < 0.0001$). Glycerol topped with the maximum conidial production of 4.92×10^4 conidia/pellet, which was 69.1% more than the control (Table 2). YEP and MEB treatments produced 4.50×10^4 and 4.45×10^4 conidia/pellet, respectively. Gelatine was the least effective among all the treatments with the lowest numbers of conidia (2.91×10^4/pellet).

In terms of conidia density generated on a 20-mm-diameter mycelial mat, the treatments varied significantly. In the first method ($F_{9,20} = 9.180$, $P = 0.0001$) of assessment in which adjuvants were added directly to the spent medium, the highest conidial production of 39.00×10^5/mycelial mat was observed in glycerol-treated fungal pellets after 30 days of incubation (Table 2). This quantity was 68.0% more than that obtained from control biomass. An increase of 62.4% and 60.6% conidia over control was observed with MEB and YEP. Gelatine-treated pellets yielded the fewest conidia. In the second method, wherein pellets were added to the adjuvant solution ($F_{9,20} = 46.00$, $P < 0.0001$), once again glycerol

Table 2 Effect of adjuvants on the growth characteristics of *Hirsutella thompsonii*

Adjuvant	No. of colonies/filter paper (±SE)	No. of conidia/pellet ($\times 10^4$) (± SE)	No. of conidia/20-mm-diameter mycelial mat ($\times 10^5$) (±SE)	
			Method 1	Method 2
Gelatine	2.3 ± 0.33 e	2.91 ± 0.23 d	20.39 ± 0.96 d	5.94 ± 0.20 d
Glycerol	19.3 ± 0.88 a	4.92 ± 0.15 a	39.00 ± 0.69 a	16.56 ± 0.48 a
Malt extract broth	10.0 ± 1.53 b	4.45 ± 0.09 bc	37.72 ± 1.59 a	14.17 ± 0.33 a
Nutrient broth	2.3 ± 0.33 e	3.07 ± 0.14 d	25.95 ± 2.04 bcd	8.56 ± 0.48 bc
Potato dextrose broth	5.3 ± 0.88 cd	3.35 ± 0.17 d	32.89 ± 4.77 abc	9.83 ± 0.10 b
Potato flour	7.3 ± 0.88 bc	3.37 ± 0.13 d	35.00 ± 2.62 ab	10.50 ± 0.91 b
Sabouraud dextrose broth	6.3 ± 0.67 bc	3.45 ± 0.09 cd	32.28 ± 0.62 abc	8.83 ± 0.50 bc
Skimmed milk powder	2.7 ± 0.33 d	3.04 ± 0.23 d	31.22 ± 0.39 abc	6.11 ± 0.49 d
Yeast extract powder	8.0 ± 0.58 bc	4.50 ± 0.13 ab	37.28 ± 1.27 ab	16.34 ± 0.33 a
Control	5.7 ± 0.67 cd	2.91 ± 0.23 d	23.22 ± 2.68 cd	6.78 ± 0.55 cd

Data in each column were subjected to one-way ANOVA. Means in each column followed by the same letter did not differ significantly (Tukey's HSD, $P > 0.05$)

treatment resulted in the maximum number of conidia (16.56×10^5/mycelial mat). Least effective was the gelatine treatment, which produced just 5.94×10^5 conidia.

Growth and conidiation of mycelial pellets on excised parts of the coconut palm

Conidiation of adjuvant-treated mycelial pellets occurred on various parts of the coconut palm but the progress of growth and conidiation was not uniform on all (Table 3). The progress of fresh fungal growth out of the pellets was the best on the nut surface or exocarp (green portion of tender nut). The spread of the fungus was very feeble on the inner and outer tepals. An unexpected shrinkage of the mycelial pellets was observed on the short peduncle as well as on the adaxial and abaxial surfaces of the leaflet.

Effect of simulated sunlight on the conidiation of *Hirsutella thompsonii*

Irradiance with simulated sunlight for 1 h resulted in reduced conidiogenesis by *H. thompsonii* (Table 4). Generally, adjuvant-treated pellets, both exposed and unexposed to simulated sunlight, produced substantial conidia compared with untreated control, irrespective of the two incubation conditions, i.e., alternating light–dark and total darkness. Better conidiation was observed under alternating light–dark regime than under total darkness in all the treatments ($F_{3,32} = 39.21$, $P < 0.0001$) (Table 4). However, there was no interaction effect ($F_{9,32} = 0.6778$, $P = 0.72$).

The three adjuvants shielded the pellets from adverse sunlight to certain extent and helped retain enough moisture to be able to undergo conidiogenesis successfully ($F_{3,32} = 19.91$, $P < 0.0001$). However, even glycerol, the best adjuvant, induced 25.6% fewer conidia per pellet after sunlight treatment and incubation under alternating light–dark conditions when compared with its conidia production due to unexposure and incubation under the same conditions.

Glycerol-treated pellets after exposure to sunlight produced 28.4% and 45.8% more conidia under alternating light-dark and totally dark regimes, respectively, when compared with water-treated, exposed pellets under the same incubation conditions.

Pathogenicity of adjuvant-treated pellets

Prior to field-testing of the fungus, the adjuvant-treated pellets were tested for pathogenicity towards the coconut mite. The adjuvants were found to significantly increase the pathogenicity of *H. thompsonii* towards the coconut mite ($F_{3,16} = 3.865$, $P = 0.0296$). Glycerol-treated pellets were the most effective in terms of the mortality caused, a 16.5% increase over control pellets, whereas mortality caused by pellets treated with the other two adjuvants was intermediate (Fig. 1).

Field trial

The pre-treatment counts of live mites per mm^2 of the nut surface just below the perianth ranged from 6.48 to 12.27 in the fourth bunch and from 3.46 to 6.65 in the fifth bunch, but there were no significant differences among the experimental palms in terms of mite population in the two bunches, both separately and collectively (Table 5).

A significant reduction in the post-treatment population of the coconut mite was observed in nut samples collected from the tagged bunch 1 ($F_{4,55} = 19.19$, $P < 0.0001$) as well as the

Table 3 Growth and conidiation of mycelial pellets of *Hirsutella thompsonii* treated separately with three selected adjuvants on different parts of the coconut palm

Plant part	Progress of growth and conidiation of mycelial pellets of *Hirsutella thompsonii* during*			
	0–24 h	24–48 h	48–72 h	72–96 h
Tepals or perianth lobes (Nos. 1, 2 & 3) (outer surface)	Hyphal formation, extension and sparse phialide initiation	Sparse to moderate conidiation	No spread of the fungus on the substrate	Shrinkage of pellets
Tepals or perianth lobes (Nos. 4, 5 & 6) (inner surface)	Hyphal formation, extension and phialide initiation	Hyphal extension, heavy phialide formation and conidiogenesis	Sparse spread of the fungus on the substrate, continued conidiation and release	Further conidiation
Nut surface or exocarp (green portion of tender nut)	Hyphal formation, extension, phialide initiation	Hyphal extension, heavy phialide formation and conidiogenesis	Spread of the fungus on the substrate, continued conidiation and release	Total maturity of conidia and release
Meristematic portion of tender nut	Hyphal formation, extension and phialide initiation	Hyphal extension, heavy phialide formation and conidiogenesis	Sparse spread of the fungus on the substrate, continued conidiation and release	Further conidiation [Shrinkage of pellets in control]
Main axis or peduncle	Hyphal formation, extension and phialide initiation	Hyphal extension and phialide formation	Conidiation	Further conidiation
Sub-axis or short peduncle	Hyphal formation, extension, phialide initiation	Hyphal extension and phialide formation	Conidiation [Shrinkage of pellets in control]	Shrinkage of pellets
Leaflet (adaxial surface)	Hyphal formation and extension	Sparse phialide formation	Conidiation [Shrinkage of pellets in control]	Shrinkage of pellets
Leaflet (abaxial surface)	Hyphal formation and extension	Sparse phialide formation	Conidiation [Shrinkage of pellets in control]	Shrinkage of pellets
Exposed root	Hyphal formation and extension	Hyphal extension and phialide formation	Conidiation	Further conidiation
Trunk bark	Hyphal formation, extension and phialide initiation	Hyphal extension and phialide formation	Conidiation	Further conidiation
Suberised or necrosed tissue of mite-infested tender nut	Hyphal formation, extension and phialide initiation	Sparse phialide formation	Conidiation	Further conidiation

* Results for the three adjuvants were similar. The deviations observed in control pellets are indicated within square brackets inside the table

Table 4 Effect of sunlight on the conidiation of *Hirsutella thompsonii* pellets treated separately with three selected adjuvants

Treatment	No. of conidia/pellet ($\times 10^4$) ($\pm$ SE)			
	Simulated sunlight (exposed)		Simulated sunlight (unexposed)	
	Alternating light-dark	Totally dark	Alternating light-dark	Totally dark
Glycerol	3.66 ± 0.13 a	3.31 ± 0.15 a	4.92 ± 0.15 a	4.30 ± 0.32 a
YEP	3.18 ± 0.20 ab	3.09 ± 0.05 a	4.46 ± 0.13 ab	4.23 ± 0.15 a
MEB	3.00 ± 0.31 ab	2.40 ± 0.09 b	4.19 ± 0.34 ab	3.47 ± 0.13 ab
Control	2.85 ± 0.16 b	2.27 ± 0.16 b	3.74 ± 0.29 b	3.09 ± 0.32 b

Means in each column followed by the same letter did not differ significantly (Bonferroni test, $P > 0.05$; after two-way ANOVA)

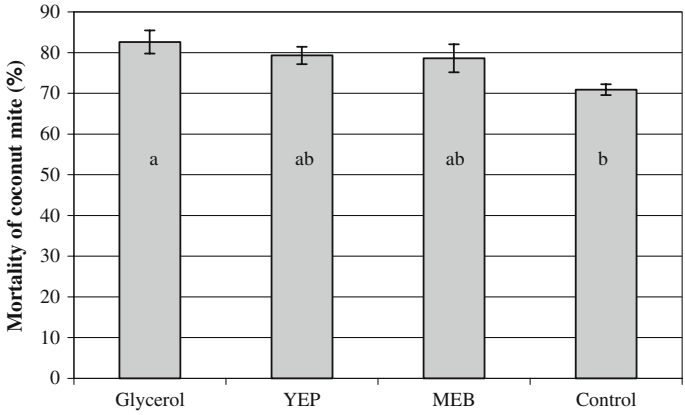

Fig. 1 Pathogenicity of *Hirsutella thompsonii* treated with three selected adjuvants against the coconut mite (mean mortality ± SE). Bars with the same letter did not differ significantly (Tukey's HSD, $P > 0.05$; after one-way ANOVA)

tagged bunch 2 ($F_{4,55} = 9.951$, $P < 0.0001$) of the palms applied with *H. thompsonii* in combination with glycerol, YEP or MEB (Table 5). The fungus was able to cause disease in the mite on all the sprayed palms as evidenced during the post-treatment sampling.

In both the tagged bunches, the fungus in the presence of MEB performed the best with an overall reduction of 97.1% in the mite population over control. This treatment was even better than the chemical, triazophos. Glycerol and YEP could also bring about an overall decline in live mites over control, namely 92.5% and 85.6%, respectively.

In terms of the pre-harvest damage grades, all the fungal treatments were on a par with the chemical and superior to control in both the tagged bunch 1 ($F_{4,55} = 18.45$, $P < 0.0001$) and the tagged bunch 2 ($F_{4,55} = 24.16$, $P < 0.0001$) (Table 5).

Discussion

The feasibility of using *H. thompsonii* in the mycelial stage itself to suppress the coconut mite has been demonstrated through the present studies. Several adjuvants have been found to have an additive effect on the performance of the fungus against the coconut mite.

Table 5 Effect of *Hirsutella thompsonii* combined separately with three selected adjuvants on the coconut mite population and pre-harvest nut damage in the field

Treatment	No. of live mites/mm^2 (±SE)						Pre-harvest nut damage grade (±SE)		
	Pre-treatment			Post-treatment					
	4th bunch	5th bunch	Mean	Tagged bunch 1	Tagged bunch 2	Mean	Tagged bunch 1	Tagged bunch 2	Mean
H. thompsonii + glycerol	6.48 ± 1.28 a	3.97 ± 0.60 a	5.23 ± 0.74 a	0.71 ± 0.71 a	0.96 ± 0.65 a	0.84 ± 0.67 a	1.9 ± 0.19 a	2.0 ± 0.22 a	2.0 ± 0.19 a
H. thompsonii + YEP	7.73 ± 1.24 a	6.65 ± 1.87 a	7.19 ± 1.30 a	1.18 ± 0.62 a	2.04 ± 0.98 a	1.61 ± 0.52 a	2.3 ± 0.26 a	2.0 ± 0.15 a	2.1 ± 0.18 a
H. thompsonii + MEB	11.91 ± 2.31 a	4.64 ± 1.33 a	8.28 ± 1.36 a	0.11 ± 0.11 a	0.52 ± 0.24 a	0.32 ± 0.15 a	2.1 ± 0.22 a	2.2 ± 0.20 a	2.2 ± 0.17 a
Triazophos	9.40 ± 1.47 a	3.46 ± 0.71 a	6.43 ± 0.91 a	0.56 ± 0.56 a	0.86 ± 0.41 a	0.71 ± 0.45 a	2.0 ± 0.18 a	2.0 ± 0.20 a	2.0 ± 0.13 a
Control	12.27 ± 2.93 a	5.36 ± 1.28 a	8.82 ± 1.86 a	11.61 ± 2.63 b	10.76 ± 3.34 b	11.19 ± 2.83 b	4.0 ± 0.09 b	4.1 ± 0.14 b	4.0 ± 0.11 b

Pre-treatment sampling, first, second and third applications of treatments, post-treatment sampling and pre-harvest nut damage grading were done sequentially on 26 & 27 September, 5 & 6 October, 19 October, 1 & 2 November, 17 & 18 November 2006 and 26 & 27 June 2007. Means in each column followed by the same letter did not differ significantly (Tukey's HSD, $P > 0.05$; after one-way ANOVA)

Out of the nine adjuvants evaluated, glycerol, YEP and MEB consistently performed well in all the laboratory experiments (Table 2) and so were further taken up to the field trial. Potato dextrose broth, potato flour and SDB, though not found to be as competent as these other three, could still be considered for field use after additional tests. The laboratory studies also indicated that not all substances with recognised nutrient or humectant qualities would augment the performance of *H. thompsonii*. For example, skimmed milk powder, gelatine and nutrient broth were either ineffective or only slightly better than control. Assigning the exact reason for the difference in performance of each of these test adjuvants seems impossible because of the differences in their macro- and micronutrient contents and their availability to the fungus.

McCoy et al. (1971) cited several advantages of using fragmented mycelium of *H. thompsonii* on citrus foliage, such as elimination of surface phase for sporulation during production, avoiding problems associated with the loss of conidial viability prior to use and after application in the field, etc. Later, McCoy and Couch (1982) demonstrated the utility of certain adjuvants to stimulate conidiation of *H. thompsonii* on citrus foliage.

In this work, the growth and conidiation of adjuvant-treated mycelial beads of *H. thompsonii* apparently varied among the different plant parts used as substrates for the fungus. The fungus was able to put forth new hyphae and conidiate profusely on several plant parts, more so on the nut surface, indicating the possible additive effect of the treatments under the actual field situation (Table 3). However, the variations could be explained as the impact of the texture of the plant surface as well as the interaction of the resident microflora with the fungus. Tank-mixed nutrients enable biopesticide fungi to grow and sporulate on the plant parts thus aiding continual infection of surviving mites. McCoy et al. (1975) used unsulphured molasses or citrus molasses as adjuvants along with fragmented mycelia against the citrus rust mite, and achieved effective control of the pest.

The three adjuvants did not affect the pathogenicity of *H. thompsonii*. The fungus treated with selected adjuvants in fact caused higher mortality of the mite in the laboratory experiment (Fig. 1). The most apparent reason for the increased mite mortality could be the ability of the adjuvants to raise the inoculum levels through enhancing the conidiation of the fungus during the incubation time.

Exposure to simulated sunlight affected the conidiogenesis of *H. thompsonii* (Table 4). Adjuvant-treated pellets, both exposed and unexposed to simulated sunlight, produced appreciable numbers of conidia compared with control, irrespective of the two incubation conditions. The pellets exposed to simulated sunlight rapidly lost considerable moisture but could recover later during the incubation putting forth numerable sporulating hyphae and a few distorted phialides. Overall, better conidiation was observed under alternating light–dark regime than under total darkness in all the treatments, which led to the application of the fungus in the early morning hours in the field. The number of mites that come in contact with the inoculum would be more during the night, by which time conidiogenesis of the fungus would have commenced. The coconut mite usually comes out of the perianth between 02:00 and 06:00 hours (a.m.) and mostly moves to other nuts (Moore and Alexander 1987), thus getting infected and at the same time spreading the disease.

Glycerol, the most effective adjuvant, has excellent hygroscopic property (Burges 1998) and may have conserved moisture better in the pellets even after the sunlight treatment to enable the fungus to conidiate post exposure. Though hyphomycetous fungi are highly susceptible to damage by solar radiation (Goettel and Inglis 1997; Inglis et al. 2001), the extraordinary ability of *H. thompsonii* to photoreactivate after damage induced by exposure to far-ultraviolet light (FUV; 200–300 nm) has been demonstrated (Kenneth et al. 1979; Tuveson and McCoy 1982). Similarly, Kenneth et al. (1979) also found no

differences between FUV-treated and untreated *H. thompsonii* mycelium. Nevertheless, in the case of *H. thompsonii* on coconuts, sunlight may not always be as critical as it may be to the fungi applied to the exposed foliage of other crops because of lower penetration of the light rays through the coconut crown.

The general incidence of the coconut mite at the field trial site had not come down during the experiment period of nine months as observed in control palms that still scored 4.0 at the end of the trial (Table 5). There was actually a slight increase of 21.2% in the population of the mite over the period of less than 2 months from pre-treatment to post-treatment analyses as detected in control palms. However, this potential increase was negated by the application of *H. thompsonii.* In the 36 palms that constituted the three fungal treatments, the combined mean post-treatment mite population was a staggering 86.7% less than that of the mean pre-treatment population. This population collapse was aided by the adjuvants presumably by their humectant, nutrient and adhesive qualities (Burges 1998). By absorbing water in the moist night and slowly losing it in the dry daytime, these nutrients might have acted as water-availability buffers for *H. thompsonii* (Burges 1998). This effect was anticipated to happen in the field on the basis of the laboratory study (Fig. 1) in which an increase in coconut mite mortality resulted due to addition of adjuvants to *H. thompsonii* inoculum. Also, the unique microclimatic conditions within the coconut crown might have supported the survival, development and initiation of disease by the fungus. While the temperature within the crown tends to be below the ground level temperature, the relative humidity tends to be higher inside the crown (Sreerama Kumar, unpublished observations).

Fragmentation of mycelium through the use of polyethylene glycol in the production medium has been achieved (Sreerama Kumar et al. 2005) but shelf-life problems, if any, need to be sorted out during the commercialisation process. Nevertheless, for a pest like *A. guerreronis* that is widespread and prevalent throughout the year on coconuts, myco-acaricide shelf-life problems could be tackled through innovative marketing and immediate use, as in the case of the mycoherbicide 'DeVine' in USA (Kenney 1986). Multilocation trials are going on in six states and the final results will be examined closely to arrive at a decision on the recommendation of mycelial application of *H. thompsonii* against the coconut mite in India.

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A tale of three acaropathogenic fungi in Israel: *Hirsutella*, *Meira* and *Acaromyces*

U. Gerson · A. Gafni · Z. Paz · A. Sztejnberg

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Abstract We review published and unpublished studies conducted in Israel with six acaropathogenic fungi, assayed in order to control the citrus rust mite, *Phyllocoptruta oleivora* (Ashmead) (CRM). *Hirsutella thompsonii* Fisher was introduced twice, killed 80–90% of the exposed mites, but due to its requirements for near-saturation humidities was deemed unsuitable for local outdoors conditions. *Hirsutella kirchneri* (Rostrup) Minter et al. and *Hirsutella necatrix* Minter et al. were also introduced and assayed against CRM and spider mites, but their efficacy was unsatisfactory. Three indigenous fungi found to be associated with mites, *Meira geulakonigii*, *Meira argovae* and *Acaromyces ingoldii*—all three recently described by Boekhout, Gerson, Scorzetti & Sztejnberg—were assayed against several mites. *Meira geulakonigii* killed 80–90% of several spider mites and of the CRM, and caused some mortality of *Iphiseius degenerans* (Berlese), one out of three phytoseiid predators assayed. Mortality was not due to parasitization; extracts from the media in which the fungi had developed caused considerable mite death, suggesting that it was a result of fungal toxins. Data from a field study indicated that spraying blastoconidia of *M. geulakonigii* on grapefruits infested by CRM significantly reduced pest-incurred damage from 23 to 13%. Applying qRT-PCR methodology indicated that *M. geulakonigii* was endophytic within sealed grapefruit flowers and in the flavedo of the fruits' peel. Neither in the laboratory nor in the field was any evidence ever obtained that this fungus damaged the plants, leading us to hypothesize that *M. geulakonigii* serves as a "body guard" of grapefruits (and perhaps other plants as well). All three fungi suffered very little mortality after being exposed to various insecticides and acaricides that are in current local use (with the exception of sulfur). The ability of *M. geulakonigii* to reduce mite numbers without affecting the host plant, the minimal fungal effect on some predatory mites, its endophytic nature

U. Gerson (✉)
Department of Entomology, Faculty of Agricultural, Food and Environmental Quality Sciences,
The Hebrew University of Jerusalem, P.O. Box 12, 76100 Rehovot, Israel
e-mail: Gerson@agri.huji.ac.il

A. Gafni · Z. Paz · A. Sztejnberg
Department of Plant Pathology and Microbiology, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, 76100 Rehovot, Israel

along with the apparent tolerance of *M. geulakonigii* to many insecticides and acaricides, suggest that this fungus could be suitable for integrated pest management (IPM) program.

Keywords Acaropathogenic fungi · *Hirsutella* · *Meira* · *Acaromyces* · Citrus rust mite

Introduction

Mites (Acari) are major plant pests whose control is increasingly becoming more problematic. Acaricides are expensive from the economic as well as environmental point of view and the target mites rapidly develop resistance to new products. Biological control by mites (Gerson et al. 2003) is an attractive possibility as are acaropathogenic fungi, which are a growing component in the non-chemical arsenal available to control pestiferous mites. Recent examples include testing such fungi against the cassava green mite (*Mononychellus tanajoa* Bondar) (Hountondji et al. 2007; Dara 2007) and against the invasive *Tetranychus evansi* Baker & Pritchard (Wekesa et al. 2006). Many of these fungi (or strains thereof) also affect insects, but a few appear to affect only mites. Van der Geest et al. (2000) compiled a list of fungi that are specific and pathogenic to mites. Of these, only a few—*Hirsutella kirchneri* (Rostrup) Minter, Brady & Hall, *H. nodulosa* Petch, *H. thompsonii* Fisher, *Neozygetes floridana* (Weiser & Muma) and *Paecilomyces eriophytis* (Massee) Leatherdale—were recorded from more than one or two acarine hosts. Chandler et al. (2000) listed fungi that were reported to infect mites, including some (e.g., *Metarhizium anisopliae* (Metchnikoff) Sorokin) that have a broad host range and may not be specific. Since then we have added three acaropathogenic species that appear to be indigenous to Israel. These are *Meira geulakonigii* Boekhout, Gerson, Scorzetti & Sztejnberg, *M. argovae* Boekhout, Gerson, Scorzetti & Sztejnberg and *Acaromyces ingoldii* Boekhout, Gerson, Scorzetti & Sztejnberg, which were assayed against several citrus mites (Paz et al. 2007a).

Here we review our past and on-going efforts to control pestiferous mites with three species of *Hirsutella*, two species of *Meira* and with *A. ingoldii*. Our main efforts were aimed at controlling the citrus rust mite (CRM), *Phyllocoptruta oleivora* (Ashmead) (Eriophyidae), the major pest of citrus in Israel, which is becoming increasingly difficult to control by chemical means (Palevsky et al. 2003). The efficacy of the fungi was also tested against several other acarine citrus pests, such as the oriental spider mite, *Eutetranychus orientalis* (Klein) and the red citrus mite, *Panonychus citri* (McGregor).

Hirsutella spp.

Hirsutella thompsonii, the best-studied species in this genus, was initially discovered and described from Florida (Fisher 1950), where it was found to attack and kill CRM. The application of this fungus against rust and bud mites, spider mites and the broad mite, *Polyphagotarsonemus latus* (Banks) was reviewed several times (Chandler et al. 2000; van der Geest et al. 2000), and more recently it was tried against the varroa mite, *Varroa destructor* Anderson & Trueman (Kanga et al. 2002). Aside from North America, the fungus has been isolated from different parts of the world, including Poland (Miętkiewski et al. 2000), India (Kumar and Anuroop 2004), The Philippines (Rombach et al. 1986), Africa (Odongo et al. 1998) and Brazil (van der Geest et al. 2000).

In the early 1970s we introduced a culture of *H. thompsonii* (accession number HTRM-3) from the University of Florida at Lake Alfred, maintained it on 3.9% w/v potato dextrose

agar (PDA) at 25°C in Petri dishes, and mass-produced the fungus on unprocessed wheat bran flakes (Kenneth et al. 1979). Its effect on mites assignable to several acarine taxa (Table 1) was assayed by placing them for 1–2 h on sporulating fungal mats and then removing them to suitable substrates or leaving them in situ (Gerson et al. 1979). The two citrus pests, *Eutetranychus orientalis* and the carmine spider mite, *Tetranychus cinnabarinus* (Boisduval), were transferred to citrus leaves maintained at 25°C and 100% relative humidity (r.h.). *Tyrophagus putrescentiae* (Schrank), *Rhizoglyphus robini* Claparède, *Tarsonemus* sp., *Nothrus biciliatus* (Koch) and *Parasitus fimetorum* (Berlese) were left on the mats. The tick *Argas persicus* (Oken) was kept within a vial placed at 100% r.h. The fungus infected and killed >80% of the citrus pests (all mortality values corrected according to Abbott 1925), whereas *T. putrescentiae*, *R. robini*, *Tarsonemus* sp. and *N. biciliatus* fed, developed and oviposited on the mycelium. In addition, the fungus had no effect on *P. fimetorum* or on the tick (Table 1).

These results led to an experiment in which 300 fungus-inoculated *T. cinnabarinus*, in batches of 100, inoculated as above, were exposed to various humidity conditions at 25°C. One batch (a) was kept for 18 h at ca. 50% r.h., followed by 6 h at saturation, approximating outdoors conditions along the coastal plain of Israel in the summer. A second batch (b) was held for 6 h at ca 50% r.h., and then for 18 h at saturation, and the third (c) was maintained throughout at 100% r.h. Mortality in the latter treatment came to 98%, but in experiment (a) it reached only 65%, and in (b) to 74%. In another experiment, intended to simulate summer greenhouse conditions, conducted like (a) but this time the mites were transferred from 25°C for 18 h at 50% r.h. to 33°C at saturation, their mortality reached

Table 1 Infection rates of mites representing pests, natural enemies, scavengers and a tick in several families and orders by *Hirsutella thompsonii* (HT; data from Gerson et al. 1979), *Hirsutella kirchneri* (HK; data from Sztejnberg et al. 1997; Gafni 1997) and by *Hirsutella necatrix* (HN; data from Gafni 1997)

Mites	Economic status	Infectivity (%)		
		HT	HK	HN
Tetranychidae				
Eutetranychus orientalis	Minor plant pest	80	70–80	16
Panonychus citri	Major citrus pest	NT	90	0
Tetranychus cinnabarinus	Major plant pest	84	70–80	17–25
Eriophyidae				
Phyllocoptruta oleivora	Major citrus pest	NT	90	NT
Tarsonemidae				
Polyphagotarsonemus latus	Major plant pest	NT	0	NT
Tarsonemus sp.	Scavenger	0	NT	0
Acaridae				
Tyrophagus putrescentiae	Scavenger	0	0	0
Rhizoglyphus robini	Major plant pest	0	0	NT
Hemisarcoptidae				
Hemisarcoptes coccophagus	Parasite of armored scales	NT	50	NT
Nothridae				
Nothrus biciliatus	Scavenger	0	NT	NT
Parasitidae				
Parasitus fimetorum	General predator	0	NT	NT
Phytoseidae				
Typhlodromus athiasae	General predator	NT	0	NT
Argasidae				
Argas persicus	A tick, parasite of poultry	0	NT	NT

NT not tested

70%. These results indicated that under the prevailing summer conditions in Israel, *H. thompsonii* would not provide satisfactory carmine spider mite control either outdoors, where relative humidities seldom reach even 80%, or in greenhouses, wherein summer temperatures may be quite high (Gerson et al. 1979). A further discouraging opinion, for similar reasons, about mite control with *H. thompsonii* under protected cultivation was expressed by Rombach and Gillespie (1988).

In the early 1990s we introduced two other isolates of *H. thompsonii*, one originating from Papua New Guinea (ARSEF 414), the other from Zimbabwe (ARSEF 255). Both were obtained from the Collection of Entomopathogenic Fungi of the USDA at Ithaca, New York, and were cultured as above. Their effect on *T. cinnabarinus* was assayed by spraying the mites with suspensions (ca. 10^7 spores/ml) of the two isolates while maintained on green lemons. The experimental mites, along with controls (sprayed with sterile water only) were then kept at 25°C and observed for 3 days. By that time isolate 414 killed ca. 80% of the mites, isolate 255 only 35% (Chernin et al. 1997).

Having found that *H. thompsonii* may reduce mite numbers only at or very close to air moisture saturation, as well as being variable in its lethality, we tried two other options in efforts to control CRM. The first was to obtain, through hyphal anastomosis, intraspecific heterokaryons that would be less susceptible to aridity. Although we obtained several heterokaryons that were distinguishable by random amplified polymorphic DNA (RAPD) markers and by different α-esterase isoenzyme patterns (Mozes-Koch et al. 1995), none were suitable for our purpose (unpublished data).

The second approach was to introduce two other reported acaropathogenic species, namely *H. kirchneri* and *Hirsutella necatrix* Minter, Brady & Hall. Both were originally isolated from the cereal rust mite, *Abacarus hystrix* (Nalepa), infesting *Lolium* in Berkshire, UK (Minter et al. 1983). Cultures of both were obtained from the International Mycological Institute, Surrey, UK, in September 1990. The culture of *H. kirchneri* was original accession number IMI 257456, and that of *H. necatrix* was IMI 252317.

Hirsutella kirchneri has been reported from several parts of the world (aside from the UK), such as Poland, infecting eriophyid mites (Miętkiewski et al. 2000), Australia, from the cyclamen mite, *Phytonemus pallidus* (Banks) (unpublished data), and Cuba (Cabrera and Dominguez 1987), where it was isolated from *P. oleivora*. The latter host report indicated that this fungus was a suitable candidate for our purpose. It was cultured and candidate host mites (some of which were challenged earlier and others that were not; Table 1) were inoculated, as above. *Panonychus citri*, *T. cinnabarinus*, *P. oleivora* and *E. orientalis* suffered high (70–90%) mortality within 3 days (Fig. 1 shows infected *P. oleivora*). The only other infected species was *Hemisarcoptes coccophagus* Meyer, the scale insect parasite (Sztejnberg et al. 1997). *Hirsutella kirchneri* killed mites at humidities somewhat lower than 100% and also differed from *H. thompsonii* in other aspects (see below).

Aside from the UK, *H. necatrix* was isolated in Poland from an eriophyid mite (Miętkiewski et al. 2000) and in China, from a plant hopper (Bao et al. 1990). We cultured this fungus as above and the candidate mites were likewise inoculated. However, in contrast to the other two species of *Hirsutella*, it caused little mortality, coming only to 16% in *E. orientalis* and 17–25% in *T. cinnabarinus*. These results are consistent with the data of Lewis et al. (1981), who observed that *H. necatrix* (initially believed to be *H. thompsonii*) caused 16% mortality of the cereal rust mite.

Believing that the chitinolytic activity of these fungi may shed some light on their pathogenicity to mites, we looked at this activity in *H. necatrix* and two isolates of *H. thompsonii* (414 and 255, noted above). All three produced chitinase, but 414 and 255 produced more than *H. necatrix*. In addition, the latter failed to produce elastase and differed from the

Fig. 1 *Hirsutella kirchneri* growing out of the body of the citrus rust mite, *Phyllocoptruta oleivora*, and detail. Note the form of the conidiogenous cells bearing the conidia (from Doron-Shloush 1995)

H. thompsonii isolates in the pattern of its chitinases and proteases. We hypothesized that these enzymatic differences are consistent with the lower lethality of *H. necatrix* to mites. The (albeit reduced) ability of this fungus to kill mites is probably due to enzymes that in part substitute for its elastase and chitinase deficiency (Chernin et al. 1997).

All three fungi can be cultured on bran and also grew saprophytically on the cadavers of various insects, which they probably invade via the thin intra-segmental membranes or the tracheae, and may be one means that enables their survival in the field.

To conclude, our efforts with the three species of *Hirsutella* indicated that they were inadequate for the control of *P. oleivora* in our region.

Meira and *Acaromyces*

Laboratory studies

Our failures with *Hirsutella* spp. led us to search for and examine cadavers of *P. oleivora* and other phytophagous mites that had died in the field without any apparent cause, e.g., in unsprayed citrus orchards. These efforts culminated in the finding, isolating and identifying of the indigenous fungi *M. geulakonigii* and *A. ingoldii* from cadavers of *P. oleivora*, and *M. argovae* from *T. cinnabarinus* and *P. oleivora* (full details in Boekhout et al. 2003). In a preliminary experiment *M. geulakonigii* (10^9 blastoconidia/ml) was sprayed onto *P. oleivora* and *Pa. citri*, maintained on citrus seedlings in the laboratory, causing ca. 80% mortality within 1 week. This led us to explore the effect of all three fungi on these and other mites. The target mites included three species of citrus, *E. orientalis*, *Pa. citri* and *P. oleivora*, along with *T. cinnabarinus* and *T. urticae*, who seldom infest citrus, but are major pests of other crops. All were maintained on 2-months-old (two-leafed) sour orange (*Citrus aurantium*) seedlings in the laboratory, at 25°C and under a 12L:12D photoperiodic regime. The mites were separately sprayed (10^8 blastoconidia/ml) with each of the three fungi and observed after 1 week and after 2 weeks. In addition, the fungi were assayed against the non-target *R. robini* and the predatory phytoseiid mites *Phytoseiulus persimilis* Athias-Henriot, *Iphiseius degenerans* Berlese and *Neoseiulus californicus* (McGregor). Individuals of *R. robini* were placed on cultures of the three fungi maintained on PDA in Petri dishes. The predators were kept on young citrus seedlings, *P. persimilis* was supplied with prey (*T. cinnabarinus*), and the other two with common reed (*Typha domingensis*) pollen. All three were sprayed as above and examined 1 week later.

The results with the five phytophagous species (Fig. 2) show that all tested fungi affected all mites, with the exception of *M. argovae* being harmless to *T. urticae*. The

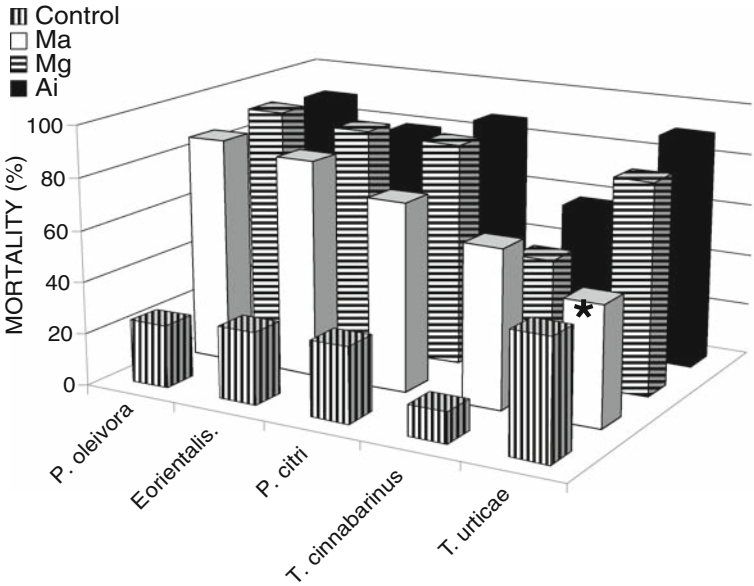

Fig. 2 Percentage mortalities of *Phyllocoptruta oleivora*, *Eutetranychus orientalis*, *Panonychus citri*, *Tetranychus cinnabarinus*, and *Tetranychus urticae*, sprayed either with *Meira argovae* (*Ma*), *Meira geulakonigii* (*Mg*) or with *Acaromyces ingoldii* (*Ai*) (all at 10^8 blastoconidia/ml). Asterisk on column showing effect of *M. argovae* on *T. urticae* denotes the only result that is not significantly different from the controls (Modified from Paz et al. 2007a)

pattern of lethality was variable for the fungi as well as for the five target mites, variation that is expressed when the data are separately observed after 1 week and after 2 weeks. Mortality rates for *P. oleivora*, *E. orientalis* and *Pa. citri* did not change much by the second week, but those of *T. cinnabarinus* increased two- or threefold as a result of being exposed to all three fungi, and those of *T. urticae* almost threefold after being sprayed by *M. geulakonigii* (Paz et al. 2007a). These results indicate that there is much selectivity in the effect of the fungi on susceptible mites, which could be exploited when using them either alone or in combination with other natural enemies.

Of the other mites assayed, *R. robini* fed on the mycelium of all three fungi, suffering no ill-effects. The predators *P. persimilis* and *N. californicus* were unaffected by all three fungi. On the other hand, *I. degenerans* showed considerable mortality after being exposed to the two *Meira* spp., but was unaffected by *A. ingoldii* (Fig. 3). The variability in these data is consistent with the variable effects of these fungi on the phytophagous mites, probably due to the different toxins that they produce.

A unique feature of all three fungi was that they did not invade the bodies of the affected mites, which usually died without physical contact with the various fungi. However, the fungi did grow on the mites' cadavers (Paz et al. 2007a).

A field study

The effect of *M. geulakonigii* on *P. oleivora* was assayed in the same organic grapefruit orchard whence the fungus was initially discovered. Observations conducted over several years showed that the pest occurred there only for a limited period of time, from mid-summer on, and was at most causing minimal damage (Z. Barkay, personal communication). Several trees were sprayed in our assay by 1-l suspension (containing 10^8 blastoconidia/ml)

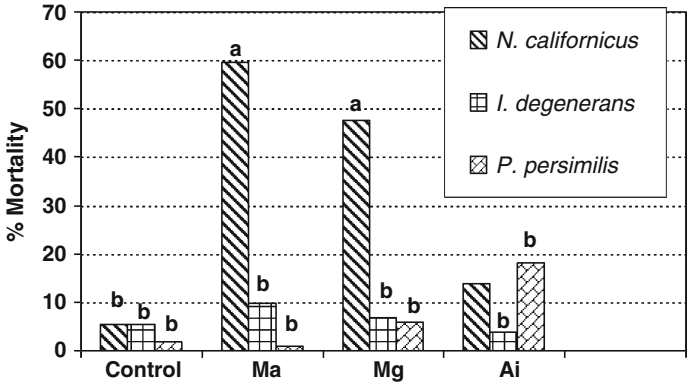

Fig. 3 Mortality (%) of *Neoseiulus californicus*, *Iphiseius degenerans*, and *Phytoseulus persimilis*, sprayed with *Meira argovae*, *Meira geulakonigii* or *Acaromyces ingoldii* (all at 10^8 blastoconidia/ml). Different letters indicate significant differences between treatments ($P < 0.05$; from Paz 2007)

per tree, either once a month (for five consecutive months), or once a season; control grapefruits received only water. Mites were counted within two randomly chosen areas of 1 cm^2 from each of five fruits collected arbitrarily. At the end of the season fruits from all three treatments were assessed for damage (extent of russeting). Mite numbers were similar on fruits sprayed every month and on those treated only once per season, but were significantly lower than on the control fruits (Table 2). Damage was similar on fruits of both groups of sprayed grapefruits (ca. 12.5%), as compared to almost 23% in the control (unsprayed) fruits. A supplementary spray of *M. geulakonigii* may thus protect grapefruits against the injurious *P. oleivora* and increase their market value (Paz et al. 2007a).

Grapefruit leaf, flower bud and fruit samples were regularly taken throughout the trial and separately examined for the presence of *M. geulakonigii* in external washings and within the leaves and the fruits. Using conventional mycological methods, the fungus could be isolated neither from the phyllosphere nor from the fruits' surface. Given that mite damage was clearly reduced, this conundrum led to the hypothesis that the fungus may be endophytic, not epiphytic (Paz et al. 2007b). Three methods, suggested by Stone et al.

Table 2 The effects of a single (seasonal) or of monthly applications of 1-l of *Meira geulakonigii* suspension (10^8 blastoconidia/ml) per tree on the populations of *Phyllocoptruta oleivora* that infest grapefruits, during two summers

Year	Month	Single application	Monthly applications	Control
2003	Early July	0.07	0.07	0.07
	Late July	0.4	0.4	17.1
	Late August	64.3	56.0	137.6[a]
	September	82.0	83.0	45.0
2004	Mid-July	0.7	0.7	0.7
	Early August	7.8	7.8	9.4
	Mid-August	76.5	105.4	198.0[a]
	Early September	72.6	63.4	131.5[a]
	Mid-September	9.7	3.0	5.1

Mites were counted in two randomly chosen areas of 1 cm^2 grapefruit from five fruits collected haphazardly

[a] Control values that are significantly different within the same row (modified from Paz et al. 2007a)

(2000), were used to test this hypothesis: (1) isolating the fungus from the internal parts of grapefruit peel; (2) visualization of peel cross sections by scanning electron microscopy, and (3) use of a fungus-specific primer for PCR amplification. The latter detected fungal traces in the peel flavedo (exocarp), and SEM images and fungal isolations confirmed this result. A suitable PCR and a quantitative real time (qRT)-PCR-based method were also developed to assess and to quantify the occurrence of *M. geulakonigii* inside the grapefruit peel.

Use of the qRT-PCR methodology enabled us to confirm the presence of the fungus in sealed flower samples and in the flavedo of the fruits' peel, and it was also detected in washings of grapefruit leaves, but not within their tissues. The possibility of inoculating *M. geulakonigii* into pristine grapefruits was explored by using plant material obtained from a commercial orchard where the absence of *M. geulakonigii* was first determined by PCR. The plant material was sprayed with 30 ml suspensions of *M. geulakonigii* (10^8 blastoconidia/ml) per grapefruit. *Meira geulakonigii* was detected on the fruit skin and in the flavedo from the first day and up to 60 days after application, although at and after 30 days the fungal population declined (Paz et al. 2007b).

A comparative experiment was conducted with a similar suspension of *M. argovae*, but no trace of this fungus was found in the inoculated grapefruits using species-specific primers (unpublished data).

No evidence was obtained either in the laboratory or in the field studies that these fungi caused any damage to the plants.

How do the fungi kill mites?

No evidence was found that *M. geulakonigii* invades the bodies of the affected mites, which often died without obvious physical contact with the fungi. This led us to examine the hypothesis that mite mortality may be due to toxic metabolites secreted from this fungus. Crude extracts of *M. geulakonigii* applied against *P. oleivora* caused their rapid, total mortality (100% after 24 h). In addition, *M. argovae* also secreted some metabolites and a spray thereof was toxic to *P. oleivora*. Work is continuing on its isolation by using HPLC methodology (unpublished). In consequence, it appears that the fungi (at least the two *Meira* spp.) kill mites by their toxic secretions.

Effect of pesticides

With the view of using *A. ingoldii*, *M. argovae*, and *M. geulakonigii* within integrated pest management (IPM) programs, their susceptibilities to a series of insecticides and acaricides (all currently used in Israel), was tested by applying the methodologies of Torgeson (1967). Colony growth was assessed by placing an 8 mm disk of PDA, covered by the mycelium of the tested fungus, in the center of a Petri dish with PDA, to which each of the appropriate pesticides (Table 3) were formerly added. Susceptibility was assessed by measuring the diameter of each colony's growth after 6 weeks at 25°C. Spore germination was determined by placing 0.1 ml of a spore suspension (containing 10^6 spores/ml, from every fungus) in a Petri dish with 10 ml PDA, to which one of each of the tested pesticides was added. The dishes were incubated at 25°C for 24–48 h, after which spore germination was assessed. Untreated controls consisted of PDA with deionized water. Each treatment was replicated four times, and the entire experiment was repeated twice.

The results (Table 3) indicate that almost all tested pesticides (except sulfur, which seemed to inhibit germination) were compatible with the three fungi. Some compounds, like buprofezin and dimethoate, adversely affected colony growth and/or spore germination,

Table 3 The effect of several insecticides and acaricides on the colony growth and spore germination of *Acaromyces ingoldii*, *Meira argovae* and *Meira geulakonigii*

Generic name	Recommended a.i. dosage	*Acaromyces ingoldii*		*Meira argovae*		*Meira geulakonigii*	
		Colony growth	Spore germination	Colony growth	Spore germination	Colony growth	Spore germination
Abamectin	9–22	97	131	86	97	69	NT
Azadirachtin	9,700	124	108	99	112	98	105
Buprofezin	250	113	84	85	80	99	53
Chlorfenapyr	96–160	108	98	62	79	85	86
Dimethoate	75–150	41	112	78	105	83	98
Fenpyroximate	25–100	115	101	88	95	91	80
Imidacloprid	175	96	101	100	67	101	100
Methoxyfenozide	96	104	105	101	102	99	104
Pyrazophos	300–500	67	128	51	64	68	59
Sulfur	2,800–7,000	93	28	86	63	81	1
Teflubenzuron	75–150	NT	NT	100	110	62	NT

Pesticide concentrations assessed were according to the active-ingredient (a.i.) dosages (in ppm) recommended for field use in Israel; variation in ranges results from usage on different crops at high- or at low-volume applications. Mean colony growth and spore germination values were calculated in percentages from the results in the untreated control

NT not tested

A. Gafni and A. Sztejnberg, unpublished

but not to the extent that this would affect fungal survival in the field. The increased growth and germination values obtained after exposure to some pesticides probably stems from the ability of the fungi to metabolize specific molecules (or their components).

Discussion

Hirsutella spp.

A comparison between *H. thompsonii* and *H. kirchneri*, as based on our data, reveals three main differences: (1) the pathogenicity of the former seems to be restricted to plant mites, whereas *H. kirchneri* infected a greater variety of acarine species; (2) at 25–27°C *H. thompsonii* developed on PDA much faster than *H. kirchneri*, forming a colony of 70 mm diameter in 18 days, as compared to a colony of only 25 mm after 30 days by the latter; and (3) *H. thompsonii* infested and killed mites only at or near saturation, whereas *H. kirchneri* affected mites at ≥80% r.h. Due to the low pathogenicity of *H. necatrix* to plant mites, it will not be discussed further.

Our results with both *H. thompsonii* and *H. kirchneri* were obtained from isolates that represent only a narrow genetic pool. The former fungus is known to be very pleomorphic, with many isolates collected and identified from different parts of the world (Boucias et al. 1982; Mozes-Koch et al. 1995; Aghajanzadeh et al. 2007). Such isolates may arise through geographical isolation but also by anastomosis (unpublished data). The variability inherent in the entity currently called *H. thompsonii* is consistent with its reported attack on members of the family Tarsonemidae and on the varroa mite. As to *H. kirchneri*, its failure to infect the broad mite was unexpected, because this fungus was initially collected from a member of the same mite family (cited by Minter et al. 1983). The parasitic mite *H. coccophagus*

appears to be the only member of the Astigmata affected by these fungi. The possible adverse effect of the susceptibility of this natural enemy to *H. kirchneri* may be offset by the fact that *H. coccophagus* is usually located under the shield ('scale' or 'armor') of its host, reducing its chances of becoming infected in the field (Sztejnberg et al. 1997).

Meira and *Acaromyces*

Meira spp. and *Acaromyces* are hard to isolate, slow-growing fungi and difficult to identify by conventional morphological methods; in fact, their determination required a variety of physiological and molecular methods (Boekhout et al. 2003). This may explain the paradox of their being common (we collected several isolates of *M. argovae* in various regions of Israel), while not having hitherto been found, despite much local work on citrus fungi. Our discovery of the three new species in this country, within a narrow geographical range, suggests that additional, related taxa may also be found.

The discovery of the endophytic presence of *M. geulakonigii* within closed grapefruit flowers and in its fruit peel throughout the season raises intriguing questions. These include the origin of the inoculation and its mode of transmission (via seeds or at grafting? Could they be windborne or do they adhere to arthropods?); does the fungus also occur in other citrus species and varieties, and in other plants? Should the association between citrus and this fungus be found to be prevalent, another interesting query would be about the benefits for either partner. From our data we speculate that the *M. geulakonigii*–grapefruit relation may be of mutual benefit. The fungus lives in or on various parts of its host plants throughout the year as a saprophyte, affecting mites as these become numerous enough to come in touch with the fungus or its secretions. At this time the fungus may possibly act as a 'bodyguard' (Elliot et al. 2000), reducing the damage that CRM causes the grapefruit. This association is similar to that of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin, which forms endophytic relationships with various plants without damaging them, thus providing alternate options for biological pest control (Lewis et al. 2001; Akello et al. 2007).

As noted, no evidence was obtained that the fungi damage the plants in any way, no relevant phytotoxicity was observed, nor did the augmentative sprays, whether seasonal or monthly, reduce fruit weight or size distribution.

Fungi that attack insects are parasitic, invading the bodies of their hosts (Diaz et al. 2006). This also holds for acaropathogenic fungi, e.g. *H. thompsonii* and *N. floridana*, as well as for mite and insect pathogenic fungi (e.g. *Beauveria* spp. and *Metarhizium* spp.), which penetrate the mites' bodies and then secrete toxic compounds (Chandler et al. 2000). *Meira* and *Acaromyces* thus appear to be unique in being non-parasitic antagonists of mites. As neither *Meira* spp. nor *A. ingoldii* are parasitic (Sztejnberg et al. 2004), we assumed that their mode of action, e.g. the cause of mite death, was due to toxic secretions. A related fungus, *Pseudozyma flocculosa* (Traquair, Shaw et Jarvis) Boekhout et Traquair, which is antagonistic to phytopathogenic fungi, exerts its effect by the secretion of toxic fatty acids (Avis et al. 2001). The putative toxins secreted by *Meira* spp. and *A. ingoldii* affect a wider range of organisms, because they are also antagonists of powdery mildew diseases (Sztejnberg et al. 2004; Gerson et al. 2005), other phytopathogenic fungi and bacteria (unpublished data).

The ability of *M. geulakonigii* to reduce mite numbers without affecting the host plant, its low toxicity to some predatory mites, its endophytic nature along with the apparent tolerance to many insecticides and acaricides, suggest that this fungus could be suitable for IPM programs. Notwithstanding our limited field trial, more out-of-doors data are needed

in order to evaluate the commercial potential of this species, as well as that of the other two fungi, as biological control agents.

Finally, do *Hirsutella*, *Meira* and *Acaromyces* interact with other natural enemies (Roy and Pell 2000)? In our experience with species of *Hirsutella*, the single case of an adverse effect was *H. kirchneri*, affecting *H. coccophagus*. As to *Meira*, both its species reduced the numbers of one of the predatory mites (Fig. 3).

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Lessons from interactions within the cassava green mite fungal pathogen *Neozygites tanajoae* system and prospects for microbial control using Entomophthorales

Fabien C. C. Hountondji

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Abstract Most fungal pathogens lack the capacity to search for their host but rather develop sit-and-wait strategies that favour contact with them. The success of these strategies depends upon the interactions of the pathogen with its host, the host plant and the environmental conditions, which altogether determine its transmissibility. Given the limited success that has characterized application of sustainable microbial control, particularly using Entomophthorales, interaction studies have been conducted with the entomophthoralean fungus *Neozygites tanajoae*, pathogenic to the cassava green mite (CGM), *Mononychellus tanajoa*, to help understand differences observed between laboratory and field performances of this pathogen. Reciprocal pathogen-host interactions as well as tritrophic interactions involving the host plant were studied. It was found that herbivory triggers the release of volatiles that promote sporulation of isolates of *N. tanajoae*, whereas the host mite avoids haloes of spores of this pathogen. However, the host mite does not avoid the pathogen when inside the mummified fungus-killed cadaver. The status of microbial control of CGM in Africa is reviewed and implications of these interactions are discussed for prospective application of microbial control using Entomophthorales.

Keywords Avoidance · Epizootic · Individual-level interaction · *Manihot esculenta* · *Mononychellus tanajoa* · Population-level interaction · Tritrophic interactions · Virulence

F. C. C. Hountondji (✉)
Biological Control Centre for Africa, International Institute of Tropical Agriculture, Cotonou, Republic of Benin
e-mail: fabienho@yahoo.com

Present Address:
F. C. C. Hountondji
Expertise, Action and Research for Development (EAR-Development), P.O. Box 2015, Abomey-Calavi, Republic of Benin

Introduction

A few sustainable microbial control programs using entomophthoralean fungal pathogens to control arthropod pests have been very successful (Hajek et al. 2005, 2007). The well-known case of sustainable microbial control success using Entomophthorales is that of *Entomophaga maimaiga* against the gypsy moth populations in North America. However, whether the early success of this pathogen was the result of release efforts or due to accidental introduction is still unclear (Hajek et al. 1995a, 2007). Moreover, in other programs that resulted in establishment, subsequent performance of established Entomophthorales was generally erratic, too slow or unpredictable (Milner et al. 1982; Wilding et al. 1990; Dara and Semtner 1996; Bidochka et al. 1997).

The reasons behind low performance in microbial control are still poorly understood. Epizootics are largely driven by pathogen-host characteristics and environmental conditions such as the capacity of the pathogen to overcome host defences, its transmissibility, the initial pathogen inocula, host density and abiotic conditions (Hajek and St. Leger 1994). Most epizootiological studies focus on aspects of primary pathogen-host interactions and the effects of host plant food on within-host dynamics of the pathogen (e.g. Hajek and St. Leger 1994; Hajek et al. 1995b; Cory and Hoover 2006; Raymond and Hails 2007). However, information on direct interactions between the host plant and the entomopathogen is scarce (Brown et al. 1995; Baverstock et al. 2005; Munster et al. 2005), and potential of the genetic diversity in host-pathogen systems is poorly explored and utilized. Candidate fungal isolates in many cases failed to demonstrate the performance they show in the laboratory, at the individual-level, under field conditions, at the metapopulation-level (e.g. Hajek et al. 1996; Inglis et al. 2001). In many cases, selection for microbial control candidates has been carried out in laboratory by treating single or a group of individual host(s) with the pathogen in a confined environment and screening them, based on virulence parameters such as time and dose mortalities. In such bioassays aspects related to the host plant, such as indirect signalling of host presence and habitat abiotic conditions, are not taken into account, which excludes population-level interactions that seem important for virulence assessment (Elliot et al. 2002a).

An example of such interactions is the microbial control of the cassava green mite (CGM), *Mononychellus tanajoa*, a pest of cassava, *Manihot esculenta* Crantz, in Africa, with the fungal pathogen *Neozygites tanajoae* (Delalibera, Hajek and Humber). The trophic system *N. tanajoae–M. tanajoa*–cassava is illustrated in Fig. 1. While Brazilian and African isolates of *N. tanajoae* do not differ in virulence under laboratory conditions, Brazilian isolates are characterized by regular epizootics in the field (Hountondji et al. 2007). In an attempt to understand the differential performance of this pathogen, tritrophic interaction studies between fungus, host mite and cassava were carried out.

Effectiveness of *N. tanajoae* does not only depend on abiotic factors and host density; it can also be influenced by biotic factors related to the host and/or the host plant (Hountondji 2005). The host plant may influence sporulation of entomopathogens, e.g. by delaying sporulation until susceptible hosts are available as observed in other entomopathogens (Brown et al. 1995; Klingen et al. 2002), or possibly also by releasing volatiles that promote production of spores following plant feeding by the herbivorous host (Baverstock et al. 2005; Hountondji et al. 2005). Apart from the influence of the host plant on sporulation, the herbivorous mite may be attracted or learn to avoid haloes of spores. These hypotheses have been tested for the *N. tanajoae-M. tanajoa*-cassava system. Integrated analyses of results from these studies can provide a better insight to management of sustainable control using Entomophthorales.

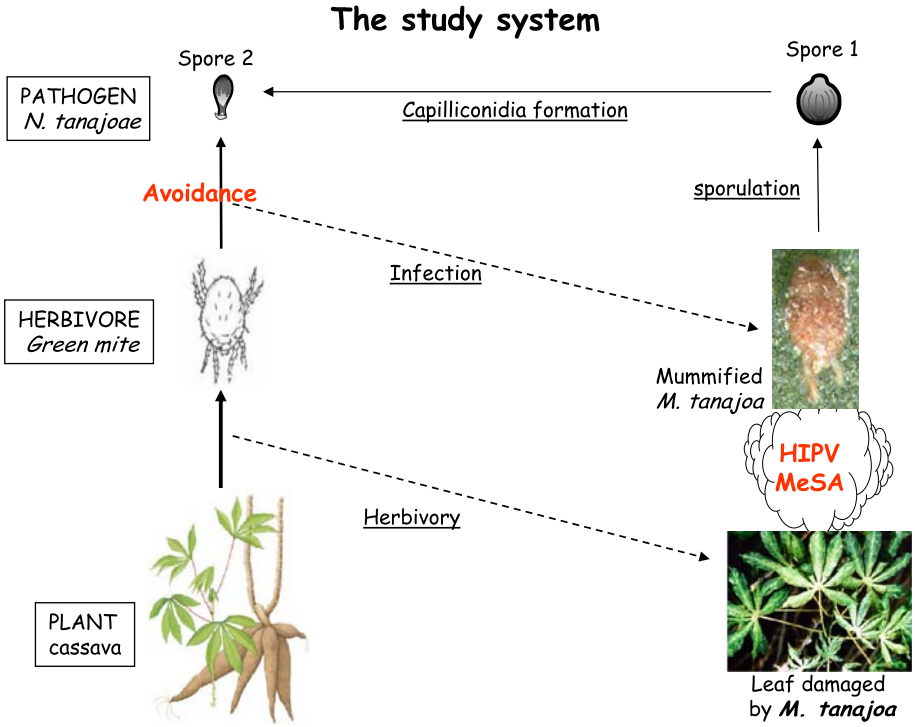

Fig. 1 Diagram of the study system with the trophic chain. The different levels (rectangles) are represented on the left-hand side; the products of the interactions between the different trophic levels are represented on the right-hand side; specific interactions are indicated in gothic characters; the different elements of the system are connected by arrows with the dotted ones pointing at the products of interactions between two trophic levels. *HIPV* Herbivore-induced plant volatiles; *MeSA* Methyl salicylate (based on Hountondji 2005)

Here, it is demonstrated that interactions between *N. tanajoae*, *M. tanajoa* and the host plant can indeed influence the microbial control and the implications are discussed.

Description of the study system

The fungus

The entomophthorale *N. tanajoae*, previously known as *N. floridana* and renamed by Delalibera Jr. et al. (2004), is a pathogen of *M. tanajoa*. It infects the mite through body contact and emission of a germ tube that penetrates the cuticle of the mite and develops into hyphal bodies, which multiply through binary division and progressively fill the haemocoel of the mite. Infected mites can still move the first and the second day, and may thus help in the dissemination of the pathogen. They die within 3–5 days at ca. 28°C (Oduor et al. 1995a). Freshly dead infected mites dry out and become mummified (hence called 'mummies') in a few hours, after which they sporulate if conditions are favourable.

Optimum sporulation conditions are fulfilled in the dark when humidity is near saturation and temperature around 18–23°C (Oduor et al. 1996). Sporulation starts with the process of conidiation which results in ejection of non-infective spores (conidia) from the

mummified infected host and ends with development of conidia into infective spores (capilliconidia), each on top of a slender capillary tube. Capilliconidia possess a glue-like end serving to attach to the mite. Both conidia and capilliconidia are displayed on the substrate within 5 mm around the sporulating mummy and are easily recognized as they form a halo around the mummy. Host mites get infected when they pass through a halo of spores and get capilliconidia attached to their body. Infectivity was found to be age-dependent (Elliot et al. 2002b) whereas time to mortality was influenced by the number of attaching capilliconidia (Oduor et al. 1997a). Production and distribution of haloes of spores and host density are therefore important factors of the transmissibility of *N. tanajoae*.

The acaropathogen *N. tanajoae* is difficult to grow in vitro, on artificial medium (Leite et al. 2000; Delalibera Jr. et al. 2003). Significant work was done by Delalibera Jr. et al. (2003) who developed a procedure for hyphal body culturing using IPL-41 medium enriched with yeastolate, lactalbumin and fetal bovine serum. However, further research is still needed to improve the yield of hyphal body production and sporulation from artificially produced hyphal bodies for practical applications. Production, release and storage of *N. tanajoae* therefore still rely on the natural host.

The mite

The tetranychid mite *M. tanajoa* is a pest of cassava, an important staple food crop in Africa (Yaninek and Herren 1988). It is preferably associated with cassava as host plant and is rarely found inhabiting other plants. It mainly lives on the underside of cassava leaves where it feeds on the leaf parenchyma. The developmental life of *M. tanajoa* is mainly influenced by temperature and relative humidity and was found to be 12.5 days under controlled conditions of 28°C and 60% RH (Yaninek et al. 1989).

Accidentally introduced to Africa in the early 1970s, *M. tanajoa* has become one of the major cassava pests (Nyiira 1972; Lyon 1973; Yaninek and Herren 1988). Heavy infestations have been observed on the continent with up to 80% yield loss and occasional plant death. In South America, its area of origin, *M. tanajoa* is kept under control by a complex of natural enemies (Yaninek and Herren 1988). A few indigenous predators attack *M. tanajoa* in Africa but their action is not sufficient to prevent pest outbreaks. The International Institute for Tropical Agriculture (IITA) initiated a classical biological control programme whereby natural enemies from South America are imported to Africa to control *M. tanajoa* (Yaninek and Herren 1988). Two phytoseiid predators, *Typhlodromalus manihoti* and *T. aripo*, were successfully introduced against the pest (Yaninek et al. 1993, 1998; Yaninek and Hanna 2003; Hanna et al. 2005). However, the impact of these phytoseiids depends on the cassava variety (particularly for *T. aripo*) and their establishment in dry savannas and in some mid-altitude regions in Africa is still a challenge (Hanna and Toko 2003).

Dynamics and within-plant distribution of mite populations are found to be influenced by the nutritional status of the leaves (Yaninek et al. 1989) as well as the presence of predators (Magalhães et al. 2002; Onzo et al. 2003). However, main dynamics are driven by upwards migration due to formation of new leaves as the cassava plant grows; this explains the higher densities observed on younger cassava leaves (Yaninek et al. 1991). The migration behaviour of the host determines its within-plant distribution and dynamics; it is thus an important factor to consider for sustainable microbial control applications.

Cassava plant

Cassava is a euphorbiaceous plant which originated from South America, particularly from Brazil (Olsen and Schaal 1999). It was introduced to Africa in the sixteenth century and has become one of the major staple food crops in sub-Saharan Africa (Yaninek and Herren 1988; Herren and Neuenschwander 1991). Cassava is propagated through vegetative cuttings and is harvested between 8 and 36 months after planting (Cock 1985). It is attacked by several pests amongst which *M. tanajoa* and another tetranychid mite *Oligonychus gossypii* (Gutierrez and Bonato 1994), the latter being indigenous to Africa where it is a minor pest in some areas. Infection of *O. gossypii* by a *Neozygites* species was also observed in the field but at extremely low levels; however, it appears not to be the same species as *N. tanajoae* (Delalibera Jr. and Hajek 2004).

In Africa, cassava is extensively cultivated and cassava fields are closely installed particularly in high-production areas, exhibiting sometimes contiguous cassava vegetation over large areas. At early ages (1–4 months), cassava fields have limited shading and plants are mostly isolated. However, at later stages, shading becomes increasingly important due to canopy development ensuring contacts between plants. Shading and cassava plant distribution condition host distribution and dynamics; these factors may influence epizootic establishment and dispersal of *N. tanajoae*.

Status of *Neozygites tanajoae*-based microbial control of the cassava green mite

Host specificity of *Neozygites tanajoae*

Host range is an important characteristic in the selection of biocontrol candidates. It determines the level of specialization of the biocontrol candidate with the host and may play a role in its persistence in the field. Preliminary work by de Moraes and Delalibera Jr. (1992) demonstrated the non-pathogenicity of *N. tanajoae* to closely related tetranychid mite species (*Tetranychus urticae* and *T. bastosi*). In order to introduce South American isolates of *N. tanajoae* to Africa for controlling *M. tanajoa*, additional pathogenicity tests were conducted with predators and parasitoids of the cassava agroecosystems, silkworms and bees. None of the species tested was a suitable host for the development of *N. tanajoae*, which demonstrates its safety for introduction into Africa (Hountondji et al. 2002a). Further bioassays and DNA work by Delalibera Jr. et al. (2004) also confirmed the specificity of *N. tanajoae* and support the speciation proposed by these authors.

Natural occurrence of *Neozygites tanajoae*

Occurrence of *N. tanajoae* is poorly investigated due to geographically limited research on the acaropathogen. *Neozygites tanajoae* was first discovered as a cause of infection in *M. tanajoa* populations in Venezuela in the mid 1980s (Agudelo-Silva 1986). Later, severe epizootics were reported in other countries of South America, e.g. Colombia (Alvarez Afanador et al. 1993) and particularly Brazil where more frequent epizootics have been observed in the North East (Delalibera Jr. et al. 1992). The fungus was also found in Africa, particularly in Kenya (Bartkowski et al. 1988), in Benin (Yaninek et al. 1996), in Ghana and in Tanzania (Hountondji and Hanna, unpublished data). It is not known whether *N. tanajoae* was introduced with its host to Africa or not, since nothing is known about its dispersal and to date, its distribution is only partially assessed on the continent. However,

given the specificity of *N. tanajoae* to its host, one of the serious hypotheses to be considered about its origin is that of its host (i.e. South America).

Prevalence of *N. tanajoae* shows great variation between geographical areas. In South America, high-prevalence has been observed in Columbia and particularly in Northeastern Brazil (Delalibera Jr. et al. 1992; Alvarez Afanador et al. 1993). In Africa, however, lower prevalence has generally been observed with especially very low infection levels in Benin before the releases of the acaropathogen in 1999 (Yaninek et al. 1996; Dara et al. 2001). Nevertheless, exceptionally, infections above 10% have been observed on this continent in the absence of releases or far away from the release areas.

Releases and post-release prevalence of *Neozygites tanajoae*

The epizootic potential of Brazilian isolates of *N. tanajoae* has led research at IITA to consider introducing Brazilian isolates against CGM. Brazilian isolates were imported to Benin through the University of Amsterdam. Preliminary virulence studies in laboratory failed to reveal significant differences between these isolates and the indigenous Beninese isolate. This prompted some field experimental releases in order to measure the performance of the isolates under natural conditions (Hountondji et al. 2002b), where field age, cassava variety, cultural practices, host densities at releases, and abiotic conditions were similar.

Due to the difficulty to culture the fungus in vitro, an in vivo release procedure was used to release Brazilian isolates in two agroecological zones, in early 1999 in Southeastern Benin, where the fungus is endemic and later during the same year in Northeastern Benin, where it had never been found. Post-release monitoring in Southeastern Benin, 11 months later, showed epizootics in three fields where Brazilian isolates were inoculated out of the 20 release fields, with infection levels between 20 and 35%. In Northeastern Benin, epizootics were also observed with infection levels between 15 and 70%, 10 months following the releases. Consistently heavier infections were observed in fields inoculated with Brazilian isolates compared to those inoculated with the Beninese isolate. Post-release surveys conducted after observing the first epizootics indicated the prevalence of higher *N. floridana* infections, more pronounced around the release sites compared to the pre-release situation. Although these observations credit the establishment and potential dispersal of Brazilian isolates, characterization studies are needed to confirm this. Development of molecular markers capable of differentiating between *N. tanajoae* isolates is underway to assess the relative performance (as far as establishment, distribution and dispersal are concerned) of *N. tanajoae* isolates for wider and more rational control of *M. tanajoa* in Benin and other African countries.

Interaction studies

Interaction between a pathogen and its arthropod host can take place at several scales related to the density and spatial distribution of both species. However, for the sake of simplistic representation of these interactions, the individual scale and the population scale may be considered. The individual-level interactions involve interactions between a single host or a group of hosts and one or a group of spore(s) of the pathogen, whereas the population-level interactions involve populations of the host or the pathogen or both.

Individual-level interactions

In this section, individual-level interactions as revealed through laboratory virulence studies are presented as well as infochemical-based interactions.

Laboratory virulence

Virulence is commonly defined as the power of a pathogen to produce disease in the host (Shapiro-Ilan et al. 2005). Virulence therefore refers to the capacity (including a dimension of time) of the pathogen to harm the host, which involves mainly physiological, physical and chemical interactions. Preliminary work conducted by Oduor et al. (1997a) demonstrated the ability of a single spore of *N. tanajoae* to infect a host; however, the proportion of infected hosts was found to increase with pathogen inoculum size (Oduor et al. 1997a).

Laboratory bioassays conducted in closed-dish environment to study virulence of *N. tanajoae* did not show significant differences in infection levels between two South American isolates known for causing severe epizootics and two African isolates with generally low-prevalence (Dara, Hountondji and Lomer, unpublished data). Other studies conducted by Delalibera Jr. and Hajek (2004) on 23 isolates including the two Brazilian isolates and one of the African isolates found that most isolates were highly virulent, causing >90% infection. These authors suggested considering the rate of mummification, which revealed rather important differences as high as 25% between the African isolate and one of the Brazilian isolates, as a plausible parameter for selection of *N. tanajoae* isolates for biological control. However, better traits are still expected from a virulent isolate after mummification, which are, e.g. related to conidiation, production of capilliconidia, contact with host, penetration and host invasion.

Role of infochemicals

Arthropods use chemical information to locate their food, victims, or hosts, and enemies in their environment. Herbivores may use cues from plants to locate their host plant and cues from their natural enemies to develop avoidance behaviour. Similarly, natural enemies may locate their victims or hosts through their cues. Beyond these direct interactions between successive levels of a trophic system, indirect interactions can also be observed in a tritrophic system, i.e. plants can promote the effectiveness of natural enemies such as predators and parasitoids, e.g. through the release of HIPV when attacked by herbivores (Price et al. 1980; Dicke and Sabelis 1988; Dicke et al. 1990; Turlings et al. 1990). Little is known about the effect of HIPV on entomopathogens, particularly fungi (Hountondji et al. 2005; Baverstock et al. 2005). *Neozygites tanajoae* is one of the entomopathogens whose interactions have been studied up to the third trophic level.

Response of Neozygites tanajoae *to cues* *Neozygites tanajoae* sits and waits for its host; hence cues cannot help it find its host. However, they may influence the production of spores by the acaropathogenic fungus and thus promote or demote its transmissibility. The role of infochemicals in the interactions between *N. tanajoae* and *M. tanajoa* was studied by Hountondji et al. (2005). Effect of green leaf volatiles (GLV) and HIPV on conidiation and production of capilliconidia was mainly tested. Effect of herbivore cues alone was not tested as the condition of occurrence of the mite cues alone is less likely to happen in the nature. The volatiles tested have an influence on the conidiation of *N. tanajoae*, and the importance of the effect varied with the isolates. On the one hand, it was found that GLV

from—mechanically damaged—cassava leaf discs used in the experiments significantly inhibit the production of conidia (428 ± 41 vs. 275 ± 47 conidia per mummy for clean air and GLV, respectively, Hountondji et al. 2005). Amongst the main volatiles produced by cassava leaves in absence of herbivory is (trans)-(*E*)-2-hexenal (Hountondji et al. 2005), which is known to have an inhibitory effect on the production of spores by fungi (Brown et al. 1995). Whether the effect of GLV is a delay in conidiation or a complete inhibition is still to be investigated.

On the other hand, when exposed to HIPV from *M. tanajoa*-infested cassava leaves versus clean air the production of conidia was increased by 37% and 14% for an African and a South American isolate, respectively (Hountondji et al. 2005). Little or no effect was observed for the production of capilliconidia except for one South American isolate where the production was promoted by 75.2% in the presence of HIPV ($t_{16} = 2.23$, $P = 0.02$; Hountondji et al. in press). Amongst the volatiles produced following herbivory is methyl salicylate (MeSA), a compound found to elicit behavioural responses of various predators and parasitoids to their victims or hosts. It may thus function as an indicator of herbivore damage and is evaluated for its role in promoting conidiation of *N. tanajoae*. It was found that the African isolate produced 37% more conidia in an environment with MeSA than without MeSA (306 ± 53 vs. 223 ± 38 conidia per mummy, respectively, Hountondji et al. 2006); no consistent response was found for the South American isolate. The variability observed between the whole HIPV blend and MeSA suggests the specificity of the responses of *N. tanajoae* isolates which may depend on the concentration of the blend/volatile and may well involve other volatiles than MeSA in the HIPV blend.

GLV inhibit conidiation of *N. tanajoae* whereas HIPV promote it. From a functional point of view, when on the leaf, *N. tanajoae* will profit from a delay in conidiation until release of HIPV signalling the presence of the herbivorous mite. However, this leaves unexplained why conidiation readily takes place in clean air, if under dry conditions the fungus inside the mummy could survive a few days or more (Oduor et al. 1995b; Elliot et al. 2002c; Van der Geest et al. 2000). It is hypothesized that the fungus does not gain by delaying sporulation in an environment without cues from plants and it may only successfully infect in the event of an unlucky herbivore passing by.

The response of *N. tanajoae* to volatile cues questions the olfactory perception in this fungus. Signalling is known to play an important role during penetration of the host by fungi (Kulkarni et al. 2005), e.g. through chemical and topographical recognition of the host surface (Hajek and St. Leger 1994). It is not excluded in the case of *N. tanajoae* that mummies capture volatiles from the environment, which interfere with the biochemistry of the release of conidia by, for instance, promoting spore release by conidiogenous cells. Advances in molecular studies have allowed detecting in a few fungi G-protein coupled receptors—GPCRs- and GPCR-like receptors, which are able to bind with ligands such as odorants and pheromones to generate processes such as olfactory sensations (Kulkarni et al. 2005). It is not known whether these receptors are also present amongst Entomophthorales. However, the responses of *N. tanajoae* and *Pandora neoaphidis* to volatile cues (Hountondji et al. 2005, 2006; Baverstock et al. 2005) suggest the existence of olfactory receptors in these fungi.

Avoidance of Neozygites tanajoae *spores by* Mononychellus tanajoa Besides the apparent 'infochemical conspiracy' between the plant and the fungal entomopathogen to promote the production of spores by the entomopathogen, the behaviour of the herbivorous mite is determining for the propagation of *N. tanajoae* amongst hosts. A series of

experiments was conducted to study the behaviour of *M. tanajoa* in the presence of the acaropathogen (Hountondji 2005; Hountondji et al. in press). One experiment used cassava leaf discs and tested the habitat preference and the oviposition behaviour of *M. tanajoa* in a two-choice unit with spores or infected hosts versus a control. Another experiment used cassava leaves and tested the habitat preference of *M. tanajoa* between lobes with and lobes without spores of *N. tanajoae*.

Evidence was found for a South American isolate that naïve mites avoid leaf discs with spores of *N. tanajoae* (41 ± 3% of migrating mites went to the disc with spores). The avoidance behaviour was somewhat less obvious for one African isolate (44 ± 4%). However, *M. tanajoa* laid significantly fewer eggs on the discs with spores than on discs without spores for all isolates. When mites was given prior experience with *N. tanajoae*—either by exposing them to live infected, not yet sporulating, conspecifics or to dead infectious conspecifics (presence of infective spores)—the avoidance tendency decreased with experience and even disappeared. Moreover, egg production was not affected by the presence of spores for the experienced mites. The only exception was observed with mites that had previous experience with spores of the African isolate; they consistently produced fewer eggs. Avoidance of *N. tanajoae* spores was also observed in the experiment using whole leaves, particularly for the South American isolate for which 43% less migration towards spores was observed. Although fewer migrating mites were generally observed on the lobes with spores than on those without spores for the Beninese isolate, the difference (17%) was not significant. Pathogen distribution amongst leaf lobes appears to influence the importance of avoidance depending on the isolate. Avoidance was more pronounced when spores were displayed on two lobes than on three lobes for the South American isolate, whereas it is the opposite for the Beninese isolate. Concentration of cues may therefore differentially influence *N. tanajoae* isolates.

When infected, not yet sporulating, hosts were used instead of spores, naïve *M. tanajoa* showed neither avoidance/repellence nor difference in egg production. *M. tanajoa* does not recognize the pathogen when it is inside the mite. It is hypothesized that live infected mites carry *N. tanajoae* to highly infested patches where they settle and stay unnoticed until they release spores. It is suspected that *N. tanajoae* induces this behaviour in the live infected mite and so it is referred to as a 'Trojan horse hypothesis'.

Population-level interactions

Individual interactions provide basic understanding of interactions between organisms, but cannot alone explain field population dynamics. Population-level interactions seem important for better understanding of interactions within systems, particularly pathogen-host systems where transmission is an important factor for pathogen performance. For sit-and-wait pathogens such as *N. tanajoae*, whose use as a biopesticide is not yet possible, population-level interactions between the pathogen and the mite may thus be a key factor to consider in the selection of isolates for virulence.

Greenhouse virulence

Population-level virulence conducted in the greenhouse showed that both a high-prevalence South American isolate and a low-prevalence African isolate developed epizootics and that the epizootic level of the isolates depended on the inoculum density (Hountondji et al. 2007). The African isolate which has low field performance developed a more severe epizootic at the high inoculum, whereas the South American isolate with high field

performance did so at the low inoculum. Dispersal from the inoculated plants to downwind, clean plants was also evaluated and showed more mites dispersed when upwind plants were inoculated with the South American isolate than when inoculated with the African isolate. These results demonstrate that differences in virulence of entomopathogenic fungi can be revealed at the patch (local population) level even when not detected at the individual-level and that suboptimal (inoculum, host density or weather) conditions may be important in differentiating isolates.

Prediction on the microbial control of Mononychellus tanajoa

Predictions on the *N. tanajoae–M. tanajoa*-cassava system were developed by Oduor et al. (1997b) to explain the dynamics of *N. tanajoae* in local CGM populations. They found that *N. tanajoae* alone was unable to drive local CGM populations to extinction. However, post-release observations on the dynamics of *N. tanajoae* and its host have shown evidence of control of the mite populations in the greenhouse as well as in the field (Hountondji et al. 2002b, 2007). The failure of the model to predict control of the mite by *N. tanajoae* has led to reconsider relevant parameters in the model to re-evaluate the predictions. Misestimating of two of the model parameters, namely the per capita rate of loss of infectiousness μ and the per capita transmission rate β, is viewed as the possible cause of the mismatch. The value of the rate of loss of infectiousness is actualized ($0.048 < \mu < 0.071$, instead of 0.004 day^{-1}) based on recent observations, which showed that capilliconidia can only survive 2–3 weeks under natural conditions on cassava plant (Hountondji and Hanna, unpublished data), instead of 233 days assumed by Oduor et al. (1997b). The new value of the rate of loss of infectiousness is used to make new predictions based on possible β solutions for extinction of local mite populations to occur (Hountondji 2005). It was found that the likely threshold β beyond which extinction can occur varies between 0.062 and 0.067 cm^2/day under field conditions, using exact solutions of a 'pancake' version of the model, whereas it varies between 0.085 and 0.091 cm^2/day using iterations based on the full model, which includes weather conditions. These values of β are at least 1.5 times as high as the β value in Oduor et al. (0.039 cm^2/day). In addition, similar iterations conducted to obtain extinction of mite populations as early as 3 weeks after inoculation (as observed in greenhouse experiments) indicated even higher β values between 0.103 and 0.110 cm^2/day. The model predicts potential control of the host mite by the pathogen provided that the transmission rate is twice as high as that estimated by Oduor et al.

In the model developed by Oduor et al. (1997b), effects of biotic factors related to the host and/or the host plant—such as plant volatiles, HIPV and pathogen avoidance by the host—were not considered in the experiments to estimate parameters of the model. The model was based on the assumption of constant transmission rate, where population-level interactions capable of modifying the transmission rate of the pathogen were not included. The new prediction resulting from improved model parameters matches with observations subsequent to the application of the microbial control of the mite using *N. tanajoae* in Africa as yet observed following releases (Hountondji et al. 2002).

Implications for sustainable microbial control

Application of sustainable microbial control has been a challenge for researchers and scientists for several reasons. Firstly, most microorganisms are difficult to detect by non-

specialists and are feared for the threat some of them have represented to human being through epidemics, veterinary and plant diseases. These characteristics make them difficult to market for research applications; e.g. regulations for importation and releases of microbial control agents have been delayed and are constraining (Hajek et al. 2007). Secondly, knowledge about epizootiology of insect diseases is still limited and a few species-cases of sustainable microbial control with particular reference to fungal pathogens have proved to be 'very' successful (Hajek et al. 2007). Thirdly, microbial control research is relatively expensive and requires advanced technologies for sound and safe research such as molecular techniques for strain-level identification. Fourthly, unlike arthropods, most pathogens—except nematodes to some extent—are not capable of moving to their host, which may be a reason for unsuccessful establishment of released pathogens such as Entomophthorales or a limitation for long-term control by those formulated as biopesticides such as hyphomycetous fungi. Instead, pathogens develop adaptive strategies (e.g. production of large numbers of spores, strategic discharge/dispersal of spores, 'ambush' strategy, and production of persistence structure), which mostly rely on biotic and abiotic factors. Once the abiotic conditions for the development and seasonal cycling of a pathogen are fulfilled, the success of a microbial control relies on the interactions of the pathogen with its host in the environment. Main interactions involved may be behavioural, chemical, physical, physiological, or their combination (Hajek and St. Leger 1994). These interactions may happen at the individual-level or at the (meta) population-level and may involve more than two trophic levels with reference to tritrophic interactions.

In the *N. tanajoae–M. tanajoa*-cassava system, individual interactions start with the behavioural patterns of both *N. tanajoae* and the mite, which are influenced by infochemicals released in the environment by cassava, the mite, the pathogen and/or other natural enemies, as a result of herbivory, predation, physiological or pathological processes. Several scenarios can be sketched out regarding ways interactions may influence efficacy of an entomophthorale, with reference to the *N. tanajoae* system. Firstly, healthy mites may move to more nutritious and more secure patches of the leaves following nutrient depletion (Yaninek et al. 1989) or threat of natural enemies (Magalhães et al. 2002; Onzo et al. 2003). In these movements, they may avoid to some extent dense patches of *N. tanajoae* spores but may settle in the vicinity of infected mites as they fail to recognize the pathogen inside the host. Secondly, the pathogen may produce more spores following HIPV emission (Hountondji et al. 2005; Baverstock et al. 2005) or may manipulate infected hosts to drive it to densely infested patches, thereby increasing the chances of contact with new hosts. Thirdly, in the absence of immediate hosts, GLV may inhibit/delay the spores until a signal of host presence (Brown et al. 1995; Hountondji et al. 2005). Fourthly, behavioural interactions lead to contact between the pathogen and the host with subsequent initiation and development of infection through physical, chemical, and physiological processes leading to exploitation of the host body (Hajek and St. Leger 1994). Although this was not studied for the *N. tanajoae* system, host plant quality can influence development of the pathogen inside the host (Hajek et al. 1995b; Cory and Hoover 2006; Raymond and Hails 2007). Also, infection processes end up with preparation of sporogenous structures, which therefore suggest a possible role of host plant quality in future sporulation; nutritional quality is known to influence fungal sporulation (Dahlberg and van Etten 1982).

Development and multiplication inside the host may sometimes lead to production of resistant structures such as resting spores. However, in the case of *N. tanajoae*, resting spores are rarely found and specific factors triggering their production are still unclear (Elliot et al. 2002c; 2002d), unlike other entomophthoralean pathogens such as

Entomophaga maimaiga (Hajek and Humber 1997; Kogan and Hajek 2000). Changes in factors such as host population, host plant quality, and abiotic factors are suspected to cause such physiological shift (Elliot et al. 2002c). Further studies are needed to understand resting spore formation, as this is important for long-term establishment of the pathogen and subsequent control efficacy.

Population-level interactions have been overlooked in the evaluation of virulence of insect pathogens as can be inferred from the commonly accepted definition of 'virulence' and from applications made of it in conducting microbial control (refer to 'individual-level interactions' above). These parameters are commonly measured at the individual-level, under optimum abiotic conditions, where the pathogen is directly inoculated to the host, sprayed or provided on confined arenas with limited host movement. Pathogen strains are commonly selected through these procedures and applied in the field for microbial control purposes. However, the results of the interaction studies exposed earlier suggest the importance of other dimensions of interactions at the population-level. Three types of such interactions may be added to the individual-level interactions to produce the outcome behaviour of the system at the population-level:

1. Host–host interactions at the population-level, which condition host numerical response related to exploitation of host plant resources and avoidance/escaping of natural enemies;
2. Pathogen–pathogen interactions at the population-level, which determine attack, invasion and killing strategies regulating host death. Such interactions can be suggested by the non-linear killing rate of the host at different inoculum sizes of a single strain (e.g., *N. tanajoae*; Oduor et al. 1997a) and possible multiple infection trades between strains (Gandon and Michalakis 2002; Nowak and Sigmund 2002), e.g. between local and exotic strains;
3. Host–pathogen interactions at the population-level, which condition tritrophic interactions, resting spore formation and possibly the Trojan horse strategy.

These interactions may well explain the unpredictable field results of microbial control efforts when abiotic host and inoculum conditions of epizootics are fulfilled. As shown earlier, interaction studies revealed the importance of cue concentration in the expression of the isolates. Effect of cues would be more expressed at the population than at the individual-level. Population-level interactions can thus better predict the behaviour of a pathogen/host/host plant system, particularly under suboptimal (host, host plant and/or pathogen) conditions, which commonly happen in the field. Efficient selection of candidates for microbial control needs therefore to consider testing population-level performances of candidate pathogens besides tests at the individual-level. Population-level investigations of the performance of pathogen strains should also consider appropriate inoculation time and conditions amenable to successful establishment and dispersal of the pathogen.

Conclusions

Insight within the *N. tanajoae–M. tanajoa*-cassava system has revealed reciprocal pathogen–host interactions, as well as tritrophic interactions, that may have profound consequences at the population-level and consequently for the field performance of the pathogen. These interactions could well explain the differential field performance of *N. tanajoae* isolates, despite the similar laboratory virulence of the same isolates.

Interactions within a pathogen–host system are determining for the transmissibility of the pathogen and thus its epizootic potential. The outcome of these interactions may vary depending on the pathogen, host, host plant and environmental conditions prevailing, whether optimal or suboptimal for the development of the pathogen.

The example of the system studied sheds some light on the application of sustainable microbial control, with special reference to the use of Entomophthorales. Particularly, selection of pathogenic strains and knowledge of appropriate inoculation conditions for a given system would better pay if population-level tests are added to individual-level investigations.

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Failure of the mite-pathogenic fungus *Neozygites tanajoae* and the predatory mite *Neoseiulus idaeus* to control a population of the cassava green mite, *Mononychellus tanajoa*

Simon L. Elliot · Gilberto J. de Moraes · John D. Mumford

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Abstract Monitoring of a population of the phytophagous cassava green mite, *Mononychellus tanajoa* (Bondar), and its natural enemies was undertaken in central Bahia, Brazil, in mid-1996. In spite of the presence of extremely high densities of the predatory phytoseiid mite *Neoseiulus idaeus* Denmark & Muma, the phytophagous mite population reached such high densities itself that there was total overexploitation of the cassava plants, leading to total leaf loss. Meanwhile, the mite-pathogenic fungus *Neozygites tanajoae* Delalibera, Humber & Hajek did not affect the *M. tanajoa* population in its growth phase as there was no inoculum present, even though we predict from a simple regression model that there was the potential for epizootics at that time. Soon after the *M. tanajoa* population crashed due to defoliation, there could have been an epizootic but there were simply no mite hosts to infect. These data demonstrate the ineffectiveness of one natural enemy (the predator) in terms of prey population regulation and demonstrate the importance of timing in the possible effectiveness of the other (the pathogen). For the pathogen, this probably explains its sporadic effect on host populations as previously reported. We conclude that the fungus is likely to be most useful as an adjunct to biological control with predatory mites other than *N. idaeus*.

Keywords *Neozygites tanajoae* · Cassava green mite · Epizootiology · Fungal pathogen · Biological control · Predatory mites

S. L. Elliot (✉)
Department of Animal Biology, Federal University of Viçosa, Vicosa, MG 36570-000, Brazil
e-mail: selliot@ufv.br

G. J. de Moraes
Depto. Entomologia, Fitopatologia e Zoologia Agrícola, Escola Superior de Agricultura Luiz de Queiroz, 13418-900 Piracicaba, SP, Brazil

J. D. Mumford
Centre for Environmental Policy, Faculty of Natural Sciences, Imperial College London, South Kensington Campus, London SW7 2AZ, UK

Introduction

Of the fungi which are pathogenic to insects and mites, the Entomophthorales are perhaps the group with the most potential within pest management strategies which seek to harness or promote natural epizootics (Pell et al. 2001). In spider mites (Acari: Tetranychidae), epizootics of the mite-pathogenic fungi *Neozygites floridana* Fisher and *N. tanajoae* Delalibera, Humber & Hajek (Entomophthorales: Neozygitaceae) can decimate host populations within a few weeks (Delalibera et al. 1999; Elliot et al. 2002a). With respect to the cassava green mite *Mononychellus tanajoa* Bondar, an important pest of cassava in northeastern Brazil and in Africa, this has led to a long-running effort to harness this epizootic potential for pest management (note that the fungus previously reported as *N. floridana* and affecting *M. tanajoa* was recently described as *N. tanajoae*; Delalibera et al. 2004). This effort has focussed on classical biological control involving the introduction of Brazilian isolates into Africa where the cassava system is exotic (de Moraes and Delalibera 1992; Delalibera et al. 1992; Yaninek et al. 1996; Elliot et al. 2000; Hountondji et al. 2002a, 2002b; Delalibera and Hajek 2004).

Approaches other than classical biological control have been considered, however. One is inundative control, an approach which is principally reliant upon the action of the initial biological material introduced as a biopesticide. This is probably not a realistic option due to difficulties of in vitro culturing (but see Leite et al. 2000; Delalibera et al. 2003) and the inaccessibility of even conventional pesticides (and spray equipment) to resource-poor cassava growers. Strategies with more potential are inoculative control, which relies principally upon reproduction of the pathogen and the impact of subsequent cycles of the pathogen (Thomas 1999; Eilenberg et al. 2001; Elliot et al. 2002d), or conservation bio-control in which the environment is manipulated so as to favour the action of the fungus. These last two could be of interest in their own right as well as when acting as components of a classical biological strategy.

Although the potential for natural epizootics of *N. tanajoae* to control populations of *M. tanajoa* has been demonstrated, the variability of this control has also been noted (Oduor et al. 1997; Elliot et al. 2000). Attempting either to harness this in new areas via the establishment of exotic strains or to improve upon it by the artificial introduction of inoculum or conservation is dependent upon the conditions being adequate for cycling of the fungus, both in terms of the abiotic conditions and the availability of potential hosts (Oduor et al. 1997; Elliot et al. 2002a). The sensitivity of the pathogen to abiotic conditions, particularly saturation deficits, has been demonstrated in laboratory (Oduor et al. 1995a, b, 1996a, b, 1997) and field studies (Elliot et al. 2002a). In the latter case, we followed a field population of *M. tanajoa* so as to identify the potential for the introduction of an inoculum in this sort of setting.

Here, we monitored mite and pathogen populations on leaves as well as airborne fungal inoculum. In addition, we used experimental measures of the potential for horizontal transmission in the field. We combine these data with estimates of epizootic potential calculated from field data and a simple regression model. The latter is a field-derived regression model which related mortality of new hosts to the quantity of inoculum available and night-time saturation deficits (Elliot et al. 2002a). Combined these give us several avenues to identify periods when the introduction of inoculum might have led to an epizootic and control of the pest mite population.

Materials and methods

Monitoring *M. tanajoa* population levels

This study was conducted in a grower's cassava field ("Field A") in Piritiba, central Bahia, northeastern Brazil, from 11 April to 9 September, 1996. Every three or four days, samples of thirty apical (first fully-formed) and thirty median (eighth fully-formed) cassava leaves were collected from randomly chosen plants while crossing the field in three zig-zags. As such, the procedure followed that of Elliot et al. (2002a): leaves were collected in the morning and transported in paper bags for examination of the undersides under binocular microscopes. Counts were made of *M. tanajoa* (eggs, nymphs and adult females—larvae and adult males were counted but the data are not presented here) on the central lobe of each leaf (Nachman et al. 1990, 1993; Elliot et al. 2002a), while predatory mites and *Neozygites*-killed *M. tanajoa* cadavers ("mummies") were counted on the entire leaves. By measuring the length of the central lobe and counting the number of lobes, leaf areas could be estimated so that all of these counts could be converted into densities per 100 cm^2 of leaf area (Elliot et al. 2002a), this figure approximating the area of the cassava leaves. Of these leaves, the number which presented 100% chlorosis through feeding injury was recorded. Meanwhile, following the observation of heavy *M. tanajoa*-induced leaf loss from 20 May, 100 plants were chosen randomly on each sampling date and the presence or absence of leaves on the first and eighth nodes was recorded.

Sentinel plants

In the same Field A as monitoring of mite populations was conducted, sentinel plants with healthy mites were placed in the field to detect airborne infective stages of *N. tanajoae*. From 24 May to 2 August, plants infested with uninfected adult female *M. tanajoa* (taken from a different field in the region) were placed among the cassava plants in Field A every 6 days and were examined after three and six days. These were potted cassava plants on to which healthy adult female *M. tanajoa* had been placed ca. three days prior to the placement of the plants in the field. To ensure that these mites were not contaminated with the pathogen, twenty individuals from the same source were mounted on slides in Amman's Blue/Hoyer's mixture to examine microscopically—at no stage were signs of disease noted. At monitoring, five leaves per plant were examined for mummies and five live mites from each leaf were mounted on a microscope slide for microscopic examination for the presence of capilliconidia and/or hyphal bodies (Steinkraus et al. 1999; Elliot et al. 2002a, c).

Horizontal transmission

In a second cassava field ("Field B"), approximately 1.5 km distant from Field A, weekly tests were conducted to assess the capability of the pathogen to be transmitted from an infected host to a new host. In each test, twenty clip-cages were placed on the undersides of the middle lobes of median leaves of cassava plants. The cages were cylindrical (2.5 cm high $\times$ 2 cm diameter), wire-framed and covered with mite-proof gauze; the rim of the open ends bore a foam ring to provide a seal and cages were fixed to the leaves with wire clips. Three laboratory-produced *N. tanajoae*-killed mite cadavers (mummies) and six adult female mites were placed on the leaf surface prior to fixing each cage in position. To ensure that these mites were not contaminated with the pathogen, twenty individuals from the same source were mounted on slides in Amman's Blue/Hoyer's mixture to examine

microscopically—at no stage were signs of disease noted. Ten control mummies were kept for 24 h in a dark incubator at 18°C and 100% RH: on all test dates, at least eight of the ten mummies produced a substantial number (i.e. >>100) of primary conidia. After six days, leaf lobes with cages were taken to the laboratory where mummies and mites were mounted as above for microscopic examination.

Climatic data

Hourly temperature and relative humidity data were recorded using a hygrothermometer kept in a Stevenson screen. These were converted to hourly saturation deficits from which the minima for each night were recorded (this parameter best explained variations in infection levels in Elliot et al. 2002a). From these data, three-day means of saturation deficit minima were calculated. A pluviometer was used to measure rainfall in a nearby yard. These instruments were <1 km from Fields A and B.

Estimates of epizootic potential: regression model

In a concurrent study (Elliot et al. 2002a), monitoring was undertaken of a nearby field (<1 km distant) which contrasted with Fields A and B reported here in that an epizootic of *N. tanajoae* did occur. Those data were used to generate a regression model which explained 70% of the variance in *N. tanajoae*-induced host mortality according to inoculum levels (mummy density) and 3-night saturation deficit minima ($SD\min_{3d}$) one week prior to sampling (Elliot et al. 2002a). (Note that host density was not a significant parameter in the original regression. Further, although densities here sometimes exceeded those in the original study, which could leave to a partial overestimation of transmission, this would not alter conclusions that an epizootic could occur.) This model is:

$$\text{Mortality}_{\text{wk1}} = \left(\sin\left(0.1537\ \text{mummy density}_{\text{wk0}} - 0.1594\left(\overline{SD\min_{3d}} \times \text{mummy density}_{\text{wk0}}\right)\right)\right)^2$$

The sine function and squaring represent back-transformation of an initial arc-sine transformation of mortality (a percentage) to obtain normality in the data. (Note that in the original paper, Elliot et al. 2002a, the square function was omitted and this mistake was not spotted.) The measured climatic data were used to estimate, for each sample date, the infection potential of *N. tanajoae*, expressed as the proportional mortality of pooled nymphs and adult females. Note that the great majority of infection of *M. tanajoa* occurs in these stages for reasons discussed by Elliot et al. (2002c). Calculations were made of the mortality expected from the introduction of 1, 5 or 25 mummies per 100 cm^2 leaf area (N.B. most leaves were only slightly larger than this and the range of the original regression was 0–28 mummies per 100 cm^2). These expected mortalities were multiplied by the densities of potential hosts (nymphs and adult females) to estimate expected mortality of these introductions in those populations.

Results

There was a peak of *M. tanajoa* densities in May, at over 1,000 adult females and nymphs (the life stages most likely to be infected in the field; Elliot et al. 2002c) per 100 cm^2 on apical leaves and over 200 per 100 cm^2 on median leaves (Figs. 1a and 2a). This peak was

accompanied by a corresponding peak of nearly 25,000 eggs per 100 cm^2 on the apical leaves (Fig. 1a). Towards the end of this peak, there were high densities of the predatory mite *Neoseiulus idaeus* Denmark & Muma (no other predatory mites were detected),

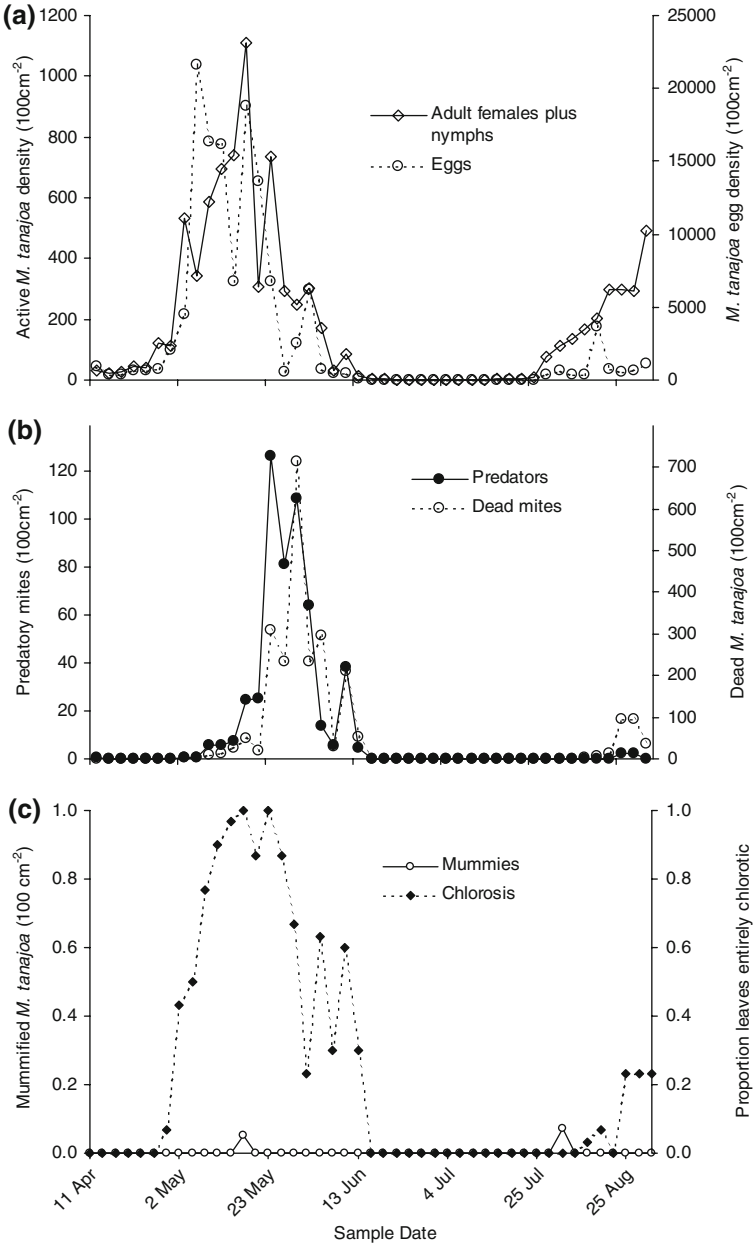

Fig. 1 Monitoring *Mononychellus tanajoa* densities on apical leaves of cassava, Piritiba, Bahia, Brazil, 1996. (**a**) Densities of active *M. tanajoa* life stages and eggs; (**b**) densities of phytoseiid predators and dead *M. tanajoa*; (**c**) densities of cadavers of *M. tanajoa* killed and mummified by *Neozygites tanajoae* and leaf injury (as proportion of leaves which were 100% chlorotic)

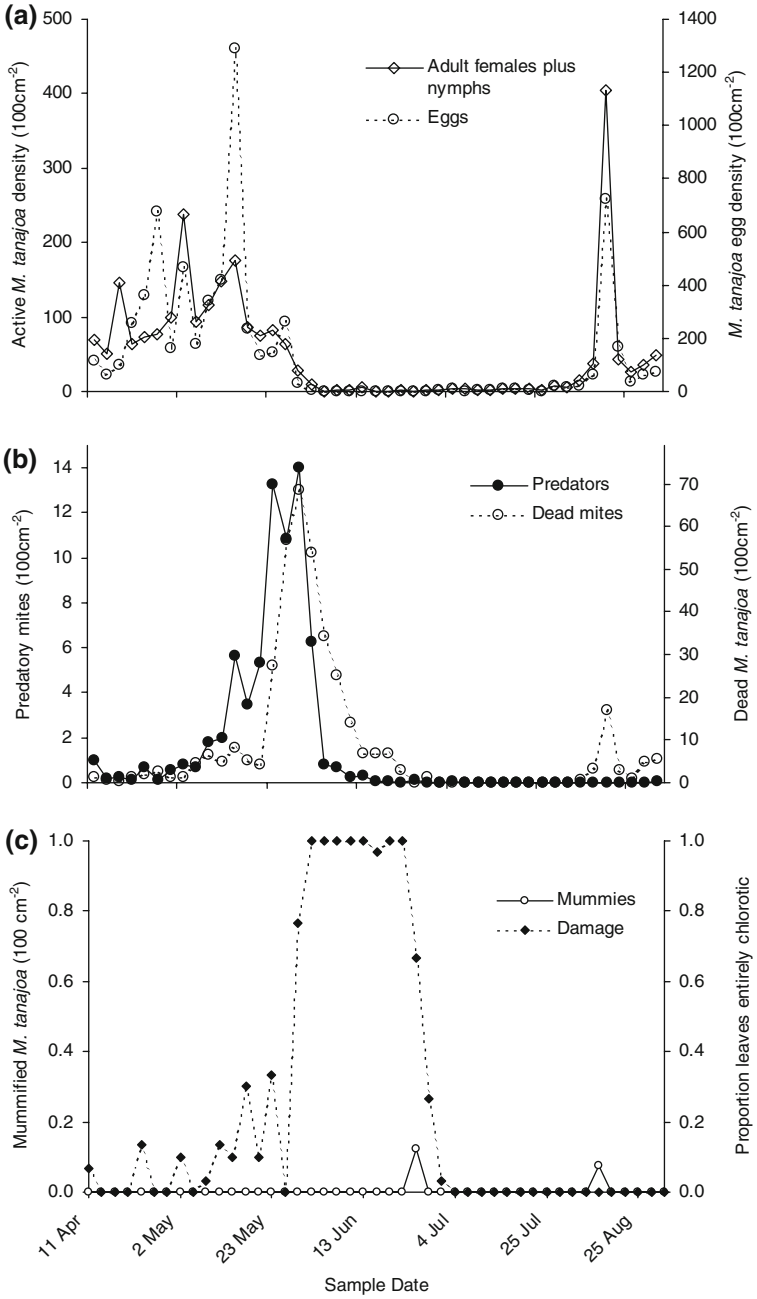

Fig. 2 Monitoring *Mononychellus tanajoa* densities on median leaves of cassava, Piritiba, Bahia, Brazil, 1996. (**a**) Densities of active *M. tanajoa* life stages and eggs; (**b**) densities of phytoseiid predators and dead *M. tanajoa*; (**c**) densities of cadavers of *M. tanajoa* killed and mummified by *Neozygites tanajoae* and leaf injury (as proportion of leaves which were 100% chlorotic)

coinciding with a drop in *M. tanajoa* egg densities and an increase in predated *M. tanajoa* cadavers (Figs. 1b and 2b). Immediately prior to this, however, there was 100% chlorosis of leaves (Figs. 1c and 2c), followed by total leaf loss of the cassava plants (data not shown). Having crashed to zero, mite populations began to recover in early August.

Meanwhile, the pathogen *N. tanajoae* was only observed at very low levels well past the first peak in *M. tanajoa* densities, whether in the natural population (Figs. 1c and 2c) or on the mites on the sentinel plants (Table 1). The results of experimental tests of transmission from early May to mid-July are presented in Table 2. During the *M. tanajoa* peak (mid-May), whilst percentage mummy sporulation was mostly high, few mites were observed bearing capilliconidia. These were found more from the end of June on and full transmission, to the production of hyphal bodies, was only observed in July. This period corresponds with low levels of saturation deficit (Fig. 3a) and also with the period in which the pathogen was observed in the nearby field described by Elliot et al. (2002a).

In April and early May, when *M. tanajoa* populations were increasing, 3-day means of saturation deficit were around 0.9 mmHg (Fig. 3a). This dropped to approximately 0.5 mmHg at the end of May when the *M. tanajoa* population was crashing and persisted at around this level until the end of the study. Using the regression model, percentage mortalities resulting from three inoculum densities were estimated on the basis of these saturation deficit minima (Fig. 3b). This pattern was similar to the saturation deficit

Table 1 Structures of *Neozygites tanajoae* observed in *Mononychellus tanajoa* spider mites on sentinel plants in a field in Piritiba, central Bahia, 1996

Date		Mites bearing capilliconidia	Total number of capilliconidia	Mites with hyphal bodies	Total mites ccollected
May	24	0	0	0	293
	27	0	0	0	222
	30	0	0	0	642
June	2	0	0	0	375
	5	0	0	0	348
	9	1	1	0	307
	11	0	0	0	375
	14	0	0	0	375
	17	0	0	0	375
	20	0	0	0	375
	24	0	0	0	269
	26	0	0	0	191
	30	2	4	0	336
July	02	4	5	2	320
	05	8	21	1	285
	08	2	0	2	353
	11	0	0	0	300
	15	1	1	0	371
	17	3	2	0	356
	21	1	1	0	370
	24	4	7	0	360
	26	0	0	0	372
August	02	0	0	0	345
Total		26	42	5	7,915

Table 2 Transmission of *Neozygites tanajoae* to new *Mononychellus tanajoa* hosts in experimental clip-cages in a field in Piritiba, central Bahia, 1996

Date	Mummies		*M. tanajoa*			
	Sporulation (%)	*N*	Bearing fungal structures (%)	Capilliconida per mite	With hyphal bodies (%)	*N*
1–7 May	66	47	1	0.04	0	72
7–13 May	62	45	3	0.03	0	78
16–22 May	0	48	0	0.00	0	61
22–28 May	92	25	15	0.20	0	40
30 May–6 Jun	80	54	40	3.33	4	79
7–13 Jun	91	53	46	2.41	0	82
12–20 Jun	90	52	12	0.53	0	57
27 Jun–3 Jul	96	53	24	0.79	15	73
6–12 Jul	98	56	19	1.10	11	64
12–18 Jul	74	46	14	0.20	9	67
Total	75	479	20	1.05	5	673

pattern, but amplified. Combining these predictions with the numbers of adult females and nymphs on leaves, we see three periods when we would have expected to see further cycling of the pathogen in the host *M. tanajoa* population, given inoculum: mid- to late April then May and finally August (Fig. 3c, d). The greater of these, in May, corresponds with declining *M. tanajoa* population levels. The other two periods, early May and late August, corresponded with rising *M. tanajoa* population levels, but were of a lesser magnitude.

Discussion

Of three factors which might have controlled populations of the herbivorous mite *M. tanajoa*, predation, parasitism and resource depletion, it is clear that it was the latter which was most important on this occasion. Densities of active mites peaked at 1,600 individuals per 100 cm^2 of leaf so it is unsurprising that leaf loss ensued. This density is far above those used by Oduor et al. (1997) to model epizootics in this system (they took 440 mites per 100 cm^2 as a characteristic density, probably a quite realistic figure during population growth and prior to overexploitation). The crash in the prey population cannot be attributed to the predators, however, as the cassava leaves were entirely chlorotic and massive leaf loss was observed, such that it even became difficult to find sample material. It is apparent, then, that the predators did not control the herbivorous *M. tanajoa* populations before the latter had depleted their resources (leaves) through overexploitation. Meanwhile, the population of predatory mites reached a mean of ca. 130 active individuals per leaf but only by the time over 70% of the plants' leaves had been lost.

It is noteworthy, then, that neither natural enemy controlled *M. tanajoa* populations to any great degree and certainly in no way of possible interest for use in pest management. For the pathogen *N. tanajoae*, considering that it apparently did regulate *M. tanajoa* populations in a nearby field in the same season (Elliot et al. 2002a), this observation requires explanation.

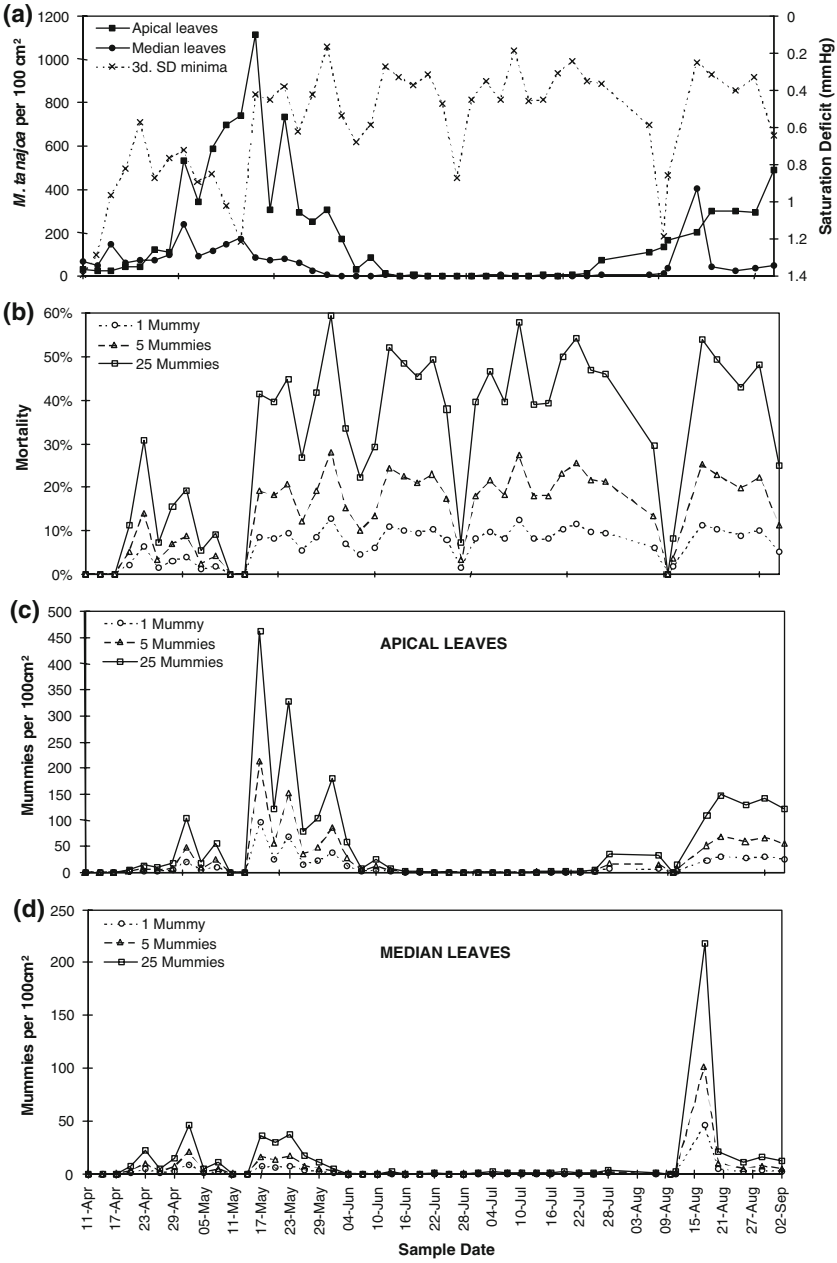

Fig. 3 Modelling epizootic potential in field populations of cassava green mite (*Mononychellus tanajoa*), Piritiba, Bahia, Brazil, 1996. (**a**) Field monitoring of densities of *M. tanajoa* adults and nymphs (pooled) on apical and median leaves of cassava (see Figs. 1 and 2) and 3-day minima of saturation deficits; (**b**) Predictions from modelling (see text) of percentage mortality of *M. tanajoa* expected if inocula of 1, 5 or 25 mummies (*M. tanajoa* killed by *Neozygites tanajoae*) present per 100 cm^2; (**c** and **d**) Absolute mortalities expected in *M. tanajoa* populations on apical and median leaves, from percentage mortalities predicted

During the first phase of *M. tanajoa* population growth (until the end of May), the fungus *N. tanajoae* was not detected on mites on sentinel plants (Table 1). Meanwhile, in the assays of transmission potential in the clip-cages (Table 2), there was some sporulation of mummies but very little to no transmission (note that the cages would be expected to increase transmission). At the same time, night-time saturation deficits were at their greatest (i.e., it was dry; Fig. 3a). This is reflected in limited epizootic capacity according to the regression model (Fig. 3b–d), except in late May when there is some potential for transmission. This potential is, however, unrealised in the field, probably due to a lag between favourable conditions and germination of fungal resting spores in the environment (Elliot et al. 2002b). From June onwards, transmission was possible according to the clip-cage experiments (Table 2) and according to model predictions (Fig. 3b), but the fungus was only observed consistently on sentinel mites from the end of June. In June, however, there were simply no mites for the pathogen to infect (Figs. 1, 2, and 3c, d). It appears that each of the stages (mummy sporulation, capilliconidium pick-up, and hyphal body production) was in some way limited, as there is a stepwise progress from early May when sporulation of mummified cadavers was lower and few capilliconidia were found, to late May/early June, when sporulation and capilliconidium pick-up were high, but few mites had hyphal bodies, and on to July, when full transmission was observed at higher levels. Put simply, the fungus arrived in the system too late.

Once the cassava plants began to recover from leaf loss, and the herbivorous *M. tanajoa* population also began to recover on the new foliage, abiotic conditions were briefly favourable for the pathogen again (Fig. 3), but humidity soon decreased and *N. tanajoae* did not reappear until several months later (data not shown).

There were, therefore, three periods when there was epizootic potential but no pathogen inoculum (Figs. 3c, d). According to the data inputted to the regression model, it was indeed only after defoliation that we could have expected most fungus-induced mortality (Fig. 3b): one mummy per leaf is predicted to yield 50–100 new mummies, while higher densities of the pathogen would immediately decimate the host population.

This sort of dynamic has to be seen as a limitation for this fungus as a biological control agent, in this system at least and on this occasion, and probably explains in part the variability of its effectiveness as a source of population regulation (Elliot et al. 2000). Under such situations, the obvious solution would be to find a way to introduce inoculum in periods when there is epizootic potential, but one must question this sort of strategy in a system of very low economic inputs such as cassava, and in which it would be difficult to predict periods of epizootic potential. Also, this would require an economically viable means of production of inoculum.

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The effects of *Pseudomonas putida* biotype B on *Tetranychus urticae* (Acari: Tetranychidae)

H. Murat Aksoy · Sebahat K. Ozman-Sullivan · Heval Ocal · Nuray Celik · Gregory T. Sullivan

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Abstract This study investigated *Pseudomonas putida* biotype B as a potential biological control agent of *Tetranychus urticae*. The bacteria were isolated from greenhouse soil from Carsamba, Turkey. The experiment was carried out in a completely randomized plot design under laboratory conditions. For this purpose, spraying and dipping applications of a suspension of *P. putida* biotype B (10^8–10^9 colony forming units/ml) were applied to newly emerged, copulated females. Dead mite and egg counts were started on the 3rd day after treatments, and observations were continued daily until all the mites had died and egg hatching had finished. Both types of bacterial application significantly reduced total egg numbers and egg hatching, compared to their respective controls. Bacterial spraying was significantly more effective than dipping—the spray application demonstrated 100% efficacy and resulted in the fewest viable eggs. The results of this study indicated that *P. putida* biotype B has a strong efficacy in causing mortality in *T. urticae*.

Keywords Fluorescent bacteria · *Pseudomonas putida* · *Tetranychus urticae* · Biological control

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is the most polyphagous species of spider mites. Recent checklists of host plants include about 1,200 species (Bolland et al. 1998; Zhang 2003). After overwintering, the females migrate to weeds and other herbaceous plants. Direct damage of the mite is due to feeding punctures; the leaves become spotty, and then dry out. If attacks are heavy, the plant may die. The

H. M. Aksoy · S. K. Ozman-Sullivan (✉) · H. Ocal · N. Celik
Department of Plant Protection, Faculty of Agriculture, Ondokuz Mayis University,
55139 Samsun, Turkey
e-mail: sozman@omu.edu.tr

G. T. Sullivan
Ondokuz Mayis University, OYDEM, 55139 Samsun, Turkey

development of *T. urticae* is fastest between 23 and 30°C, and at a relative humidity of less than 50% (Jeppson et al. 1975; Hussey and Scopes 1985; van de Vrie 1985). Fecundity and fertility of *T. urticae* are age-specific functions, strongly dependent on factors such as host plant, food quality, temperature, population size and density. Adaptive strategies of the two-spotted spider mite, as a colonizing type of species, are based on high fecundity in the young females and a female-biased offspring sex ratio, leading to a rapid population increase (Carey and Bradley 1982; Sabelis 1985).

Conventional control treatments for the two-spotted spider mite require the application of broad spectrum acaricides which, in addition to controlling the pest, also eliminate most predators, including predatory mites. Moreover, acaricides cause the development of pesticide-resistant strains of *T. urticae* and residue problems (Muir and Cranham 1979; Cho et al. 1995; Goka 1998; Devine et al. 2001; Nauen et al. 2001; Kim et al. 2006). To alleviate these problems, alternative strategies for spider mite control can utilise biocontrol agents, including parasites, predators and pathogens. Biological control of spider mites by predators is widely used, especially by phytoseiid mites, for example *Phytoseiulus persimilis* A.-H. About 20 phytoseiid species are currently being mass reared and sold worldwide (Zhang 2003; Gerson et al. 2003; Gerson and Weintraub 2007).

Most research on mite pathogens deals with fungi; among them potential mycoacaricides are the species *Hirsutella thompsonii* Fisher, *Neozygites floridana* Weiser and Muma, *Beauveria bassiana* (Balsamo) Vuillemin, and *Verticillium lecanii* (Zimm.) Viégas (Askary et al. 1998; Chandler et al. 2000, 2005; van der Geest et al. 2000). As for bacteria, *Bacillus thuringiensis* Berliner is a well-known control agent, but this species is not a pathogen *sensu stricto* (van der Geest et al. 2000). Some fluorescent pseudomonads have been shown to be potential biocontrol agents of plant root diseases (Scher and Baker 1982; Park et al. 1988; Walsh et al. 2001). In addition, Aksoy and Mennan (2004) demonstrated the effects of a fluorescent *Pseudomonas* sp. isolate against nematodes. None of the fluorescent pseudomonads has yet been used as a biocontrol agent of mites. Neverthless, an infection that caused disease in *T. urticae* was identified as *Pseudomonas aeruginosa* (Poinar and Poinar 1998). This is an example of the increasing interest in pathogens of mites, as indicated by the large number of reviews on this subject (McCoy 1996; Poinar and Poinar 1998; Chandler et al. 2000; van der Geest et al. 2000).

Pseudomonas putida Trevisan, the subject of this study, is a gram negative, rod-shaped, saprophytic soil bacterium (Anzai et al. 2000). It demonstrates a very diverse metabolism, including the ability to degrade organic solvents such as toluene, and the compounds naphthalene and styrene oil (Marques and Ramos 1993; Gomes et al. 2005; Ward et al. 2006). *Pseudomonas putida* has also demonstrated potential biocontrol properties, as an effective antagonist of damping off diseases such as *Pythium* and *Fusarium* (Amer and Utkhede 2000; Validov et al. 2007).

In this study, the potential of *P. putida* biotype B as a biocontrol agent of *T. urticae* was examined by determining its effects on mortality, fecundity and egg hatching.

Materials and methods

Experiments were conducted in a growth room at 25 ± 1°C, L16:D8 photoperiod, and 55% r.h., at the Department of Plant Protection of Ondokuz Mayis University, Samsun, Turkey. All observations were made with a stereo-binocular microscope.

Soil sampling

A total of 30 soil samples was collected from tomato greenhouses in villages of Çarşamba in Samsun province, Turkey in 2004–2005. Relative to the size of greenhouse, 3–12 samples were randomly collected from 0 to 20 cm depth and mixed. A sub-sample of ~1 kg of soil was then taken per greenhouse and stored in sterile, polyethylene bags at 4°C for 2–3 days before processing.

Isolations

Stored soil samples were used to isolate fluorescent *Pseudomonas* isolates. Firstly, each soil sample was sieved through a 1-mm-mesh sieve, mixed at a ratio of 1:10 with sterile, distilled water, shaken thoroughly on a rotary shaker at 150 rpm at 24–26°C for 60 min, and serial dilutions (10^{-2}–10^{-4}) were prepared. Diluted samples were placed on King's B Agar (KBA) and incubated at 24–26°C for 24–48 h. Identification of bacterial isolates was based on colony morphology and fluorescent character, according to the standard diagnostic methods (Lelliott and Stead 1987; Kiewnick and Sands 2001). One of the fluorescent *Pseudomonas* isolates, FPin2 (Fluorescent *Pseudomonas* isolate number 2) was selected for this study after a preliminary study. It was later identified by using the computer-assisted microbial identification system (MIS) which employs gas–liquid chromatographic analysis of bacterial fatty acids.

The bacterial isolate was also re-isolated from the dead *T. urticae* from the experiment to verify that the same bacterial isolate was the cause of death. For this purpose, the dead mites were surface-sterilized in 0.5% sodium hypochlorite (NaOCl), rinsed several times in sterile water, gently blotted dry on paper towels, placed on KBA and incubated at 24–26°C for 24–48 h. This pure isolate, which was later identified using MIS and shown to be identical to the isolate described previously, was used to inoculate healthy *T. urticae* and shown to cause the same disease symptoms and mortality.

Bioassay

Newly emerged, copulated females of *T. urticae* and bean leaves (*Phaseolus vulgaris* L.) were used for the experiments. Bean plants were grown in pots, and *T. urticae* was reared as a stock culture in the laboratory on the beans. For the applications, freshly cut, 3-cm-diameter bean leaf discs were placed upper surface down on water-saturated sponges in 10-cm-diameter Petri dishes. Stikem Special™ was painted around each leaf disc to form a barrier to escape. The experiment was carried out in a completely randomized plot design with four treatments (spraying, dipping and a control for each treatment) and 10 replications, and with five mites in each replication. Spraying and dipping applications of the bacterial suspension of *P. putida* biotype B at 10^8–10^9 colony forming units (CFU) ml^{-1} were applied to newly hatched, copulated adults. For the spraying application, after the mites had been placed on the leaf discs, the suspension was applied from a distance of 25–30 cm with a hand spray atomiser of 50-ml capacity until the leaf surface was just wetted with very fine droplets. For the dipping application, the leaf discs were dipped in the suspension for 5 s. *Tetranychus urticae* were then transferred to these leaf discs. Sterile water was applied for the controls. Counting of dead mites and eggs started on the 3rd day after treatment, and daily counting continued until all individuals of the original cohorts had died and egg hatching had finished. After being counted daily, the eggs for each replication were transferred to an untreated leaf disc for hatching observations.

Statistical analysis

Mite mortality and egg hatching were expressed as percentages for the statistical analysis. To determine the efficacy (%) of *P. putida* biotype B on *T. urticae*, mortality data were analysed using the Henderson-Tilton formula. Total egg numbers were $\sqrt{x}$-transformed, while egg hatching, mortality and efficacy data were arcsin$\sqrt{x}$-transformed, because the data could not be assumed to be normally distributed. One-way ANOVA was not performed because of heteroscedasticity. Instead, differences among the treatments were analysed with the nonparametric Kruskal–Wallis test, for total egg number, egg hatching and mortality. Dunn's multiple comparison tests were then applied to determine any further differences among the groups. The Mann–Whitney *U*-test was performed to test for differences in efficacy between the spraying and dipping bacterial treatments. All computations were performed using Minitab (Minitab 2000, V. 13.20).

Results

Identification of fluorescent *Pseudomonas* isolates

A total of seven distinct fluorescent *Pseudomonas* isolates were obtained from 30 soil samples taken in Carsamba, Turkey; three were from Damlatas village, four from Karabahce village. Isolate FPin2 from Damlatas, which had been effective in pre-testing on *T. urticae*, was later identified using MIS as *P. putida* biotype B. Colonies of *P. putida* biotype B on KBA were whitish-grey, raised, and with diffusible, yellowish-green pigment, and they fluoresced under ultraviolet light (366 nm) (Fig. 1). Re-isolated, pure culture of the suspected pathogen from

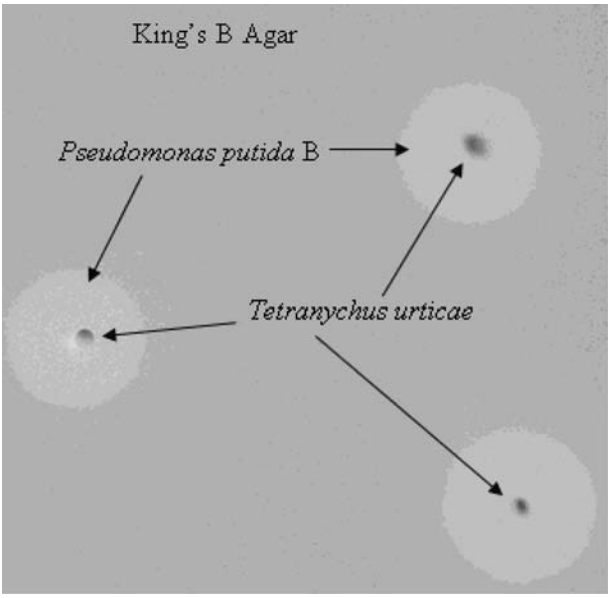

Fig. 1 *Pseudomonas putida* biotype B colonies growing from dead *Tetranychus urticae*

Table 1 The effects (mean ± standard error) of *Pseudomonas putida* biotype B (PpB) on *Tetranychus urticae**

Treatments	Mortality (%)	Efficacy (%)	Total egg number	Egg hatching (%)
Spraying control	20 ± 3.0 c		106.7 ± 35.22 ab	100 a
Spraying PpB	100 a	100 a	2.6 ± 0.76 c	46.3 ± 13.86 c
Dipping control	6 ± 3.1 d		163.7 ± 53.98 a	97.4 ± 1.84 a
Dipping PpB	80 ± 5.2 b	78.5 ± 5.67 b	37.8 ± 5.97 b	93.4 ± 1.35 b

* Different letters within a column signify difference ($P < 0.01$)

dead *T. urticae* was reintroduced to healthy *T. urticae* in which it again showed the same pathology, and was again identified as *P. putida* biotype B.

The effects of *Pseudomonas putida* biotype B on *Tetranychus urticae*

After *P. putida* biotype B applications on *T. urticae*, the symptoms were: slowing down of movements, cessation of feeding, reduced egg laying, and then occurrence of brown-black coloration. The earliest deaths occurred within the first day after the treatments. All the mites in the spraying bacterial treatment died within 4 days, so statistical analysis for mortality and efficacy were done using the data on day 4. There were highly significant differences in adult mortality among the treatments (Table 1). The highest mortality (100%) was for the spraying bacterial treatment and the lowest was 6% for the dipping control. Bacterial dipping caused 78.5% mite mortality, significantly lower than the effect of bacterial spraying. Because no mites had survived more than 4 days after bacterial spraying, the total egg numbers (2.6 eggs) were much lower than after dipping (37.8 eggs), where some mites remained alive and continued to lay eggs (Table 1).

Almost all eggs hatched in the two controls, but egg hatching was reduced to 46% in the spraying application (Table 1). In 3 of the 10 spray replicates, no eggs were laid due to the premature deaths of all the mites. In those three cases, 0% egg hatching was assumed.

Discussion

The results of this study indicate that *P. putida* biotype B has a strong efficacy in causing mortality of the phytophagous mite *T. urticae*, but the mechanisms are not clear. However, there is evidence that the genus *Pseudomonas* contains virulent species. General support for our results comes from the effects on insect pests of different *Pseudomonas* strains, either as bacterial suspension or through different formulations (Zehnder et al. 1997; Broadway et al. 1998). In a specific case, Vodovar et al. (2006) reported that *Pseudomonas entomophila* exhibits virulence against *Drosophila melanogaster* Meigen that may be due to strong hemolytic activity, involving proteins such as lipases, chitinases and/or hydrolases. It may be that one or more of these factors also contributed to the mortality of *T. urticae* caused by *P. putida* biotype B in the current study. Because chitin is a structural component of the gut lining of insects and mites, chitin metabolism is considered to be an excellent target for selective pest control. Research on bacteria-mediated insect control has indicated that bacterial chitinases may hydrolyze the insect's chitin (Kramer and Muthukrishnan 1997; Broadway et al. 1998). Various modes of action have been demonstrated for *P. putida*'s antagonistic effects against soil-borne diseases. They include the production

of secondary metabolites and antifungal compounds (Dowling and O'Gara 1994; Maurhofer et al. 1998; Ramamoorthy et al. 2001; Raaijmakers et al. 2002), and the production of the lytic enzymes chitinase and glucanase which act on fungal cell wall components (Singh et al. 1999). Wilson et al. (2002) reported that bacterial hemolysins are exotoxins that attack blood cell membranes and cause cell rupture by poorly defined mechanisms. This may also be involved in the pathogenicity of *P. putida* biotype B against *T. urticae*, especially given the rapidity of death of *T. urticae* in the current study.

In the spraying application in the current study, the vegetative cells of *P. putida* biotype B were applied directly to the body surface of the mites. According to Vodovar et al. (2006), pathogenic bacteria rely on a variety of cell surface-associated virulence factors that allow adhesion to the host surface and promote effective colonization. If applicable to *P. putida* biotype B, this adhesion could enhance the incursion of proteases, chitinases, lipases and hydrolases through the cuticle, stigmas and body orifices of *T. urticae*, initiating the rapid death of the mite. The mites' body surfaces were wetted and provided an ideal environment for the survival, colonisation and reproduction of *P. putida* biotype B. However, further research is necessary to elucidate the mode of action of *P. putida* biotype B; for instance, the enzymes produced by the pathogen need to be identified.

In comparison to spraying, mortality after dipping was lower and more protracted. This may be attributable to the body surface of *T. urticae* not being wetted, providing a less conducive environment for bacterial colonisation, and cells of *P. putida* biotype B only being introduced to the body surface indirectly, e.g. by brushing the ventral surface of the body against the leaf disc surface and cleaning the mouthparts. Infection by *P. putida* biotype B via dipping would therefore have been later and arguably at lower levels of inoculation, hence the lower levels of mortality spread over a longer period. Separately, the spraying control mortality was higher than for the dipping control. We cannot explain this, given that the very low spraying pressure was unlikely to have caused physical damage to the mites. However, there was a highly significant difference between the bacterial spray and its control, so clearly something caused mortality, distinct from the spraying method.

Despite the fact that the mechanisms are unclear, *P. putida* biotype B caused substantial mortality of *T. urticae* in a relatively short time, especially in the spray application, and also reduced egg numbers and egg hatching. However, further experiments are needed to assess the efficacy of *P. putida* biotype B and other biotypes against *T. urticae* at lower concentrations and in glasshouse and field experiments, in order to quantify its potential as a biological control agent. The compatibility of *P. putida* biotype B with biocontrol agents currently employed to control *T. urticae* would also need to be carefully assessed.

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Overwintering and prevalence of *Neozygites floridana* (Zygomycetes: Neozygitaceae) in hibernating females of *Tetranychus urticae* (Acari: Tetranychidae) under cold climatic conditions in strawberries

Ingeborg Klingen · Gunnar Wærsted · Karin Westrum

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Abstract To evaluate overwintering strategies of the fungus *Neozygites floridana*, an important natural enemy of *Tetranychus urticae*, hibernating *T. urticae* females were investigated for the presence of fungal structures throughout one winter (October 12, 2006 to February 19, 2007) in field-grown strawberries in a cold climate in Norway (min. ambient temp −15.3°C). *Neozygites floridana* was present as hyphal bodies inside live, hibernating females in *T. urticae* populations throughout the sampling period. The lowest percentages of hibernating females with hyphal bodies were found at the two first dates of sampling at 5.5 and 0% on October 12 and 19, respectively. The prevalence then increased and peaked at 54.4% on January 14. Resting spores (immature) were also found in live hibernating females at some dates, but at lower prevalence than for hyphal bodies and predominantly only until November 8. Prevalence of resting spores in live hibernating females ranged from 2.5 to 13.8%. Total number of *T. urticae* was also recorded, and most mites of all four categories (nymphs, males, non-hibernating and hibernating females) were found at the first sampling date. At this date non-hibernating females were the most abundant. A sharp decrease in non-hibernating females, nymphs and males was, however, seen from mid-October to mid-November; also numbers of hibernating females decreased, but not as fast. The relative abundance of hibernating females compared to non-hibernating females increased from 32.2% at the first collection (October 12) to 97.7% at the last collection (February 2). This study confirms that *N. floridana* survives the winter as a semi-latent hyphal body infection, protected inside live hibernating females. It is therefore ready to develop and sporulate as soon as climatic conditions permit, resulting in early season infection of *T. urticae*.

I. Klingen (✉) · G. Wærsted · K. Westrum
Plant Health and Plant Protection Division, Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Høgskoleveien 7, 1432 Aas, Norway
e-mail: ingeborg.klingen@bioforsk.no

G. Wærsted
Department of Plant and Environmental Sciences, Norwegian University of Life Sciences, PO Box 5003, 1432 Aas, Norway

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Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) has a broad host plant range and is an annual pest of many crops throughout the world (Greco et al. 2005), including strawberries (Cross et al. 2001; Easterbrook et al. 2001; Garcia-Mari and Gonzalez-Zamora 1999; Raworth 1986). *Tetranychus urticae* lowers the potential yield of strawberries due to a reduction in photosynthesis and transpiration in leaves, caused by its feeding on mesophyll and parenchyma cells (Sances et al. 1979, 1982; Veerman 1985). Use of acaricides is the main strategy to control *T. urticae* in field crops, and development of resistance in *T. urticae* populations is common (Cranham and Helle 1985; Devine et al. 2001). Resistance may lead to increased use of acaricides that may in turn increase production costs, appearance of secondary pests and negative effects on public health and environment. Public concern on the negative effects of pesticides has resulted in a focus on the development of biological control methods and conservation biological control may be one way of controlling *T. urticae*. Most studies on conservation biological control of *T. urticae* focus on predators (Klingen and Westrum 2007), but the mite-pathogenic fungus *Neozygites floridana* (Weiser and Muma) (Zygomycetes: Neozygitaceae), an important natural enemy of *T. urticae,* is also a good candidate (van der Geest et al. 2000).

Neozygites spp. develop inside spider mites as hyphal bodies, kill their host, penetrate the cuticle and produce spores (primary conidia) on conidiophores. Primary conidia are actively ejected from swollen brown mite cadavers, referred to as mummies, and these conidia germinate to form the infective and more persistent capilliconidia that infect new mites (Carner 1976; Delalibera et al. 2006; Elliot 1998). *Neozygites* spp. are considered by many authors to be a major factor causing decline in populations of *T. urticae* in different crops when microclimatic conditions are right (Boykin et al. 1984; Carner and Canerday 1970; Dick and Buschman 1995; Klubertanz et al. 1991; Smitley et al. 1986b). Pesticides may limit the survival and efficacy of *N. floridana* and laboratory studies on the effect of pesticides on *N. floridana* have been conducted, resulting in suggestions on how to manage pesticide application to reduce negative effects on this beneficial fungus (Klingen and Westrum 2007; Klubertantz et al. 1991). Further, a successful extension-based integrated pest management (IPM) strategy has been established for the related insect pathogenic fungus *Neozygites fresenii* (Nowakowski), infecting cotton aphid, *Aphis gossypii* Glover, in which growers withhold insecticide applications for aphids when *N. fresenii* epizootics are predicted to control the cotton aphid (Steinkraus 2000).

Overwintering of pathogens through long periods of unfavourable conditions is another factor that may limit the survival and efficacy of a pathogen in a pest insect or mite population, and can be critical for their use in biological control (Elliot et al. 2002). *Tetranychus urticae* is known to overwinter as hibernating females (Veerman 1985), and these partly inactive females may harbour *N. floridana*. The mite exhibits a facultative diapause in adult females only, and the diapause is induced during the larval and nymphal stages by short-day photoperiods (Veerman 1977a), but temperature and nutrition may also contribute to this induction (Veerman 1985). Termination of the diapause depends on the period of chilling and on the photoperiod, at least during the first 3 or 4 months of diapause (Veerman 1977b, 1985).

Little is known about the distribution of hibernating *T. urticae* females in strawberry fields during the winter in Norway. One study in Norwegian strawberry fields reported,

however, that aggregation of hibernating females was seen in dark places such as below wilted leaves and under the plastic cover (Stenseth 1976). No hibernating females were found directly on the soil. According to Veerman (1985), *T. urticae* females that have been induced to diapause feed only very little before they leave the host plant in search of dark hibernation sites. It is not well known how *N. floridana* survives the harsh conditions throughout a cold winter, and only a few studies report fungal structures that may survive. Only one of these studies is conducted throughout the winter, but under warmer climatic conditions than at our study site (Brandenburg and Kennedy 1981; Klubertanz et al. 1991; Mietkiewski et al. 1993). In previous studies in Norway, however, females of *T. urticae* infected by *N. floridana* were found as early as March 18 in 2003 (Nordengen and Klingen 2006). The mean temperature at this time of year was low (1.7°C) (http://lmt.bioforsk.no/agrometbase/getweatherdata.php), and the resulting development of an infection was probably very slow. This indicates that such an early infection could not have been initiated by resting spores from plant debris but rather was a latent infection of *N. floridana* present in hibernating females. The aim of this study was therefore to investigate whether *N. floridana* may be present inside living hibernating females of *T. urticae* throughout the winter season, and if so, in what prevalence and what stage of its fungal life cycle. The occurrence of *T. urticae* hibernating females, non-hibernating females, males and nymphs found in samples throughout the winter was also investigated.

Materials and methods

Field information and climatic recordings

The study was conducted in a south-sloping 1-ha strawberry field (cultivar Corona, planted in 2004) in Lier in southeastern Norway (59°47′ N, 10°16′ E). Strawberries were grown conventionally, and the acaricides hexythiazox and methiocarb were used once in spring and after harvest, respectively. The insecticide esfenvalerate was used once before bud break, and the fungicides fenhexamid, cyprodinil + fludioxonil and tolylfluanid were used twice each during summer. The sampling site was established autumn 2006 along the border at the north end of the field and measured 10 m downslope × 10 double rows. Poles of 60 cm were inserted into the ground for every 1 m downslope. To facilitate collection of strawberry leaves from the field after snowfall, the sampling site was covered by a Polyfelt® polypropylene cover (140 g/m^2) placed on top of the poles from October 26. The part of the strawberry field that was not used for sampling was naturally covered with at least 10 cm fluffy snow from mid-January to mid-February. Temperature below and above the cover were recorded from January 15 to March 23 with Tiny Tag® loggers (INTAB Interface-Teknik AB, Stenkullen, Sweden). Recordings from a weather station in Lier, located 2 km east of the field, were also used for more comprehensive weather data for the whole winter (http://lmt.bioforsk.no/agrometbase/getweatherdata.php). RH was only measured at the weather station.

Sampling mites in the field

Hibernating females of *T. urticae* were obtained by weekly collection of green trifoliate strawberry leaves, from October12, 2006 to January 30, 2007, except the three last weeks of December. During October and November, one trifoliate strawberry leaf was collected from each of the 10 double rows at 10 sampling points downslope, giving a total of 100 leaves per

sampling date. To obtain sufficiently high mite numbers when the mite population started to decline (from December 6), two leaves were taken from each double row and each sampling point, giving a total of 200 leaves per sampling date. Leaves were collected randomly from all parts of the plant. When not enough fresh green leaves were present in the field (February 5, 12 and 19) mites were collected by taking a handful (approximately 0.1 l) of plant debris from the ground surrounding the strawberry plants in each row and at each sampling point. The collected plant debris consisted mainly of wilted strawberry leaves and stems, plus some straw. One handful of debris for each double row and at each of the ten sampling points resulted in 100 handfuls (10 l) of debris for each date. Samples were placed in paper bags and brought immediately to the laboratory for processing.

Extraction of mites from samples

Washing out mites from green leaves

Samples from each sampling point (ten green trifoliate leaves) were placed in individual plastic boxes (2 l) filled with soapy water at ca. 40°C (0.5 ml washing detergent in 1.5 l water). Leaves were immersed in the soapy water overnight at room temperature and mites were washed out the next morning and placed in a glass vial containing 20 ml 80% ethanol as described by Nordengen and Klingen (2006).

Extracting mites from plant debris

Mites from samples of plant debris were extracted by using a Berlese funnel method (Hutchins 1994), except that no gauze was used to prevent upward movement of mites. About 1 l plant debris from each sampling point was placed on top of a metal sieve (3 × 3 mm mesh) in individual Berlese funnel. The tip of the funnel was inserted through the lid of a plastic cup containing 20 ml 80% ethanol. A 40 W incandescent light bulb was mounted on top of the funnel 10 cm above the debris. Funnels were left like this for 7 days for mites to crawl out of the plant debris, away from the light bulb, and to fall into the cup containing the ethanol.

Recording mite numbers

Ethanol samples were investigated for presence of *T. urticae* immediately after processing. The mites were counted and categorized as nymphs, males, and hibernating and non-hibernating females under a compound microscope (6.3–50×) using a counting grid consisting of 8 equal sectors. The term hibernation for females of *T. urticae* (when an orange-red body colour appears) is generally used to denote a state of dormancy (diapause or quiescence) which occurs during winter months (Veerman 1985), and this is also how we use the term in this paper.

Neozygites floridana infections

Live Method A: infections in hibernating females from ethanol samples

Due to practical constraints (peaks in work load) it is an advantage to use a method where samples can be processed as time permits. In the present study, we therefore used a method to quantify hyphal bodies of *N. floridana* in *T. urticae* stored in ethanol (Nordengen and

Klingen 2006) and adapted it to include observation of all fungal stages in hibernating females. All hibernating females that looked like they had been alive when collected (orange-red in colour, not injured and having a body full of "content") were mounted in 50% lactic acid and observed under the microscope (200–400×) for the presence of *N. floridana* (see Table 1 for numbers of hibernating females (n) mounted at each date). This method is called Live Method A below. Hibernating females were classified as infected when resting spores or hyphal bodies were present. They were also classified as infected when at least one germinated capilliconidium was attached to the mite body. Previous studies have shown that it only takes one attached capilliconidium to produce a lethal infection (Oduor et al. 1997), and that capilliconidia attached to the mite body indicate a strong infection potential and hence a good estimate for the infection level (Delalibera et al. 2000). Humber (1997) and Keller (1991) were used for identification of *N. floridana* fungal structures.

Live Method B: infections in hibernating females directly mounted

To ensure that the infection level of *N. floridana* recorded by Live Method A was not an artefact of the method, a direct method was used as a control. In the control method, *N. floridana* infection was investigated in live *T. urticae* hibernating females transferred directly from green leaves to a slide by a small paint brush. This method is called Live Method B below. Leaf samples were randomly collected from the same sample site as for Live Method A on 9 sampling dates between October 26 and February 5. One collection was also conducted on March 23. When enough mites were present (see Table 1 for numbers), 50 live mites were mounted directly in lactophenol cotton blue (LPCB, with 0.0016% cotton blue) and checked for hyphal bodies and resting spores under a microscope (200–400×). To test whether hyphal bodies were viable, 20 live hibernating females collected on February 12 were also incubated individually in vials on strawberry leaf disks as described in Klingen and Westrum (2006).

Resting spores of Neozygites floridana in dead mites

To quantify the prevalence of resting spores of *N. floridana* in dead *T. urticae*, ethanol samples were also inspected for cadavers filled with black resting spores using a compound microscope (6.3–50×) with contrast illumination. Fresh leaves collected for Live Method B were also observed under a compound microscope (6.3–50×) to search for dead mites with resting spores directly on the leaf.

Statistical analysis

To compare infection levels obtained by the two methods (Live Method A and B) a pairwise t-test was used. Statistical analyses were conducted with MINITAB® (2006).

Results

Neozygites floridana in hibernating females, Live Method A and B

Hibernating females (Live Method A) were found with hyphal bodies of *N. floridana* on all sampling dates, except October 19. The lowest percentages of hibernating females with hyphal bodies were found in the beginning of the collection period; 5.5 and 0% on October

Table 1 Percent hibernating females of *Tetranychus urticae* infected with *Neozygites floridana* during the winter 2006/2007 in Lier, Norway. Live Method A and B with hyphal bodies/capilliconidia (Hyp/Cap) or with immature resting spores (IRestSp). Last column presents numbers of dead *T. urticae* with mature resting spores (MRestSp)

Date	Live method A[a]				Live method B[b]				Dead mites[c]
	# Mounted (n)	% Hyp/Cap	% IRestSp	% Total infection	# Mounted (n)	% Hyp/Cap	% IRestSp	% Total infection	# With MRestSp
12.10.06	91	5.5	8.8	14.3	–	–	–	–	0
19.10.06	58	0	13.8	13.8	–	–	–	–	0
26.10.06	61	23.0	9.8	32.8	43	48.8	9.3	58.1	10
02.11.06	52	25.0	9.6	34.6	50	56.0	8.0	64.0	6
08.11.06	40	37.5	2.5	40.0	–	–	–	–	4
14.11.06	38	34.2	0	34.2	–	–	–	–	8
22.11.06	40	27.5	0	27.5	–	–	–	–	4
30.11.06	49	42.9	0	42.9	37	54.1	0	54.1	1
06.12.06	70	32.9	0	32.9	50	50.0	0	50.0	1
01.01.07	29	37.9	0	37.9	–	–	–	–	0
08.01.07	71	46.5	0	46.5	50	58.0	2.0	60.0	0
14.01.07	92	54.3	0	54.3	49	65.3	0	65.3	3
22.01.07	54	53.7	0	53.7	26	53.9	0	53.9	2
30.01.07	40	32.5	0	32.5	45	53.3	0	53.3	0
05.02.07	53	52.8	0	52.8	30	46.7	0	46.7	0
12.02.07	80	53.8	0	53.8	–	–	–	–	0
19.02.07	84	44.0	0	44.0	–	–	–	–	0
23.03.07	–	–	–	–	50	28.0	0	28.0	–

– = No observation

[a] Live Method A: Mounted live looking mites from ethanol samples. Mites were obtained from green strawberry leaves until January 30. After this date mites were obtained from plant debris from the ground surrounding the strawberry plants

[b] Live Method B: Mounted live mites picked directly from green leaves only

[c] Dead mites: Dead mites were observed for mature resting spores in ethanol samples. Same samples as those used for Live Method A

12 and 19, respectively. The prevalence of hyphal bodies peaked at 54.3% on January 14. Only immature resting spores were observed in live hibernating females, and these were only found at the first five dates of collection. Prevalence of resting spores was generally lower than for hyphal bodies, ranging from 2.5 to 13.8% (Table 1).

Hibernating females (Live Method B) were found with hyphal bodies of *N. floridana* at all 10 sampling dates, but resting spores were found at three sampling dates only. The highest prevalence of hyphal bodies was found on January 14 (65.3%). Also with this method, live hibernating females were found with immature resting spores only. These were detected at the first two collection dates. Further, a single observation of one mite with immature resting spores was made on January 8. The prevalence of immature resting spores ranged from 2.0 to 9.3% and was generally lower than for hyphal bodies (Table 1).

The pairwise t-test revealed that the Live Method B detected a higher total prevalence of *N. floridana* ($T = 3.59$, $P = 0.007$) than Live Method A. During collection of live hibernating females from leaves, sporulating cadavers were also observed on leaves on November 2 and December 6. Out of the live mites incubated on February 12, 60% were found with *N. floridana* fungal structures at death, and out of these 30% sporulated.

Resting spores in cadavers and on leaf surface

Cadavers containing mature resting spores were found from October 26 till January 22 (Table 1). Leaves with a mass of resting spores from disintegrated cadavers were observed on January 8 (Fig. 1.6).

Recently killed mites filled with resting spores found on fresh leaves were dark brown to black, swollen and had a raspberry-like textured cuticle that was not easily broken when handled, even when dry (Fig. 1.1). Most cadavers found at Norwegian locations have thread-like rhizoids, but rhizoids with disk-like holdfasts have also been observed (Fig. 1.5.). Rhizoids were present on each side of the mite body. Cadavers containing several of the fungal stages (hyphal bodies, resting spores, conidiophores, primary conidia and capilliconidia) in the same individual were found quite frequently.

Occurrence of *Tetranychus urticae*

The highest numbers of mites of all four categories (nymphs, males, non-hibernating and hibernating females) were found at the first sampling (October 12). A sharp decrease in non-hibernating females, nymphs and males was seen from mid-October to mid-November (Fig. 2A). Numbers of hibernating females also decreased, but not as fast as for the three other *T. urticae* categories. The relative abundance of hibernating females compared to non-hibernating females increased from 38.9% at the first collection (October 12) to 97.7% at the last collection (February 2). Numbers of hibernating females increased from 20 (January 30) to 80 (February 5) when the substrate for collecting mites was changed from green leaf samples to plant debris from the ground. Both non-hibernating and hibernating females were found at all sampling dates, while nymphs and males were not observed at the last three dates when the plant debris method was used (Fig. 2A).

Climatic conditions at weather station and below cover

The temperature below the cover was generally higher than at the weather station, and the mean difference measured for the collection period was 3.9°C. When the lowest temperature recorded at the weather station was −15.3°C (January 22) the temperature below the

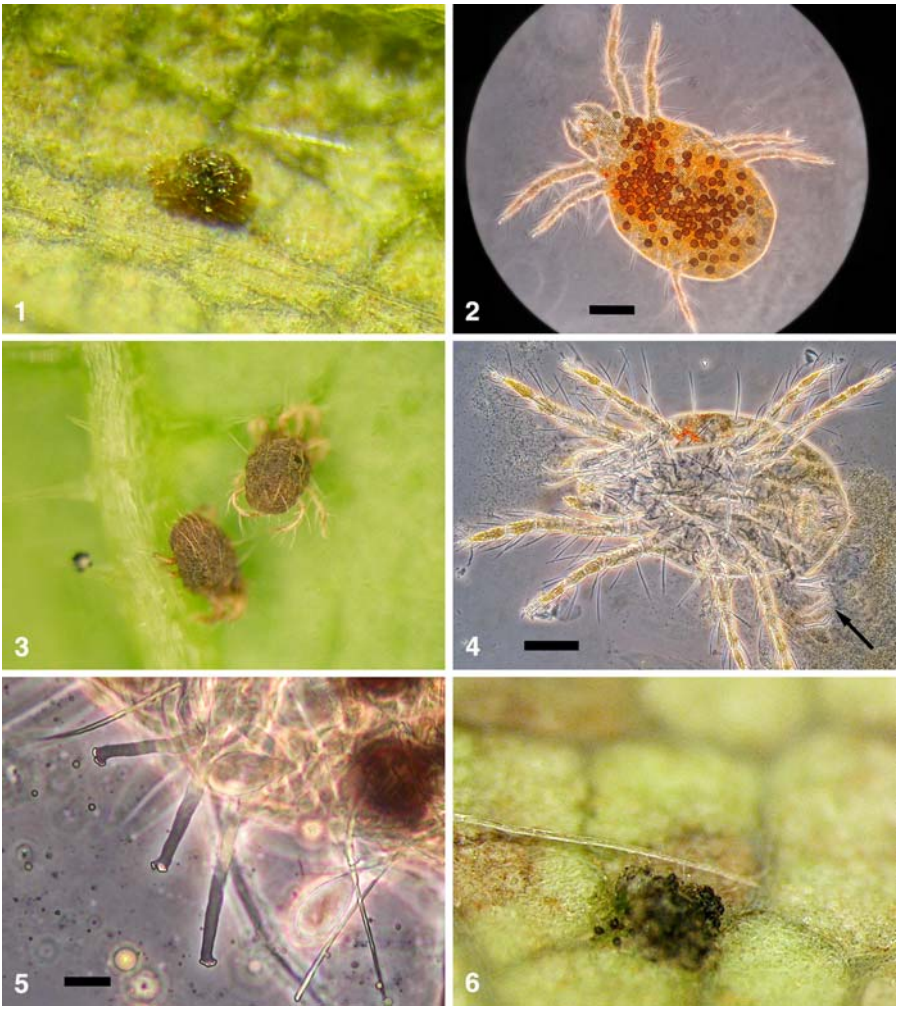

Fig. 1 (1) Resting spore cadaver of *Tetranychus urticae* killed by *Neozygites floridana.* (2) Hibernating female of *T. urticae* infected with resting spores of *N. floridana.* Scale bar = 100 µm. (3) Hyphal body cadavers (mummies) of *T. urticae* killed by *N. floridana.* (4) Hibernating female of *T. urticae* infected with hyphal bodies of *N. floridana.* Scale bar = 100 µm. (5) Three rhizoids with disk-like holdfasts on resting spore cadaver of *T. urticae* killed by *N. floridana.* Scale bar = 10 µm. (6) Disintegrated cadavers leaving just a mass of resting spores on the leaf surface

cover was −1.0°C. The lowest temperature recorded below the cover was −5.2°C (February 12) and on this date the temperature at the weather station was −10.0°C. On both these dates, live hibernating females with hyphal bodies were found. The highest temperature recorded below the cover was 2.9°C (February 5). On this date, the temperature above the cover was 3.4°C. Temperatures recorded at the weather station, when sporulating cadavers were found, were −6.9°C (low) and 4.3°C (high) on November 2, and 1.0°C (low) and 6.5°C (high) on December 6. The highest mean RH recorded at the weather station was 96.4% (November 14), the lowest 64.9% (January 14). However,

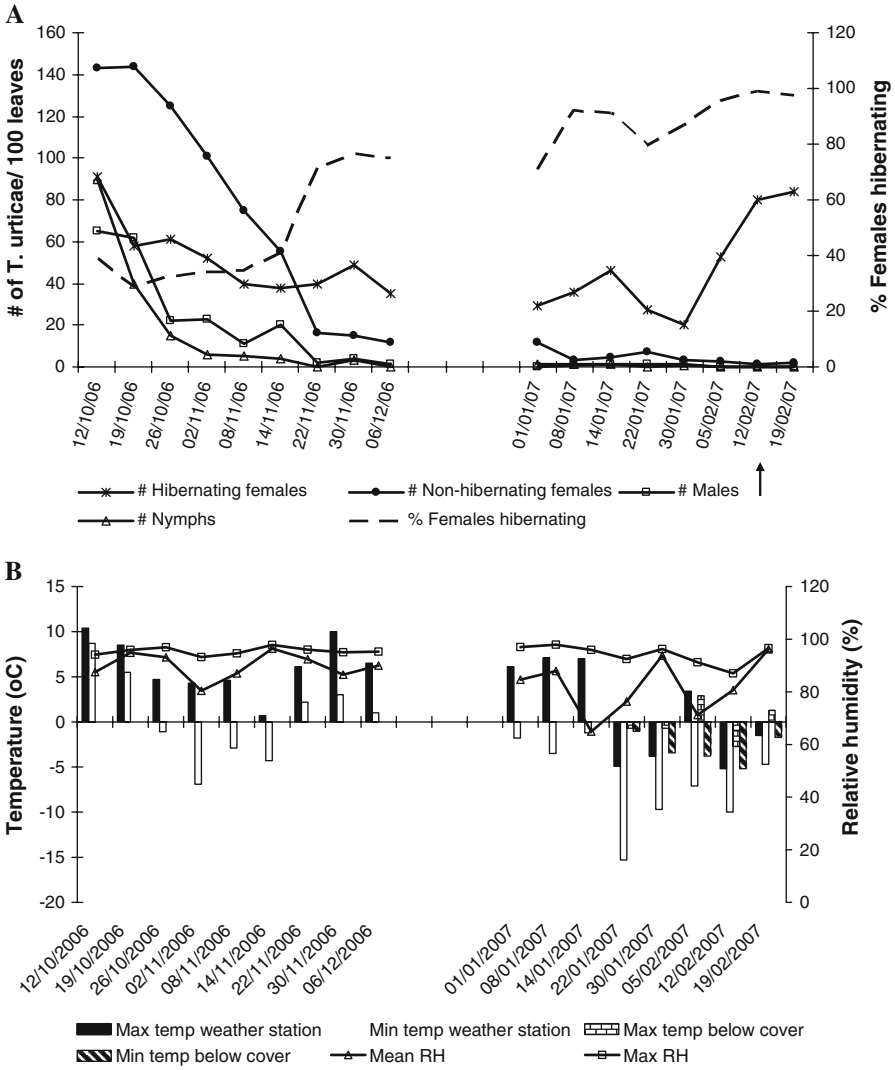

Fig. 2 (**A**) Number of *Tetranychus urticae* hibernating females, non-hibernating females, males and nymphs found per 100 leaves and % females hibernating per sampling date during the winter 2006/2007. Mites were obtained from green strawberry leaves until January 30, after this date mites were obtained from plant debris from the ground surrounding the strawberry plants. The arrow indicates this. (**B**) The max and min temperatures below snow cover in the sampling site, and at a nearby weather station during the winter 2006/2007. The relative humidity was recorded at the weather station only

RH is known to vary greatly throughout a 24-h cycle; the max RH during a 24-h cycle was always higher than 87% and mostly above 96% (Fig. 2B).

Discussion

Prevalence of hyphal bodies in hibernating females found in this study was high. The hyphal bodies were viable and not a dead end for the pathogen, and a 30% sporulation of

live hibernating females incubated on February 12 confirmed this. *Neozygites floridana* transfers its inoculum from one season to another efficiently by overwintering as hyphal bodies inside live mites. The pathogen remains dormant and protected inside its host during the winter and is ready to infect other mites as soon as conditions are favourable.

An early-season introduction of *N. floridana* to control *T. urticae* in strawberries is important, since *T. urticae* is known to cause reductions in strawberry yield at much lower population levels in early season than in late season (Sances et al. 1981). For *N. floridana* to control *T. urticae* populations early in the spring, factors important for sporulation and dissemination of the fungus need to be favoured. The adapted use of pesticides, especially fungicides (Klingen and Westrum 2007) might therefore be very important at this time of the year. Further, the climatic conditions for sporulation and dissemination need to be suitable for the fungus. A sporulating cadaver in this study was found as late as November 2 and December 6. In this period ambient temperatures between −6.9°C (low) and 10.0°C (high) were measured. This indicates that isolates of *N. floridana* from this location in Norway can tolerate quite low temperatures and still be able to sporulate during warm periods of the winter. However, to obtain precise knowledge on the minimum temperature requirement for sporulation of this isolate, a controlled laboratory study is needed. Sporulation of *N. floridana* at low temperatures in laboratory studies has been reported by others, and Brandenburg and Kennedy (1981) showed that an isolate from North Carolina could sporulate at 5°C. Further, Smitley et al. (1986a) reported that the minimum requirements for sporulation for an isolate from North Carolina was 4.4°C. Climatic observations below the cover in our study showed that the cover probably enhanced the temperature with on average about 3.9°C, and this probably improved the conditions for sporulation in *N. floridana*. The presence of snow is known to modify temperature fluctuations, and normally the temperature below fluffy snow is higher than above the snow during periods of the winter when air temperature is below 0°C (Helen K. French, pers. comm.). In our study, the field was covered with snow from mid-January to mid-February. In the area of Norway where this study was conducted, an unstable snow cover is quite common, but years with earlier and more stable snow cover may also occur.

Live hibernating females were observed with immature resting spores only. In a study conducted by Delalibera et al. (2000), only immature resting spores of *Neozygites* sp. were found in live females of the cassava green mite, *Mononychellus tanajoa* (Bondar) (Acari: Tetranychidae). Further, hyphal bodies of other fungal species (*Entomophaga maimaiga* Humber, Shima zu & spoer and *E. gryllii* (Fresenius) Batko (Zygomycetes: Entomophthorales)) are known to begin maturation to the final double-walled resting spore stage after death of their hosts (Hajek and Humber 1997; Tillotson and Margolies 1990). Day length, temperature, host age/stage and fungal isolate are factors known to affect the production of resting spores (Hajek 1997; Thomsen et al. 2001; Nilsen and Steenberg 2004), but knowledge concerning factors important for the production of resting spores of *N. floridana* in *T. urticae* under cold climatic conditions is limited. Mietkiewski et al. (1993) reported, however, an increasing number of dead *T. urticae* filled with resting spores from the beginning of September in Poland. Hours of light in Poland at this time of year is about 14 (http://www.heavens-above.com/) and corresponds to the period for induction of hibernating females in central Europe (Helle 1962). Hence, the state of the host may play an important role for the induction of resting spores. Our study was started too late to observe a potential increase in resting spore formation during autumn, but it suggests that resting spore formation is terminated in early November. In that part of the season both hours of light and temperature are still falling and the hours of light is about 8 (Brahde 1970).

Cadavers filled with resting spores were only found until January 22. It is likely that these cadavers had disintegrated, as suggested by Elliot et al. (2002). Overwintering in the form of resting spores is a strategy which enables the fungus to maintain itself outside a living host (Hajek 1997), and in our studies we found leaves with disintegrated cadavers leaving just a mass of resting spores on the leaf surface (Fig. 1.6) until January 8. Hence, resting spores later in the season would probably only have been obtained by recovering them from plant debris or from the soil as described for the entomophthoralean fungi *E. grylli*, *E. maimaiga* and *Conidiobolus obscurus* (Hall & Dunn) Remaud & Keller (Hajek 1997). The finding of cadavers in our study was similar to what Elliot et al. (2002) described for *N. floridana* (syn. *N. tanajoa* Delalibera Jr., Humber & Hajek) in *M. tanajoa*, except that *M. tanajoa* cadavers with resting spores were easily broken when dry even with gentle handling. The cadaver filled with resting spores found in our study was not easily broken probably because of the strong cuticle that hibernating females have. Carner (1976) describes *T. urticae* cadavers with resting spores of *Entomophthora* sp. near *floridana* to be swollen and shiny black and fragile. When broken, it released a dark liquid into the leaf. No such liquid was seen from cadavers with resting spores in our study. Rhizoids with disk-like holdfasts were also observed on a resting spore cadaver in our study. Keller (1991) reported, however, that rhizoids of *N. floridana* have unspecialized endings. In contrast to what Elliot et al. (2002) found, we found cadavers containing both resting spores and conidiophores in the same individual. Nemoto and Aoki (1975) also reported a few observations where both resting spores and the last stage of conidial formation were present in the same individual.

There are only a few previous studies on survival of *Neozygites* sp. in *T. urticae* populations under cold climatic conditions (Table 2). In North Carolina (USA), *N. floridana* was reported to overwinter as hyphal bodies in *T. urticae* (Brandenburg and Kennedy 1981). This was, however, in a region where *T. urticae* was active on wild hosts during the winter and regular infection cycles of *N. floridana* occurred. Minimum winter temperature in North Carolina is about −2°C (in January), and hence much higher than in our study. Klubertanz et al. (1991) argue that the presence of resting spores in *T. urticae* cadavers late in the summer in Iowa, USA (January low: −13°C) indicated that *N. floridana* is not dependent upon a live host for winter survival, and we support this statement. We also suggest, however, that the protection of the fungus inside the cold temperature tolerant hibernating females found in our study (Fig. 1.3 and 1.4) is an important survival strategy. Further, it is not affected by low temperature as long as it is above the freezing point for the hibernating female. Mietkiewski et al. (1993) observed dead cadavers filled with resting spores from the beginning of September. However, no observations of live mites throughout the winter were conducted.

Hibernating females of *T. urticae* were found when air temperatures were down to −15.3°C in our study. This supports previous laboratory studies stating that *T. urticae* from Norwegian populations can tolerate temperatures down to −22.4°C (Stenseth 1965). The relative abundance of hibernating compared to non-hibernating females increased throughout our study period (October 12–February 2). The critical day length for production of hibernating females in *T. urticae* populations from similar latitudes (60°N) is reported to be about 17 days (Veerman 1985). This suggests that the induction of hibernating females at our location started already in the end of July (Brahde 1970). According to Stenseth (1976), however, hibernating females are produced in the beginning of August at these latitudes in Norway. Further, Veerman (1985) stated that local climatic conditions are the most important factors determining the date of entry into diapause and temperatures later in the season may have played an important role in the induction of hibernating

Table 2 Reports of survival of *Neozygites* sp. in *Tetranychus urticae* under cold climatic conditions

Reported name of fungus	Fungal structures	Location (coordinates geographic center)	Lowest winter temperatures	References
Neozygites floridana	Hyphal bodies in live hibernating females throughout winter. Resting spores in live hibernating females until January 8. Resting spores in dead mummified mites until January 22.	Norway, Lier (59°47′ N, 10°16′ E)	January 22 Min. temp: − 15°C Max. temp: + 3°C	This study
Neozygites floridana	Resting spores in dead mites from early September to late October.	Poland, Siedlce, Biala Podlaska and Konin (from 52°03′ N to 52°21′ N and from 18°27′ E to 23°13′ E)	January Min. temp:−12°C Max. temp: +7°C	Mietkiewski et al. (1993) http://www.tutiempo.net/en/Climate/
Neozygites sp.	Resting spores in dead mummified mites in late summer.	Iowa, USA (41°57′ N, 93°23′ W)	January; Min. temp: −13°C Max. temp: −2°C	Klubertanz et al. (1991) http://www.rssWeather.com
Neozygites floridana	Hyphal bodies in active mites throughout winter. Resting spores only in November.	North Carolina, USA (35°36′ N, 79°27′ W)	January; Min. temp: −2°C Max. temp: + 8°C	Brandenburg and Kennedy (1981) http://www.rssWeather.com

females. Stenseth (1976) conducted a strawberry field study at Ås in southeastern Norway (59°42′ N, 10°44′ E) on the relative abundance of hibernating compared to non-hibernating females from August to October. The highest relative abundance of hibernating females was found at 40% in September in the first year of the study (1973). In the second year of his study (1974), more than 30% of the females found were hibernating. These numbers are similar to what we found in our study at the same time of the year. Numbers of hibernating females increased when collecting plant debris on the ground instead of leaves on the plant in our study. This was probably due to the fact that more hibernating females are found in the plant debris since hibernating females move away from the host plant in search of dark hibernation sites (Stenseth 1976, Veerman 1985). The reason we still chose to use the green leaf sampling method as long as possible was because we were concerned that the light and heat in the Berlese funnel method used for plant debris would affect the diapause of hibernating females.

The high prevalence of hyphal bodies of *N. floridana* inside hibernating females of *T. urticae* suggests that the fungus survives the winter under protected conditions. Immature resting spores were also found but at much lower prevalence. This may indicate that the major overwintering strategy is hyphal bodies in hibernating females and that resting spores are produced mainly for sexual recombination. On the other hand, a higher proportion of mites with resting spores might have occurred earlier in the season at dates that were not included in this study. To obtain the tools that may help us to manipulate the system in a way that would enhance early season control of *T. urticae* by *N. floridana* in conservation biological control, we need to know under what climatic conditions (temperature and RH) hibernating females with hyphal bodies will die and sporulate. Further it would be of great help to understand which *T. urticae* densities and distribution patterns would support an epidemic development from an early sporulating hibernating female cadaver. To understand the role of resting spores in this system there is also a need to conduct studies on how and when resting spores are able to infect healthy mites in the spring and how prevalent they are on plant debris and in the soil. Further there is a need to know when resting spore production is induced and terminated and whether they need a dormant period before germination.

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Sprays of emulsifiable *Beauveria bassiana* formulation are ovicidal towards *Tetranychus urticae* (Acari: Tetranychidae) at various regimes of temperature and humidity

Wei-Bing Shi · Ming-Guang Feng · Shu-Sheng Liu

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Abstract Aerial conidia of *Beauveria bassiana* in an emulsifiable formulation germinated by >95% after 24 h exposure to the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH. Ovicidal activities of the formulation towards two-spotted spider mite, *Tetranychus urticae*, were assayed at the concentrations of 0, 18, 160 and 693 conidia mm^{-2} sprayed separately onto fava bean leaves including 39 (25–76) eggs per capita. All the sprayed eggs on the leaves were directly exposed to the different regimes for hatch after 24 h maintenance in covered Petri dishes. Generally, hatched proportions increased over post-spray days and decreased with the elevated fungal concentrations; no more eggs hatched from day 9 or 10 onwards. Based on the counts of the hatched/non-hatched eggs in the different regimes, the final egg mortalities were 15.0–40.4%, 48.9–66.6% and 62.9–87.5% at the low, medium and high concentrations, respectively, but only 5.6–11.3% in blank controls. The RH effect on the fungal action was significant at 20 and 25°C but not at 30°C whereas the effect of temperature was significant at 51% and 74% RH but not at 95% RH. Probit analysis of the egg mortalities versus the fungal sprays generated median lethal concentrations (LC_{50}) of 65–320 conidia mm^{-2} at all the regimes, and of only 65–78 conidia mm^{-2} at 25–30°C with 74–95% RH. The results highlight ovicidal activities of the emulsifiable formulation against the mite species at the tested regimes and its potential use in spider mite control.

Keywords *Beauveria bassiana* · *Tetranychus urticae* · Fungal formulation · Ovicidal activity · Environmental effect · Spider mite control

W.-B. Shi · M.-G. Feng (✉)
Institute of Microbiology, College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, China
e-mail: mgfeng@zju.edu.cn

M.-G. Feng · S.-S. Liu
Institute of Insect Science, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, Zhejiang 310029, China

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch [including synonymous *T. cinnabarinus* (Boisduval); Ros and Breeuwer 2007], infests a large variety of economic plants worldwide (Hazan et al. 1974; Ho et al. 1997). Spider mite control in the past few decades has relied upon a number of acaricides, such as organochlorides and organophosphates (Gerson and Cohen 1989). This reliance on chemicals has generally caused mite resistance and public concerns on their high residues in products (Guo et al. 1998; Dagli and Tunc 2001). Some acaricides, such as dicofol, cyhexatin and fenbutatin oxide, have thus been prohibited from mite control on vegetables, melons, fruits and tea in China, making it necessary to search for alternative control measures. Fungal pathogens of mites are considered to be potential for the purpose (Poinar 1998; Chandler et al. 2000; Van der Geest et al. 2000).

Entomopathogenic hyphomycetes, such as *Beauveria bassiana* (Balsamo) Vuellemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith, are well-known fungal biocontrol agents (Feng et al. 1994; Faria and Wraight 2001; Roberts and Leger 2004) and have been formulated for wide application to insect control (Langewald et al. 1997; Wraight et al. 2000; Wraight and Ramos 2002; Feng et al. 2004a, b; Pu et al. 2005). They are also potential mite pathogens despite rare prevalence in the field (Chandler et al. 2000). Recently, some fungal isolates derived from host insects have proven to kill spider mite eggs under laboratory conditions and unformulated conidia of a *B. bassiana* isolate have an ovicidal LC_{50} of 548 conidia mm^{-2} (Shi and Feng 2004), which can be reduced greatly by low application rates of pyridaben included in fungal sprays (Shi et al. 2005). In other studies, the fungal insect pathogens are also found capable of infecting active stages of spider mites (Alves et al. 2002; Wekesa et al. 2005, 2006; Maniania et al. 2008; Shi et al. 2008a) and ectoparasitic mites (Shaw et al. 2002; Lekimme et al. 2006, 2008; Meikle et al. 2008).

Environmental temperature and relative humidity (RH) are known to affect conidial germination, colony growth, and host infection of the fungal pathogens (Feng et al. 1994; Roberts and Leger 2004). Appropriate temperature and high RH are usually crucial to successful infection of the fungal agents (Milner et al. 1997; Luz and Fargues 1999). Although common fungal agents in unformulated form have proven to infect various stages of mite pests under controlled conditions (Shi and Feng 2004; Lekimme et al. 2006; Wekesa et al. 2005, 2006), the possible effects of selected formulations and variable environments on their acaricidal activities have not yet been understood. This has hindered a sound evaluation of their potential in mite control. In the present study, aerial conidia of the ovicidal *B. bassiana* isolate found previously (Shi and Feng 2004) were formulated into an oil-based, emulsifiable carrier and then sprayed onto leaves where *T. urticae* eggs were laid in advance. Our goals were to evaluate ovicidal activities of the formulation at gradient application rates and to determine the effects of different temperature and humidity regimes on the hatch rates and mortalities of the mite eggs. The data presented in this paper would help to value the potential of the fungal formulation for incorporation into mite pest management systems.

Materials and methods

Preparation of aerial conidia and emulsifiable formulation

The ovicidal isolate, *B. bassiana* SG8702, was derived from a naturally mycosed aphid (Feng et al. 1990) with accession number ARSEF 2860 (USDA-ARS Collection of

Entomopathogenic Fungal Cultures, Ithaca, NY, USA). This isolate has been formulated for control of greenhouse whiteflies (Feng et al. 2004a) and tea leafhoppers (Feng et al. 2004b; Pu et al. 2005). It was preserved as a mixture of dried conidia with sterile sands at −72°C. To produce conidia in this study, the preserved conidia were used to inoculate the plates of Sabouraud dextrose agar plus 1% yeast extract (SDAY) for 7 days incubation at 25°C. The resultant conidia were suspended in Sabouraud dextrose broth (SDB) and incubated for 2 days at 25°C by shaking at 110 rpm. The resultant liquid culture was mixed with steamed rice at the rate of 10% (v/w) and the mixture was then poured into 15-cm-diameter Petri dishes (100 g per dish). After 7 days growth and conidiation at 25°C, the rice cultures were dried overnight in a ventilation chamber at 33°C and then passed through an electrically vibrating sieve (10 threads mm^{-1}) for harvest of conidia, followed by vacuum drying to ca. 5% water content at ambient temperature (Ye et al. 2006).

The dried conidial powder was uniformly suspended in a mixture of 95% (v/v) industrial paraffin as oil carrier and 5% (v/v) fatty alcohol polyethylene glycol ether 'AEO-3' as emulsifier (Xiaoshan Chemical Additives, Hangzhou, Zhejiang, China). The emulsifiable formulation was standardized to 1×10^{10} conidia ml^{-1} and used immediately or stored at 6°C in dark for bioassays below.

Viability assays at different regimes

Aqueous dilution (1×10^6 conidia ml^{-1}) of the emulsifiable formulation was prepared and 100 μl aliquots were smeared evenly onto the 90-mm-diameter plates of SDAY supplemented with 0.1% chloramphenicol to prevent possible bacterial contamination. Not covered with lids, the smeared SDAY plates were maintained in incubators at 20, 25 and 30 ± 1°C with 12:12 L:D, respectively. Each incubator included three Perspex chambers (135 × 135 × 185 mm), in which 51%, 74% and 95% RH were achieved by pumping continuously moisture-specific air from the last of three rubber-tube-connected jars into each chamber (Feng et al. 1999). Aqueous solutions of 59.8%, 31.4% and 18.8% (v/v) glycerin were separately half-filled into each set of the jars to generate the RHs at 20–30°C (Doberski 1981). The conidia smeared on the plates were thus exposed to nine treatments of temperature and RH combinations, each including three plates as replicates.

Germinated and non-germinated conidia at each of the regimes were counted after 12 and 24 h incubation under microscope at 400× magnification (three counts of >100 conidia per plate). Conidial viability at a given regime was determined as percentages of the germinated conidia (with visible germ tubes) in total.

Preparation of the mite eggs

The eggs of *T. urticae* were prepared using a detached leaf system described by Shi and Feng (2004). A laboratory population of the mite species was maintained on fava bean (*Vicia faba* L.) plants in a walk-in growth room at the regime of 23 ± 2°C and 12:12 L:D. Twenty vigorous adult females arbitrarily taken from the population were transferred to a detached leaf in Petri dish (6.5 cm diameter), in which root hairs grew from the petiole into an agar plate below the leaf. The females were allowed to lay eggs freely for 18 h and then removed. A certain number of eggs (usually 30–40) were left on each leaf to receive treatments as follows. The detached leaf system could support a mite colony for 15 days or so, warranting normal hatch of the mite eggs with no need for leaf change during a bioassay.

Ovicidal assays of *B. bassiana* at different regimes

Aqueous dilutions (1×10^8, 1×10^7 and 1×10^6 conidia ml^{-1}) of the emulsifiable formulation were sprayed onto the eggs on the leaves for inoculation using a non-touch leaf method (Shi and Feng 2004). Briefly, each uncovered dish of the detached leaf bearing the mite eggs was placed on the center of the bottom specimen dish (11 cm diameter) of an Automatic Potter Spray Tower (Burkard Scientific, Uxbridge, Middx, UK) to receive a 2-ml spray of each conidial dilution from its top nozzle at the working pressure of 0.7 kg cm^{-2} (the manufacturer's guide). Separate equal-volume sprays of the three aqueous dilutions resulted in different concentrations of the conidia deposited onto the mite eggs and leaves. Each concentration was determined as no. conidia mm^{-2} using microscopic counts of the conidia deposited onto a glass slip (20 × 20 mm; five 0.2165-mm^2 view fields per slip), which was placed beside the dish under each spray. The same-volume spray of 100-fold aqueous dilution of the liquid carrier alone (i.e., the mixture of 95% paraffin and 5% emulsifier) was included as blank control of the three fungal sprays in each bioassay.

After exposure to the fungal sprays, all the eggs on detached leaves in Petri dishes were covered with lids and maintained overnight at 25°C and 12:12 L:D to favor conidial germination. Subsequently, all the dishes with the sprayed leaves and mite eggs were uncovered and arranged randomly into the regimes of 20–30°C and 51–95% RH (humidity chambers) as described above with each regime including the three fungal concentrations as treatments. Egg hatches were daily examined until no more eggs hatched for three consecutive days at any of the regimes. All non-hatched eggs, together with the detached leaves, were examined under a dissection microscope for verification of fungal infection. Final egg mortalities in different treatments were computed based on the last-day counts of the hatched and non-hatched eggs. All the bioassays were repeated three times during a period of 75 days.

Data analysis

The 12- and 24-h germination rates of the formulated conidia exposed to the temperature and RH regimes were analyzed using two-way ANOVA. Hatched proportions of the mite eggs observed at the concentrations of 0–693 conidia mm^{-2} from the regimes of 20–30°C and 51–95% RH were plotted over post-spray days. Variation in egg mortalities at a given RH or temperature was differentiated among the fungal concentrations by two-way ANOVA. The egg mortalities caused by the fungal sprays at each of the combined regimes were corrected using background mortality in the corresponding blank control and then subjected to probit analysis. A linear concentration- mortality relationship from each analysis was used to estimate median lethal concentration (LC_{50}) and associated 95% confidence limits (CL) as an index for ovicidal activity of the fungal formulation at each regime. An updated version of DPS software (Tang and Feng 2007) was used in all the analyses.

Results

Effects of temperature and RH on the viability of oil-formulated conidia

The viabilities of *B. bassiana* conidia formulated were significantly affected by temperature (12 h: $F_{2,16} = 171.9$, $P < 0.01$; 24 h: $F_{2,16} = 57.0$, $P < 0.01$) but not by RH (12 h: $F_{2,16} = 2.6$, $P = 0.11$; 24 h: $F_{2,16} = 1.9$, $P = 0.18$) among the concerned regimes. The interaction of both

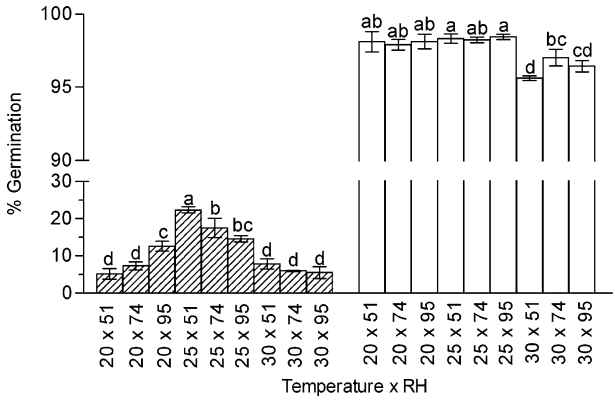

Fig. 1 Comparison of the viabilities of the formulated *Beauveria bassiana* conidia incubated for 12 (shading bars) and 24 h (white bars) on SDAY plates at the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH, respectively. Bars with different letters differed significantly in height (Tukey's HSD, $P < 0.05$). Error bars: SD

variables also had significant effect on the viabilities (12 h: $F_{4,16} = 3.3$, $P = 0.04$; 24 h: $F_{4,16} = 21.7$, $P < 0.01$). Germination ranged from 5.1% at the regime of 20°C and 51% RH to 22.4% at 25°C and 95% RH after 12 h incubation but reached 95.6–98.3% in all the regimes by 24 h (Fig. 1). Thus, the oil-formulated conidia had high viabilities despite some variation perhaps due to the temperature and RH interaction.

After12 h incubation, the overall mean germination rates (± SD) at 51–95% RH were significantly higher at 25°C (18.1 ± 3.6%) than at 20 (8.3 ± 3.5%) or 30°C (6.4 ± 1.5%) (Tukey's HSD, $P < 0.05$). By the end of 24 h incubation, however, mean germination rates in the three RH treatments were very close among the temperatures, i.e., 98.0 ± 0.5% at 20°C, 98.3 ± 0.2% at 25°C, and 96.4 ± 0.7% at 30°C. Moreover, overall mean germination rates at 95% RH (12 h: 10.9 ± 4.3%; 24 h: 97.7 ± 1.0%) did not differ significantly from those at 51% RH (12 h: 11.8 ± 8.1%; 24 h: 97.3 ± 1.4%) or at 74% RH (12 h: 10.2 ± 5.7%; 24 h: 97.7 ± 0.6%) when the three-temperature observations were pooled (Tukey's HSD, $P > 0.05$).

Hatch trends of sprayed mite eggs at different regimes

Sprays of the three conidial dilutions generated mean concentrations of 17.9 (±3.0), 160.4 (±17.1) and 693.1 (±183.6) conidia mm^{-2} deposited on the leaves with 39 (25–76) eggs per capita in the repeated bioassays. Thus, a total number of 4,218 mite eggs sprayed at 0–693 conidia mm^{-2} were exposed to the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH.

The trends of hatched proportions of the mite eggs at all the regimes are illustrated over days after each fungal spray (Fig. 2). Generally, observations within each of the regimes were dependent on both fungal concentrations and post-spray days. Most of the mite eggs in blank controls or sprayed at the low fungal concentration hatched within 7–9 days but no egg hatch was observed in the first 2 days. Differences in hatch rates were small among the fungal treatments during the first 3–4 days but became larger thereafter. Very few eggs were observed hatching from day 9 or 10 onwards. As a result, different numbers of the mite eggs were not hatched in the fungal treatments irrespective of the regimes.

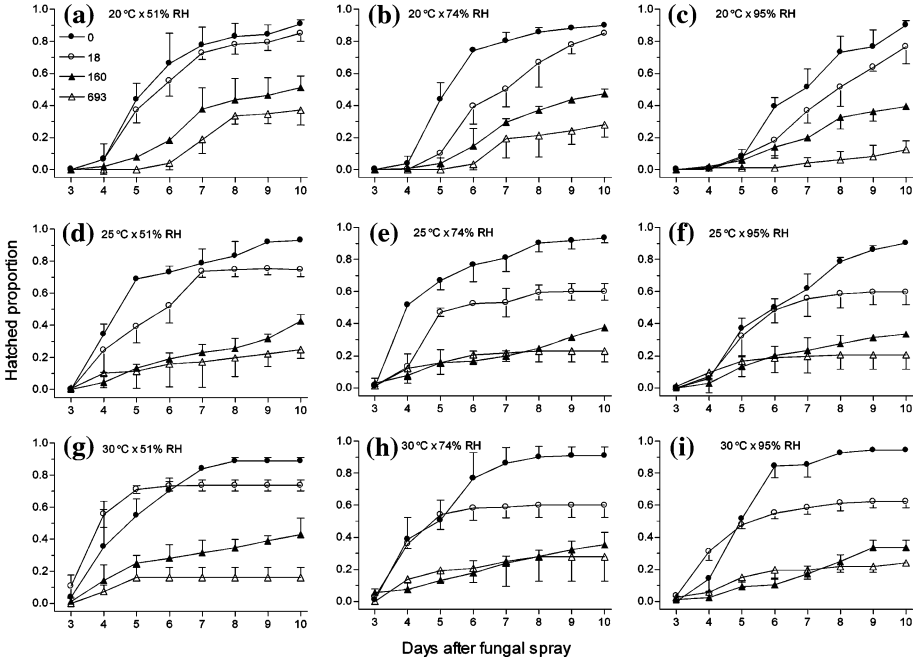

Fig. 2 The trends of the hatched proportions of *Tetranychus urticae* eggs at the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH over days after being sprayed with the emulsifiable *Beauveria bassiana* formulation at the concentrations of 0–693 conidia mm^{-2} (0 = blank control). Error bars: SD

Egg mortalities caused by fungal sprays at different regimes

Variations in the final mortalities of the mite eggs at the different fungal concentrations were differentiated by two-way ANOVA (Table 1). The fungal concentration was consistently most influential on the egg mortalities (maximal F with minimal P) at a given temperature or RH. This indicates that the egg mortalities were attributed to infection by *B. bassiana*. The RH effects on the mortalities were significant only at 20 and 25°C ($P < 0.01$) but insignificant at 30°C ($P = 0.79$). The RH and concentration interaction was not significant at a given temperature ($P > 0.05$). The effect of temperature on the mortalities was significant only at 51% or 74% RH ($P < 0.01$) but not at 95% RH ($P = 0.23$). However, a significant effect was found in the interaction of temperature with the fungal concentration at 95% RH ($P < 0.01$).

The egg mortalities caused by the fungal formulation fell in the range of 62.5–87.9% at the high concentration and of 48.9–66.6% at the medium, varying with the temperature/RH regimes (Fig. 3). These were significantly higher than the background mortalities of 5.6–11.3% in the blank controls (Tukey's HSD, $P < 0.05$). The low fungal concentration resulted in the mortalities of 15.0–40.4% but only those at the regimes of 25°C or 74–95% RH were significantly higher than the mortalities in the controls.

LC_{50}s as indices of ovicidal activities at different regimes

The linear concentration-mortality relationships determined by probit analysis generated the LC_{50} values and associated 95% CL for the tested formulation against the mite eggs at

Table 1 Variation in the mortalities of *Tetranychus urticae* eggs attributed to the sprays of *Beauveria bassiana* formulation (0–693 conidia mm^{-2}) at different temperature and RH regimes

Source of variation	Given temperature			Source of variation	Given RH		
	df	*F*	*P*		df	*F*	*P*
20°C				51% RH			
Replicate	2, 22	0.5	0.60	Replicate	2, 22	0.6	0.56
RH	2, 22	11.9	<0.01	Temperature	2, 22	10.3	<0.01
Fungal spray	3, 22	245.2	<0.01	Fungal spray	3, 22	229.0	<0.01
RH × spray	6, 22	2.3	0.07	Temp × spray	6, 22	1.8	0.15
25°C				74% RH			
Replicate	2, 22	1.0	0.39	Replicate	2, 22	0.5	0.60
RH	2, 22	7.6	<0.01	Temperature	2, 22	7.1	<0.01
Fungal spray	3, 22	335.3	<0.01	Fungal spray	3, 22	166.3	<0.01
RH × spray	6, 22	1.6	0.21	Temp × spray	6, 22	3.3	0.02
30°C				95% RH			
Replicate	2, 22	0.1	0.87	Replicate	2, 22	1.2	0.33
RH	2, 22	0.2	0.79	Temperature	2, 22	1.6	0.23
Fungal spray	3, 22	161.1	<0.01	Fungal spray	3, 22	335.4	<0.01
RH × spray	6, 22	2.3	0.08	Temp × spray	6, 22	4.3	<0.01

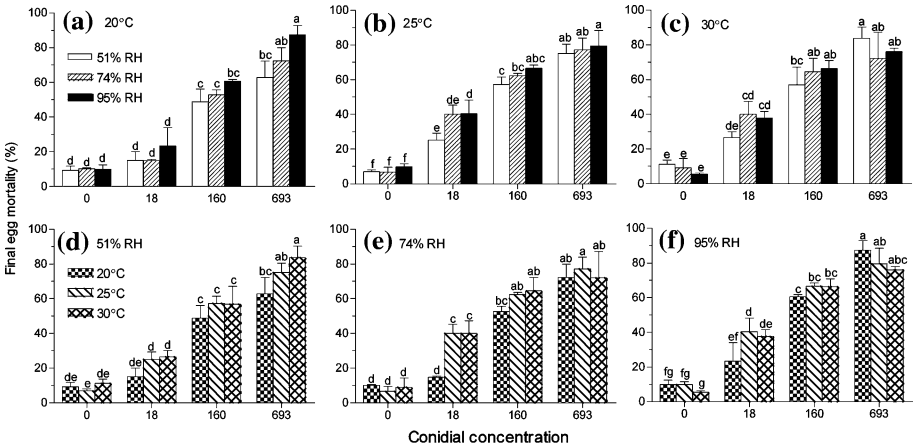

Fig. 3 Comparison of the final mortalities of *Tetranychus urticae* eggs at the different concentrations of *Beauveria bassiana* formulation (no. conidia mm^{-2}; 0 = blank control) at the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH, respectively. Bars with different lowercase letters in each graph differed significantly in height (Tukey's HSD, $P < 0.05$). Error bars: SD

all the regimes (Table 2). The fungal formulation was highly ovicidal with the LC_{50} declining with increased RH at a given temperature. The maximal LC_{50} at the regime of 20°C and 51% RH was 320 conidia mm^{-2}. Those at the regimes of 25–30°C with 74–95% RH were only 65–78 conidia mm^{-2}.

Discussion

In summary, the formulated *B. bassiana* conidia were highly viable at the regimes of 20–30°C with 51–95% RH. The hatched proportions of *T. urticae* eggs after exposure to

Table 2 The LC_{50} estimates and associated 95% confidence limits (CL) for the ovicidal activities of emulsifiable *Beauveria bassiana* formulation against *Tetranychus urticae* at different temperature and RH regimes, based on probit analysis

Temp (°C)	RH (%)	Intercept	Slope ± SE	χ^2	P*	LC_{50} with 95% CL (no. conidia mm^{-2})
20	51	2.35	1.06 ± 0.17	2.40	0.12	319.9 (209.5–593.1)
20	74	2.02	1.26 ± 0.17	2.12	0.15	233.0 (167.9–354.4)
20	95	2.36	1.29 ± 0.15	0.22	0.64	111.0 (80.5–150.9)
25	51	2.99	0.94 ± 0.12	0.10	0.75	135.7 (94.1–197.3)
25	74	3.74	0.68 ± 0.12	0.00	0.99	70.7 (35.6–118.4)
25	95	3.61	0.76 ± 0.11	0.12	0.73	66.1 (37.5–103.4)
30	51	2.47	1.18 ± 0.14	0.29	0.59	136.0 (96.5–193.4)
30	74	3.81	0.63 ± 0.12	0.99	0.32	78.7 (39.6–135.5)
30	95	3.71	0.71 ± 0.11	0.59	0.44	65.4 (36.4–103.2)

* Homogeneity for the fit was accepted if $P > 0.05$ for the χ^2 test (df = 2)

fungal sprays of 18–693 conidia mm^{-2} were generally lower than those in blank controls despite some variations. The final egg mortalities in the fungal treatments were always higher than those in blank controls irrespective of the temperature/RH regimes. The RH effect on the fungal action was significant at 20 and 25°C but not at 30°C whereas the effect of temperature was significant at 51% and 74% RH but not at 95% RH. The ovicidal LC_{50} s of the formulation spanned from 65 to 320 conidia mm^{-2} at all the regimes but fell in a very narrow range of 65–78 conidia mm^{-2} at 25–30°C with 74–95% RH. The results indicate a conspicuous ovicidal activity of the fungal formulation towards the spider mite species at the concerned regimes.

A high viability of fungal conidia in a formulation sprayed onto target pests is a prerequisite for their germination and infection. Germination *in vitro* is related to expression of fungal virulence (Jackson et al. 1989; Altre et al. 1999). Since conidial germination is known to largely depend on RH and temperature (Feng et al. 1994; Roberts and St. Leger 2004) and spider mite eggs are usually laid on the surfaces of leaves or shoots with some moisture (e.g., metabolic water, dew), the formulated conidia in this study were allowed to germinate for 24 h on uncovered SDAY plates entirely exposed to the regimes of 51–95% RH and 20–30°C. These are normal conditions for heavy infestation of spider mite pests in the field. The observed high viabilities help to interpret the high egg mortalities caused by *B. bassiana* at the same regimes. This indicates that the emulsifiable formulation would be able to act on spider mites under field conditions. Other reports have also shown substantial infections of *B. bassiana* and/or *M. anisopliae* to southern pine beetle adults at 55–94% RH (Moore 1973), elm bark beetle larvae at 51–100% RH (Doberski 1981), grasshoppers at 12–100% RH (Marcandier and Khachatourians 1987), and the Chagas' disease vector *Rhodnius prolixus* Stål at 43–97% RH (Fargues and Luz 2000), despite higher mortalities associated with higher RH.

The LC_{50} of the tested *B. bassiana* formulation against *T. urticae* eggs ranged from 65 conidia mm^{-2} at 30°C and 95% RH to 320 at 20°C and 51% RH. We think that the emulsifiable formulation has greatly enhanced ovicidal activities of the fungal agent in comparison with an LC_{50} of 548 (393–857) or 546 (406–818) conidia mm^{-2} (plain conidia suspended in 0.02% Tween-80) toward the eggs of the same mite species at 25°C under moist conditions (Shi and Feng 2004; Shi et al. 2005). This supports previous reports on oil-increased efficacy of *M. anisopliae* against whiteflies (Malsam et al. 2002) and of *B. bassiana* against aphids (Ye et al. 2005), and on improved adaptation of oil formulations to low-humidity environ-

ments for insect control (Bateman et al. 1993; Kooyman and Godonou 1997). Although possible mechanisms involved in the enhancement of fungal activities by the oil-based formulation are not clear at present, we postulate that the enhancement may result from better attachment of the formulated conidia to target pests and from improved protection of the conidia from desiccation after spray. This warrants more studies.

The ovicidal activities of the emulsifiable formulation tested at different temperature/RH regimes highlight its potential for practical incorporation into mite pest management due to its adaptation to the low-RH environments. In field trials, this formulation sprayed twice at the rate of ca. 1.5×10^{13} conidia ha^{-1} has provided significant control of citrus red mites, *Panonychus citri* (McGregor), in the orchards of east China (Shi and Feng 2006) and of cotton spider mites, mainly *Tetranychus truncates* Ehara and *T. turkestani* (Ugarov & Nikolskii), in the Tarim Basin of northwest China (Shi et al. 2008b). However, spider mites in southern China and other subtropical areas often infest crops heavily during hot summer, which is a challenge for the tolerance of the fungal formulation to outdoor thermal stress often around 40°C. If fungal candidates with greater thermotolerance and other improved traits (Ying and Feng 2004; Zou et al. 2006) are formulated into the oil-based carrier, application of fungal formulations to more stressed seasons or environments for spider mite control would be more promising. This also warrants future studies.

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Role of entomopathogenic fungi in the control of *Tetranychus evansi* and *Tetranychus urticae* (Acari: Tetranychidae), pests of horticultural crops

Nguya K. Maniania · David M. Bugeme · Vitalis W. Wekesa · Italo Delalibera Jr. · Markus Knapp

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Abstract The spider mites *Tetranychus urticae* Koch and *Tetranychus evansi* Baker and Pritchard are important pests of horticultural crops. They are infected by entomopathogenic fungi naturally or experimentally. Fungal pathogens known to cause high infection in spider mite populations belong to the order Entomophthorales and include *Neozygites* spp. Studies are being carried out to develop some of these fungi as mycoacaricides, as stand-alone control measures in an inundative strategy to replace the synthetic acaricides currently in use or as a component of integrated mite management. Although emphasis has been put on inundative releases, entomopathogenic fungi can also be used in classical, conservation and augmentative biological control. Permanent establishment of an exotic agent in a new area of introduction may be possible in the case of spider mites. Conservation biological control can be achieved by identifying strategies to promote any natural enemies already present within crop ecosystems, based on a thorough understanding of their biology, ecology and behaviour. Further research should focus on development of efficient mass production systems, formulation, and delivery systems of fungal pathogens.

Keywords Tetranychidae · Spider mites · *Tetranychus evansi* · *Tetranychus urticae* · Entomopathogenic fungi · Biological control · Integrated pest management · Horticulture

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch, and the tomato red spider mite, *Tetranychus evansi* Baker and Pritchard, are among the most important pests of horticultural crops, such as tomato, cut flowers, French beans, eggplants and cucumber. Whereas *T. urticae* has been known as a worldwide pest of a wide range of horticultural crops both

N. K. Maniania (✉) · D. M. Bugeme · M. Knapp
International Centre of Insect Physiology and Ecology (ICIPE), PO Box 30772, Nairobi, Kenya
e-mail: nmaniania@icipe.org

V. W. Wekesa · I. Delalibera Jr.
Department of Entomology, Plant Pathology and Agricultural Zoology, University of São Paulo, ESALQ Campus, Av. Pádua Dias 11 CP 9, Piracicaba, SP 13418-900, Brazil

outdoors and in the greenhouses, the importance of *T. evansi* has dramatically increased during the last decade. *Tetranychus evansi* is probably of South American origin (Gutierrez and Etienne 1986) and invaded Africa in the late 1970s (Blair 1983; Meyer 1987; Knapp et al. 2003), Europe in the mid 1990s (Ferragut and Escudero 1999; Migeon 2005) and parts of Southeast Asia in 2004 (Ho et al. 2004). It is a major pest of tomatoes in eastern and southern Africa (Knapp et al. 2003; Saunyama and Knapp 2003). If left uncontrolled under hot and dry conditions, *T. evansi* can destroy tomato plants within 3–5 weeks (Qureshi et al. 1969), and the farmer can lose his production within a week's time. The economic importance of *T. urticae* and its control were extensively reviewed by Helle and Sabelis (1985a, b). Biological control of *T. urticae* with phytoseiid predatory mites has been successful mainly in protected environments but also in open fields in many parts of the world (e.g., Gerson et al. 2003; Zhang 2003). Farmers in Africa largely rely on expensive synthetic pesticides to control *T. evansi*, but pesticide applications are frequently ineffective (Sibanda et al. 2000; Saunyama and Knapp 2003). Although no effective biological control strategy has yet been developed, a Brazilian strain of the predatory mite *Phytoseiulus longipes* Evans and the pathogenic fungus *Neozygites floridana* Weiser and Muma have recently shown promising results in laboratory experiments (Furtado et al. 2007; Wekesa et al. 2007). Detailed information on the distribution of both mite species, including maps and many relevant references, are in Migeon and Dorkeld (2006). Alternatives to chemical control need to be developed because spider mites can rapidly develop resistance to acaricides, and due to the growing concern about environmental and health risks associated with pesticide use.

Research and development of biological control options for spider mites has largely concentrated on the conservation of natural enemies and releases of predatory mites (Nyrop et al. 1998; Gerson et al. 2003; Zhang 2003). However, this is often not sufficient and supplementary sprays of acaricides are needed. Entomopathogenic fungi may play a major role in the natural regulation of spider mite populations and could be used in biological control programme, either as a stand-alone solution in replacement of synthetic acaricides that are currently in use, or as a component of integrated mite management. The diseases caused by entomopathogenic fungi in mites and spider mites were reviewed by van der Geest (1985) and by van der Geest et al. (2000). Chandler et al. (2000) reviewed opportunities of exploiting fungal pathogens for biological control of Acari, including mites. In this paper we review the use of fungal pathogens in the inundative, conservation and classical biological control of *T. urticae* and *T. evansi*.

Entomopathogenic fungi associated with *Tetranychus evansi* and *Tetranychus urticae*

Natural incidence

Entomopathogenic fungi can play an important role in the regulation of arthropod pest populations. Many reports have been published on natural incidence of entomopathogenic fungi on tetranychid mites, including *T. evansi* and *T. urticae,* and were reviewed by van der Geest (1985); Chandler et al. (2000) and by van der Geest et al. (2000) (Table 1). The fungi known to cause high infections in *T. evansi* and *T. urticae* populations belong to the order Entomophthorales and include *Neozygites* spp., the mitosporic fungi *Hirsutella thompsonii* Fisher and members of the *Lecanicillium* (=*Verticillium*) *lecanii* complex.

Identification of mite pathogenic species of *Neozygites* is still unresolved. Four species of *Neozygites* were described from spider mites: *N. floridana*, *N. tetranychi*, *N. adjarica* and more recently *N. tanajoae*. Both *N. tetranychi* and *N. adjarica* are only known from a

Table 1 Entomopathogenic fungi reported naturally on *Tetranychus evansi* and *Tetranychus urticae*

Fungal species	Host	Reference
Zygomycota		
Basidiobolus sp.	*T. urticae*	Jegina (1976) (cited by van der Geest 1985)
Conidiobolus obscurus	*T. urticae*	Jegina and Cinowski (1970) (in van der Geest 1985), Andreeva and Shternshis (1995) (cited by Chandler et al. 200
Entomophthora sp.	*T. urticae*	Kenneth et al. (1971) (cited by van der Geest 1985)
Neozygites floridana	*T. urticae*	Weiser and Muma (1966), Kenneth et al. 1972 (cited by van der Geest 1985), Smitley et al. (1986), Keller (1991), Mietkiewski et al. (1993), Shih and Shiue (1994), Dick and Buschman (1995) (cited by Chandler et al. 2000), Smith and Furr (1975) (cited by van der Geest et al. 2000)
	T. evansi	Fiaboe (2007)
Neozygites sp. near *floridana*	*T. urticae*	Ramaseshiah (1971) (cited by van der Geest 1985)
Neozygites tetranychi	*T. urticae*	Weiser (1968) (cited by van der Geest 1985)
Neozygites sp.	*T. urticae*	Cincadze et al. (1976) (cited by van der Geest 1985), Carner and Canerday (1968), Carner (1976), Brandenburg and Kennedy (1981), Klubertanz et al. (1991) (cited by Chandler et al. 2000)
	T. evansi	Humber et al. (1981)
Ascomycota		
Beauveria bassiana	*T. urticae*	Wright and Kennedy (1996) (cited by Chandler et al. 2000)
Lecanicillium (= *Verticiliium*) *lecanii*	*T. urticae*	Gams (1971) (cited by van der Geest 1985), Gillespie et al. (1982), Andreeva and Shternshis (1995) (cited by Chandler et al. 2000), Chandler et al. (2005).
Metarhizium anisopliae	*T. urticae*	D.M. Bugeme (unpubl. data)

single collection. A comparison of these four species indicates a considerable overlap of taxonomic characteristics, such as spore sizes. *Neozygites adjarica* is considered a synonym of *N. floridana* (Keller 1991; Balazy 1993). *Neozygites tetranychi* also might be *N. floridana* (Balazy 1993). In the past, *N. floridana* was called *Triplosporium floridana* (Kenneth et al. 1972), *Triplosporium* sp. (Humber et al. 1981), and *Entomophthora floridana* (Nemoto et al. 1975). More recently, authors have referred to the pathogens associated with spider mites as *N. floridana* (Keller, 1997; Kennedy and Smitley 1988; Mietkiewski et al. 2000). In this paper, we will consider all *Neozygites* species pathogenic to *T. urticae* and *T. evansi* as *N. floridana,* although we think that more studies are needed to clarify the taxonomy of this species complex.

Susceptibility of *Tetranychus urticae* and *Tetranychus evansi* to fungal infections under laboratory conditions

Fungi that are not associated with arthropod hosts in nature can be tested for their pathogenic activity against different target species in the laboratory (Hall and Papierok 1982). Experimental infections, induced under controlled conditions, allow testing of the pathogenic activity of different fungal isolates with the aim of developing them as biological control agents or biopesticides. The fungal pathogens that have been tested against *T. evansi* and *T. urticae* in the laboratory are listed in Table 2. The report in this section focuses on recently published articles published after the reviews by Chandler et al. (2000) and van der Geest et al. (2000).

Tamai et al. (2002a) screened 45 isolates of mitosporic fungi against *T. urticae*, including 32 isolates of *Beauveria bassiana* (Balsamo) Vuillemin, 10 isolates of *Metarhizium*

Table 2 Entomopathogenic fungi tested against *Tetranychus evansi* and *Tetranychus urticae* in the laboratory

Fungal species	Host	Reference
Basidiobolus sp.	*T. urticae*	Jegina (1976) (cited by van der Geest 1985)
Beauveria bassiana	*T. urticae*	Tamai et al. (1999) (cited by Irigaray et al. 2003), Alves et al. (2002), Irigaray et al. (2003), Bugeme et al. (unpubl. data)
	T. evansi	Wekesa et al. (2005), Bugeme et al. (unpubl. data)
Hirsutella thompsonii	*T. urticae*	Gerson et al. (1979), Gardner et al. (1982) (cited by Chandler et al. 2000), Chandler et al. (2005)
Lecanicillium lecanii	*T. urticae*	Gillespie et al. (1982), Chandler et al. (2005)
L. muscarium	*T. urticae*	Mineiro et al. (2004)
Metarhizium anisopliae	*T. urticae*	Chandler et al. (2005), Bugeme et al. (unpubl. data)
	T. evansi	Wekesa et al. (2005), Bugeme et al. (unpubl. data)
Neozygites floridana	*T. urticae*	Smitley et al. (1986)
Meira geulakonigii	*T. urticae*	Paz et al. (2007)
Meira argovae	*T. urticae*	Paz et al. (2007)
Acaromyces ingoldii	*T. urticae*	Paz et al. (2007)

anisopliae (Metchnikoff), one each of *Aschersonia aleyrodis* Webber, *Hirsutella* sp. and *Isaria farinosa* (= *Paecilomyces farinosus*) (Holmsk.) Fr. Among these isolates, eight *B. bassiana* and four *M. anisopliae* isolates caused >80% and 90% mortality, respectively, at concentration of 5×10^7 conidia ml^{-1}, 5 days postinfection. Applied at concentration of 1.7×10^7 conidia ml^{-1}, *Hirsutella* sp. caused 73% mite mortality. *Aschersonia aleyrodis* and *I. farinosa* were not pathogenic to the two-spotted spider mite. The authors concluded that *M. anisopliae*, *B. bassiana* and *Hirsutella* sp. were the most promising fungi to be formulated as mycoacaricides for *T. urticae* control. The authors also noted that conidia, blastospores and yeast-like cells of five isolates of *B. bassiana* were pathogenic against the same mite. Lethal concentration to 50% mortality (LC_{50}) values ranged from 4.95 to 82.1×10^6 cells ml^{-1}.

Irigaray et al. (2003) evaluated the efficacy of Naturalis-L® (a *B. bassiana*-based commercial biopesticide) against the two-spotted spider mite and obtained lethal concentration values (LC_{50}) of 3184 viable conidia ml^{-1} for the juvenile stages and 1949 viable conidia ml^{-1} for the adults. Naturalis-L® caused significant egg mortality compared to the control, with no significant differences amongst egg age classes (24-, 48-, 72-, and 96-h-old eggs) at the tested concentrations of 1,400–22,800 viable conidia ml^{-1}. In another study, Simova and Draganova (2003) evaluated the virulence of four isolates of *B. bassiana*, one isolate each of *M. anisopliae*, *I. farinosa* and of *L. lecanii*, to *T. urticae* at conidial concentrations of 2×10^9 conidia ml^{-1}. Six out of the seven isolates tested were virulent to *T. urticae* and one isolate of *B. bassiana* outperformed the others with lethal time to 50% mortality (LT_{50}) values of between 1.3 and 1.4 days. Except for *I. farinosa,* which was less virulent, the other isolates were equally virulent to *T. urticae*. Chandler et al. (2005) tested 40 isolates of anamorphic entomopathogenic fungi from six genera against *T. urticae,* along with three commercial fungus-based products: Naturalis-L®, Mycar® (*H. thompsonii*) and Mycotal® (*Lecanicillium (=Verticillium) muscarium*). Three isolates, *M. anisopliae*, *Hirsutella* spp. and *L. muscarium*, and the three commercial isolates, were pathogenic to *T. urticae*. Koike et al. (2005) also demonstrated that four isolates of *L. lecanii* (Vertalec, Mycotal, A-2, B-2) were pathogenic against *T. urticae*, but the levels of virulence differed among the isolates. Alves et al. (2002) compared the virulence of yeast-like cells and conidia of *B. bassiana* against *T. urticae* and found that their virulence was similar, causing

mortalities of 42.8–45.0% at a concentration of 10^7 cells ml^{-1}, and of 74.4–77.8% at 10^8 cells ml^{-1}.

Recently, new taxa of fungi were described as Exobasidiomycetidae of the class Ustilaginomycetes (Basidiomycota) (Boekhout et al. 2003), that include *Meira geulakonigii* gen. nov., sp. nov., *Meira argovae* sp. nov. and *Acaromyces ingoldii* gen. nov., sp. nov. Their pathogenicity was evaluated in the laboratory against herbivorous mites including *T. urticae* (Paz et al. 2007). With the exception to *M. argovae* which was not virulent, *M. geulakonigii* and *A. ingoldii* caused mortalities of 81.9–90.0% to *T. urticae,* 7 and 14 days post-application, respectively. Some isolates of *H. thompsonii* produce metabolites in broth cultures and exudates over the surface of solid cultures (Cabrera and Lopez 1977; Samson et al. 1980). These metabolites have been reported to be toxic to mites (Omoto and McCoy 1998). Rosas-Acevedo et al. (2003) recently tested the effect of an exudate of *H. thompsonii* Mexican strain HtM120I on *T. urticae* egg production and obtained 100% reduction in mite fecundity after topical application of the metabolite over the initial 6 days of the experiment. They also observed that depending on the exudate dosage, mites partially recovered within 3 and 6 days post-treatment, but produced fewer eggs. Further studies are needed to identify metabolites and quantify exudate concentration.

Wekesa et al. (2005) evaluated 17 isolates of *M. anisopliae* and two isolates of *B. bassian*a against *T. evansi.* All isolates were pathogenic to adult female mites, causing 22.1–82.6% mortalities. Isolates causing more than 70% mortality were subjected to dose-response bioassays. The LC_{50} values ranged between 0.7×10^7 and 2.5×10^7 conidia ml^{-1} and the LT_{50} values of the most active isolates of *B. bassiana* and *M. anisopliae* strains varied between 4.6 and 5.8 days. Adults were more susceptible to *B. bassiana* and *M. anisopliae* infections than immatures. Both isolates also caused egg mortality of >80% at a concentration of 1.0×10^8 conidia ml^{-1}. When deutonymphs were treated with a sub-lethal concentration of 1.0×10^5 conidia ml^{-1} of either isolate, the adults that developed from these deutonymphs laid significantly fewer eggs than untreated mites (Wekesa et al. 2006).

Field and glasshouse assessment of fungi for mite management

Relatively few field trials have been undertaken to evaluate entomopathogenic fungi against *T. urticae* and, even to a lesser extent, *T. evansi.* Dresner (1949) treated *T. urticae* with a dust formulation of conidia containing 0.5% spores of *B. bassiana* in the field and obtained 71% mortality. In a semi-field experiment using *B. bassiana* against *T. urticae* infesting chrysanthemum (*Dendranthema grandiflora*) (Ramat), Alves et al. (1998) obtained results that were better than the chemical pesticide used. Tamai et al. (2002a) reported similar results with *B. bassiana* against *T. urticae* on chrysanthemum when the fungus was applied at a concentration of 2×10^8 conidia ml^{-1}. They also observed that with four fungal sprays within 14 days, mite density was reduced from 1.8 to 0.1 mites/leaf. However, the reduction was lower in strawberry (*Fragaria* sp.) than in chrysanthemum, with a mean density of 13 mites/leaflet 21 days after application of 1×10^8 or 5×10^7 conidia ml^{-1}, compared to 43 mites/leaflet in control plots. The authors also observed an effect of strawberry varieties on the pathogen performance, with the varieties 'Campinas' and 'Princesa Isabel' having the lowest mite densities. Tamai et al. (2002a) concluded that *M. anisopliae*, *B. bassiana* and *Hirsutella* sp. were the promising fungi to be formulated as mycoacaricides for *T. urticae* control.

Chandler et al. (2005) obtained reductions of *T. urticae* populations in a glasshouse following spray applications of *B. bassiana*, *H. thompsoni*, *M. anisopliae*, *L. lecanii* and Naturalis-L®. Naturalis-L® reduced *T. urticae* numbers by up to 97%. In another glasshouse

experiment, single sprays of Naturalis-L® resulted in 98% reductions of adults, nymphs and eggs of *T. urticae* (Chandler et al. 2005). However, attempts to use an entomopathogenic fungus to control *T. urticae* in greenhouses by Andreeva and Shternshis (1995) (cited by Irigaray et al. 2003) were unsuccessful.

Wekesa et al. (2005) treated potted tomato plants artificially infested with *T. evansi* with oil and aqueous formulations of *B. bassiana* and *M. anisopliae,* and obtained reductions of the mite population in comparison with the untreated controls at 7 and 14 days post-treatment. Conidia formulated in oil outperformed the ones formulated in water. For instance, the number of mites/leaf on middle leaves was 24.2 and 5.3 in the aqueous formulations of *M. anisopliae* and *B. bassiana*, respectively, compared to 2.3 and 0.3 mites/leaf in oil formulations of *M. anisopliae* and *B. bassiana*, respectively, 14 days post-treatment.

Strategic options in the use of entomopathogenic fungi for spider mite control

Research efforts intended to develop entomopathogenic fungi as mycoinsecticides in general, and acaricides in particular, have markedly increased in recent years (Faria and Wraight 2007). Although entomopathogenic fungi can be used in classical, conservation and augmentative biological control, emphasis has been placed on their development as inundative augmentative control agents (Goettel and Hajek 2001).

Classical biological control

Classical biological control aims at the permanent establishment of an exotic agent in a new area. Pathogens used for classical biological control are extremely host specific and have great potential to persist in the environment and cause epizootics. While examples of the use of parasitoids and predators in classical biological control abound in literature, there are only few reported examples about entomopathogenic fungi (Hajek et al. 2007). Nineteen species of entomopathogenic fungi have been used in 57 classical biological control programs, but only three mite species were targets for the classical approach. *Hirsutella thompsonii* Fisher var. *synnematosa* Samson, McCoy & O'Donnell was introduced from Zimbabwe into Argentina against *Eriophyes sheldoni* (Ewing) and *Phyllocoptruta oleivora* (Ashmead) in 1985. At the same time, *H. thompsonii* Fisher var. *vinacea* Samson, McCoy & O'Donnell from North Carolina was also released in Argentina. Infection after release was high but persistence is unknown and the project was discontinued (Hajek et al. 2005).

Neozygites tanajoae Delalibera, Hajek and Humber was introduced into Benin against the cassava green mite, *Mononychellus tanajoa* Bondar, in 1998–2000 (Yaninek and Hanna 2003), where it is established (Hountondji et al. 2002b). Indeed, preliminary surveys conducted in the semi-arid region of North-eastern Brazil have identified *N. floridana* as a pathogen of *T. evansi* (Furtado et al. 2007). This pathogen has previously been reported from *T. evansi* over 25 years ago (Humber et al. 1981) and plans are underway to import this fungus to Africa as a possible agent for the classical biological control of *T. evansi* (V. Wekesa and M. Knapp, unpublished).

Inundative augmentative biological control

Use of fungi as biopesticides is considered an attractive strategy in inundation biological control, not only in the control of mites but also for the control of several agricultural pests, because the effect on the targets with this strategy is relatively fast. From a commercial

point of view, this strategy is similar to a 'chemical approach' where the fungal inoculum is applied directly to the crop or the target pest and control is achieved exclusively by the released propagules themselves (Eilenberg et al. 2001). Entomopathogenic fungi in the Ascomycota, order Hypocreales, are well suited for this inundative strategy based on their relatively wide host range, ease of production, formulation and application (Wraight et al. 2001). A major disadvantage of using fungi in this strategy is the dependence of most species on high relative humidity and success may only be guaranteed therefore where optimum humidity conditions are met. However, the fact that dry and hot conditions normally favour development of spider mites may compromise control efficiency, but this could be overcome by high relative humidity during night, favouring fungal sporulation and germination. Moreover, since epizootic development is density dependent and high mite density is common on crops, this makes fungi good candidates for spider mite control.

Because of the high strain variability and wide host range of Hypocreales, most members of this group have the potential to be developed as mycoinsecticides and mycoacaricides. However, they require many steps in their development as mycoinsecticides, including isolation, identification, strain selection, mass production and formulation, field trial, registration and commercialization (Zimmerman 1986). Strain selection is considered an essential starting point in their successful development (Soper and Ward 1981). Consequently, many isolates of entomopathogenic fungi have been screened against *T. urticae* and *T. evansi* and hold potential for their development as mycoacaricides (see Table 2). A recent review (Faria and de Wraight 2007) provides a table showing the list of the mycoacaricides, of which 17 formulations were developed to control mites of the family Tetranychidae.

Entomophthoralean fungi, on the other hand, possess very few characteristics that can fit them into the inundation biological control paradigm. A strong positive attribute of this group is their general high virulence, an attribute that makes them desirable for the inundation strategy. However, the major drawback of this group is that their infective stages are rather short-lived, making their development and use far more difficult. Another shortcoming is the difficulty associated with mass production. Recent attempts suggest that entomophthoralean fungi have high prospects in inundative strategy under greenhouse conditions (Shah et al. 2000). Production of high value crops, including several horticultural crops, is increasingly been undertaken in greenhouses, and the use of entomophthoralean fungi might become more attractive under these conditions, because environmental conditions that normally favour the efficacy of these fungi can be easily manipulated. The use of entomophthoralean fungi under greenhouse conditions has another advantage over alternative control agents in that efficient horizontal transmission, which relies on availability of susceptible hosts, may be increased and repetitive application may be unnecessary. However, reliance on horizontal transmission implies that these fungi are dependent on host population density for survival and dispersal, which means that their efficacy may be compromised at low host densities (Fuxa 1987). Intrinsic differences in mite susceptibility to *N. tanajo*ae and *N. floridana* is associated with the mite life stages, size and behaviour, as well as age (Elliot et al. 2002). Host death caused by these fungi normally occurs at night, when relative humidity is high, favorable for sporulation (Hajek and St Leger 1994). Sporulated conidia germinate to form infective capilliconidia (Fig. 1) that infest arthropod hosts during the day, when their activities are accelerated following increased daytime temperatures.

Another limitation in the use of fungal pathogens to control spider mites is the lack of appropriate formulation and application strategies for the target host. While progress has been made in the formulation of Hyphomycetes fungi, whose aerial spores can be produced

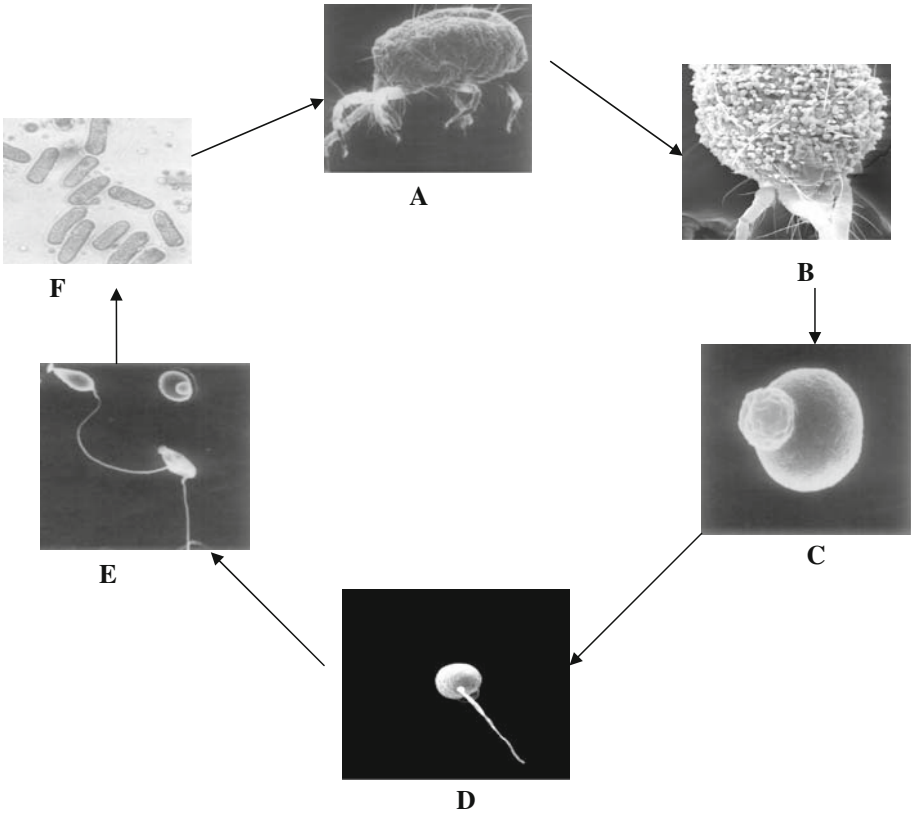

Fig. 1 Schematic diagram of the life cycle of *Neozygites floridana* infesting the tomato spider mite, *Tetranychus evansi*: mummified mite (**a**), sporulated mummy (**b**), primary conidium (**c**), germinating primary conidium (**d**), capilliconidium (**e**), and hyphal bodies (**f**). Courtesy of V.W. Wekesa

easily on common media, little progress has been made with the Entomophthorales whose members include major pathogens of spider mites. Formulation of fungal pathogens can extend shelf life, facilitate handling and application, aid in persistence due to protection from harmful environmental factors and enhance efficacy by increasing contact with the target pests (Jones and Burges 1998). The use of oil formulations in ultra low volume (ULV) spraying has recently helped to overcome many problems on the use of *M. anisopliae* to control locusts in Africa (Bateman 1997). Application of *M. anisopliae* and *B. bassiana* in oil emulsion has been tested against *T. evansi* with very promising results (Wekesa et al. 2005).

The fragility of the hyphal bodies and protoplasts from members of the Entomophthorales has made formulation difficult. Dried mycelia of *Zoophthora radicans* (Brefeld) Batko were formulated with sugar coating as a method for their long-term storage (McCabe and Soper 1985) and algination of mycelia of *Erynia neoaphidis* Remaudière & Hennebert has been demonstrated as a promising method for formulating conidia (Shah et al. 1998). Sugar coating of dry mycelia and algination of the hyphal matrix was facilitated by the in vitro culturing of the fungal species.

Conservation biological control

Conservation biological control involves "modification of the environment or existing practices to protect and enhance natural enemies … to reduce the effect of pests" (Eilenberg et al. 2001; Fuxa 1998). It does not rely on the addition of natural enemies but rather on identifying strategies to promote those natural enemies already present within crop ecosystems, based on a thorough understanding of their biology, ecology and behaviour (Gurr et al. 1998; Landis and Menalled 1998). Despite the important role played by Entomophthorales in the natural regulation of arthropod pests, little consideration has been given to understanding their ecology and function in crop ecosystems (Pell 2007). Because of their ability to persist in the target pest populations, entomophthoralean fungi may fit well in these cropping systems. Research in the UK is evaluating the potential use of arable field margins as habitat refugia in order to encourage early season multiplication of natural enemies, including entomophthoralean fungi, for dispersal into field crop to suppress pest aphids (Pell et al. 2001; Ekesi et al. 2005). The success of this approach largely depends on the presence of a succession of different pest and non-pest insects feeding on non-crop plants in the field boundaries that provide sufficient host densities for continuous infection transmission and dispersal of inoculum into the crop. Future studies should consider these practices and their influence on the biological control of *T. evansi* and *T. urticae*.

Entomopathogenic fungi as a component of spider mite IPM

Although effective in the management of many arthropod pests, the use of entomopathogenic fungi will not supersede the use of synthetic pesticides in all commercial production systems, but in many instances may be applied in conjunction with pesticides in integrated pest management (IPM) programmes. Regardless of whether an entomopathogenic fungus is to be classically introduced, conserved or augmented in an environment as part of an IPM programme, it is crucial to know how it might be affected by the synthetic pesticides commonly used in that environment, in order to determine whether the pesticide application needs to be momentarily or spatially separated from the most susceptible life stages of the fungal pathogen (Pell et al. 2001). It is therefore essential to be aware of the adverse effects that chemical pesticides may have on the efficacy of fungal biological control agents, or the adverse effects the entomopathogenic fungi can have on other natural enemies, especially on predatory mites.

Since the interactions between different pests and disease control methods constitute a key factor in IPM strategies, we shall concentrate in this section on the interactions between entomopathogenic fungi and pesticides, and on the interactions between entomopathogenic fungi and other natural enemies that are used in the same agro-ecosystems while controlling *T. urticae* and *T. evansi*.

Interactions between entomopathogenic fungi and pesticides

Several studies showed negative or positive interactions between entomopathogenic fungi and pesticides used in the same environment for controlling mite populations. While studying the effects of four concentrations of the insecticide imidacloprid (50, 100, 200 and 500 ppm) on two spider mite pathogens, *N. tanajoae* and *H. thompsonii,* Dara and Hountondji (2001) found that the insecticide significantly reduced the germination of primary conidia and the formation of infective capilliconidia in *N. tanajoae,* thus significantly reducing its

infectivity on mites. In contrast, the same insecticide, at a concentration of 100 ppm and above, increased conidial germination in *H. thompsonii*. However, no synergism between imidacloprid and *H. thompsonii* was detected on the mortality of the cassava green mite.

Suppression and reduction of the infection level of *N. floridana* in *T. urticae* populations by the fungicide benomyl (a benzimidazole) have been reported in bean and corn fields (Brandenburg and Kennedy 1982, 1983). Other fungicides, such as chlorothalonil, mancozeb and maneb also reduced the infection level of *N. floridana* in *T. urticae* populations infesting corn and peanut fields (Brandenburg and Kennedy 1982; Boykin et al. 1984; Smitley et al. 1986). Klingen and Westrum (2007) compared the effect of different pesticides (fungicides, insecticides, acaricides and molluscicides) used in strawberry plantation on *N. floridana.* Although their negative effects varied with fungicide, all fungicides tested (tolylfluanid, fenhexamid, cyprodinil + fludioxonil) were harmful to *N. floridana* and could potentially reduce its survival and efficacy, while the acaricide/insecticide/molluscicide, methiocarb, appeared to have a stimulating effect on the fungus.

Apart from *N. floridana* and *H. thompsonii*, the two major mite-pathogenic fungi, the entomopathogenic *B. bassiana, M. anisopliae* and members of the *L. lecanii* complex are also potential biological control agents of tetranychid mites. Tamai et al. (2002b) studied the toxicity of 93 products (three stickers, 36 fungicides and 54 insecticides/acaricides), normally used to control insects and diseases, to the fungus *B. bassiana*, and found much variability in the toxicity of different classes of products. All stickers were very toxic to the fungus, whereas only three out of 36 fungicides (propamocarb hydrochloride, sulphur and kasugamycin) and 24 out of 54 insecticides/acaricides (including those with the following active ingredients: abamectin, acephate, acetamiprid, betacyfluthrin, bifenthrin, ciromazine, deltamethrin, diafenthiuron, diflubenzuron, dimethoate, fenpropathrin, fenpyroximate, fenvalerate, imidacloprid, metamidophos, propargite, and tebufenozide etriclorfon) were compatible with *B. bassiana*. Wenzel et al. (2004) evaluated the compatibility of the insecticides Provado (imidachloprid) and Trigard700PM (cyromazine) to the entomopathogenic fungus *L. lecanii,* in terms of vegetative growth, sporulation, conidial viability and pathogenicity against *T. urticae*. All the insecticides tested were compatible with *L. lecanii* in all evaluated parameters and mite mortality was above 68% in all treatments. Compatibility of *B. bassiana* with triflumuron was investigated for the control of *T. urticae* by Irigaray et al. (2003). Combination of *B. bassiana* with 0.25 g Alsystin (25% triflumuron as a wettable powder) l^{-1} resulted in a significant decrease of *T. urticae* egg mortality. Triflumuron reduced mycelial growth but not conidial germination of *B. bassiana*. They concluded that *B. bassiana* is a possible candidate to be included in IPM programs of *T. urticae* with triflumuron. Shi et al. (2005) investigated the effects of 10 acaricides on *B. bassiana* in the laboratory. At field concentration rates, dicofol, chlorpyrifos, abamectin, liuyangmycin, and azocyclotin significantly reduced the germination rate of *B. bassiana* conidia, whereas pyridaben, propargite, hexythiazox, amitraz and matrine had no effect. Combinations of *B. bassiana* with pyridaben, the acaricide with the least effect on conidia germination, significantly increased the mortality of *Tetranychus cinnabarinus* (Boisduval) eggs compared to *B. bassiana* alone. According to Inglis et al. (2001), pesticides that are inhibitory in the laboratory do not always exhibit the same action in field conditions. This may be due to the concentration of the pesticide used in the field or to applying the pesticide in a manner that minimizes contact with the fungus.

Interactions between entomopathogenic fungi and other natural enemies

Pathogens may contribute to the suppression of spider mite populations in combination with other arthropod natural enemies. However, because natural enemies of spider mites have

evolved and function in a multitrophic context, it is important to assess interactions within complexes of natural enemies if they are to be exploited effectively in pest management (Ferguson and Stiling 1996; Roy and Pell 2000). Fungal natural enemies can interact either synergistically/additively (e.g., enhanced transmission and dispersal of spider mite pathogens) or antagonistically (e.g., parasitism/infection, predation and competition) (Ferguson and Stiling 1996; Roy and Pell 2000).

Only a few reports are available on the interactions between entomopathogenic fungi and other spider mite natural enemies. Ludwig and Oetting (2001) studied the susceptibility of *Phytoseiulus persimilis* Athias-Henriot and *Iphiseius degenerans* (Berlese) to *B. bassiana* (strain JW-1). The natural enemies were highly susceptible to infection by *B. bassiana* under laboratory conditions, whereas lower infection rates were observed in greenhouse trials. Ludwig and Oetting (2001) also evaluated the susceptibility of *I. degenerans* to the entomopathogenic fungi *B. bassiana* (strain GHA), *L. lecanii* and *M. anisopliae* in a greenhouse and observed that the predatory mite was least susceptible to *M. anisopliae,* followed by *L. lecanii* and *B. bassiana*. Studying the effect of *B. bassiana* on the predatory mite *Neoseiulus cucumeris* Oudemans, Jacobson et al. (2001) found that *B. bassiana* had no detrimental effect on the mite when sprayed onto excised cucumber leaves in a laboratory bioassay, or when sprayed onto glasshouse-grown cucumbers. They suggested that a mycopesticide based on *B. bassiana* could be used as a second line of defence to support preventative pest management with *N. cucumeris*. Chandler et al. (2005) evaluated the efficacy of Naturalis-L® as a supplementary treatment to *P. persimilis* for the control of *T. urticae* populations on tomato in a glasshouse. Application of *P. persimilis* on its own did not reduce numbers of *T. urticae* adults, nymphs or eggs. In contrast, application of *P. persimilis* + Naturalis-L® reduced numbers of *T. urticae* adults, nymphs and eggs compared with all other treatments. Fewer *P. persimilis* were recorded from the *P. persimilis* + Naturalis-L® treatment than from the *P. persimilis* treatment alone. Since it was not clear if this was caused by Naturalis-L® directly killing *P. persimilis,* or by lack of prey causing the predatory mites to migrate, the authors suggested that further work on the mode of action of Naturalis-L® and its compatibility with *P. persimilis* be conducted.

Recent studies on the effect of *N. floridana* on *T. evansi* and its predator *P. longipes* showed that, despite being able to attach to the body of *P. longipes*, *N. floridana* is unable to infect it (Wekesa et al. 2007). Although *N. floridana* and *N. tanajoae* have been reported to be non-pathogenic to some phytoseiids and several non-target insects (Moraes and Delalibera 1992; Hountondji et al. 2002a; Wekesa et al. 2007), Furtado et al. (1996) reported that the fungus *Neozygites acaricida* (Petch) is pathogenic to the phytoseiid mite *Euseius citrifolius* Denmark and Muma.

Conclusions and prospects for future development

Compared to other biological control agents (e.g., predators, parasitoids, *Bacillus thuringiensis*), the development of entomopathogenic fungi for inoculative or inundative and classical biological control programs is still far from satisfactory. However, entomopathogenic fungi have a considerable potential to become major components of sustainable IPM, provided there is continued investment in research, technology transfer and education (Shah and Pell 2003). There is a great potential for their use in conservation and classical biological control programs, as public pressure is growing to adopt sustainable agricultural practices, reduce synthetic pesticides and protect the environment. Successful use of entomopathogenic fungi as microbial control agents of mites will ultimately depend on

how well the strains are selected (virulence, persistence), on large scale production, formulation, compatibility with other control agents and on better systems of delivery, including timing of applications.

More research is required to make in vitro production of entomophthoralean fungi (hyphal bodies, conidia or resting spores) possible, together with development of appropriate formulations for better delivery to target spider mites. Some entomophthoralean fungi produce resting spores in submerged culture and these spores can be harvested, formulated and applied in the field for control of pests in inoculative releases (Kogan and Hajek 2000). The potential of conidia as the basis of a commercial product is limited by their rapid environmental desiccation. In contrast, resting spores are long-term survival structures that are thick-walled and robust, long-lived and environmentally stable. Resting spores, therefore, have potential as alternative commercial inocula for use in augmentation (inoculative and mycoacaricide use) approach. No studies have been undertaken with the mite-specific Entomophthorales to apply their spores in the aforesaid manner for biological control. For this group of fungi, resting spores seem to be the best stage that can be easily manipulated and attempts at their mass production should primarily be of this stage. Future studies should investigate methods for the induction of resting spore formation in *N. floridana,* either through nutritional or physical stress, followed by other tests that can enable the use of this fungus as a mycoacaricide.

Difficulties associated with the establishment of in vitro cultures of these pathogens are likely to be circumvented through selection and development of new inexpensive cell culture media. The use of genetic manipulation to overcome other limitations is promising. For instance, recombinant DNA technology can be used to expand the host range of the promising host-specific strains so that they can be used to target multiple pest mite species, as has been done with other fungal pathogens (St. Leger 2001). Bioprospecting for the discovery of fungal isolates with new traits should also be considered. For instance, surveys for more strains with varied virulence from different geographic regions and hosts will increase the possibility of obtaining a wide range of strains for use in different agroecoystems.

For the development of mycoacaricides based on entomopathogenic fungi in the Ascomycota, order Hypocreales, screening for more efficient strains is still necessary. Efficient mass production methods and formulations also need to be developed. A major problem for open field applications is the requirement of high ambient humidities for successful infection. The commercial products Vertalec and Mycotal, based on the *L. lecanii* complex and used in the control of aphids, thrips and whiteflies, are exclusively used in greenhouses where humidity can be modified to favour infection (Milner 1997). However, recent advances in formulation technology have resulted in an adjuvant that enhances the activity of Mycotal at low humidities (Shah and Pell 2003).

Entomopathogenic fungi can play an important role in IPM if used in conjunction with other strategies for sustainable pest control (Shah and Pell 2003). To achieve this, the compatibility of the mycoacaricides with other mite biocontrol agents, especially phytoseiid mites, as well as with synthetic pesticides, needs to be investigated.

The further development of entomopathogenic fungi as control agents for spider mites needs considerable investment in multidisciplinary research by the public and private sectors. When commercial interests are absent, as in the development of classical biological control and conservation strategies, especially in developing countries, long-term government support is essential.

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Effect of temperature on virulence of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to *Tetranychus evansi*

David M. Bugeme · Nguya K. Maniania · Markus Knapp · Hamadi I. Boga

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Abstract The virulence of three isolates of *Beauveria bassiana* (Bals.) Vuill. and 23 isolates of *Metarhizium anisopliae* (Metschnik.) Sorok. (Ascomycota: Hypocreales) against the tomato spider mite, *Tetranychus evansi* Baker and Pritchard (Acari: Tetranychidae), was assessed in the laboratory. The effect of temperature on germination, radial growth and virulence of selected isolates (two isolates of *B. bassiana* and nine of *M. anisopliae*) on *T. evansi* was also investigated in the laboratory. All the fungal isolates tested were pathogenic to the adult females of *T. evansi*, and there were significant differences in mortality between fungal isolates. The lethal time to 50% mortality (LT_{50}) values ranged from 4.2 to 8.1 days and the LT_{90} values from 5.6 to 15.1 days. Temperature had significant effects on germination, radial growth and virulence of the various isolates. The best fungal germination was observed at 25 and 30°C, while for the fungal radial growth it was 30°C. All the isolates germinated and grew at all temperatures, but germination and radial growth varied with isolate and temperature. The selected isolates were all virulent to *T. evansi*, but virulence varied also with isolate and temperature.

Keywords Ascomycota · *Beauveria bassiana* · Hypocreales · Germination · *Metarhizium anisopliae* · Pathogenicity · Radial growth · Tetranychidae · *Tetranychus evansi* · Virulence

Introduction

The spider mite, *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae), is an exotic mite species, probably of South American origin (Gutierrez and Etienne 1986) and was first recorded in Africa in 1979 in Zimbabwe (Blair 1983), from where it spread

D. M. Bugeme · N. K. Maniania (✉) · M. Knapp
International Centre of Insect Physiology and Ecology (ICIPE), P.O. Box 30772, Nairobi, Kenya
e-mail: nmaniania@icipe.org

D. M. Bugeme · H. I. Boga
Department of Botany, Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya

northwards. More recently, it was also reported from central, western and northern Africa (Bonato 1999; Kreiter et al. 2002; Duverney et al. 2005). It is a pest of solanaceous crops, especially tomatoes (Silva 1954; Ramalho and Flechtmann 1979; de Moraes et al. 1986). If left uncontrolled under hot and dry conditions, *T. evansi* can destroy tomato plants within 3-5 weeks, and the farmer can lose his production within a week's time. For instance, yield losses of up to 90% have been reported in Zimbabwe (Saunyama and Knapp 2003).

Due to problems related to the use of synthetic acaricides in controlling *T. evansi* (spider mite resistance and environmental contamination), the control of this pest is still a major problem for farmers and attracts a strong attention in the world of researchers. Thus, non-chemical control measures are being developed at the International Centre of Insect Physiology and Ecology (ICIPE) as alternatives to synthetic acaricides for the control of *T. evansi*. They include improved crop management, screening for resistance in commercial and wild tomato germplasm and biological control using predatory mites and entomopathogenic fungi.

Biological control of *T. evansi* with the predatory mites, *Neoseiulus californicus* McGregor and *Phytoseiulus persimilis* Athias-Henriot, was not effective (de Moraes and McMurtry 1986; Escudero and Ferragut 2005); however, a recently discovered strain of *P. longipes* Evans has shown promising results in laboratory experiments (Furtado et al. 2007). The virulence of entomopathogenic fungi including *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorok. (Ascomycota: Hypocreales) towards spider mite species have been reported by many authors (Chandler et al. 2000; Barreto et al. 2004; Wekesa et al. 2005).

However, entomopathogenic fungi are exposed to a number of biotic and abiotic factors. Temperature, humidity and solar radiation are probably the most important environmental factors affecting survival and capability to cause mortality by entomopathogenic fungi (Benz 1987; Inglis et al. 2001). Temperature affects the pathogen-its germination, growth, survival and virulence- the host and the host-pathogen interaction (Ekesi et al. 1999; Dimbi et al. 2004; Kiewnick 2006). In general, optimum temperatures for germination, growth, sporulation and virulence of entomopathogenic fungi have been reported to range between 20 and 30°C (Ekesi et al. 1999; Tefera and Pringle 2003; Dimbi et al. 2004; Kiewnick 2006). Since variation in temperature tolerance among isolates can be significant (Ekesi et al. 1999; Tefera and Pringle 2003; Dimbi et al. 2004), this study was therefore initiated to evaluate (i) the virulence of *B. bassiana* and *M. anisopliae* isolates to *T. evansi* and (ii) to test the effect of temperatures on germination, radial growth and virulence of the selected isolates to *T. evansi* in order to pick isolates with a broad temperature range for further studies.

Materials and methods

Mite cultures

A stock culture of *T. evansi* was established in the laboratory at the ICIPE Headquarters, Nairobi, Kenya. *Tetranychus evansi* was reared on tomato, *Lycopersicon esculentum* Mill. variety Cal-J at 26 ± 2°C, 60–70% RH and a 12:12 L:D photoperiod. The initial culture originated from mites collected from tomato plants at Mwea Irrigation Scheme, Kenya, in 2001. Quiescent deutonymphs were collected from the mite culture using a fine camel hair brush and placed on tomato leaf discs. Two days later, newly emerged adult female mites were selected and used in the experiments.

Fungal cultures and viability assessments

The 26 fungal isolates used in this experiment were obtained from the ICIPE Arthropod Germplasm Centre (Table 1). Conidia were harvested by scraping the surface of 3-week-old sporulating cultures grown on Sabouraud dextrose agar (SDA) in Petri dishes at 26 ± 2°C. Conidia were suspended in 20 ml sterile distilled water containing 0.05% Triton X-100. The suspension was vortexed for 5 minutes to produce homogenous conidial suspension. The viability of conidia was then determined by spread-plating 0.1 ml of the suspension (titrated to 3.0×10^6 conidia ml^{-1}) on SDA plates. A sterile microscope cover slip was placed on each plate. Plates were incubated at 26 ± 2°C and the percentage germination was determined from 100-spores for each plate using a compound microscope at 400 X magnification.

Bioassays

Virulence tests

The virulence of fungal isolates against *T. evansi* was tested by spraying 10 ml of a standard concentration of 1.0×10^7 conidia ml^{-1} on both sides of tomato leaf discs (25 mm diameter) using the Burgerjon's spray tower (Burgerjon 1956) (INRA, Dijon, France), corresponding to 3.8×10^6 conidia cm^{-2}. In the control treatments, leaf discs were sprayed with sterile distilled water containing 0.05% Triton X-100. The leaf discs were then air-dried under the laminar flow cabinet for 20 min and placed on wet cotton wool in Petri dishes. Twenty 1–2-day-old adult female *T. evansi* were then placed onto each of the treated tomato leaf discs. Mites were maintained in an incubator at 25 ± 2°C and transferred onto untreated leaf discs after 4 days. Mortality was recorded daily for 10 days. Dead mites were transferred to Petri dishes lined with moist filter paper to allow the growth of fungus on the surface of the cadaver. Mortality caused by fungus was confirmed by microscopic examination. Treatments were arranged in complete randomized blocks and replicated six times. The same experimental procedure was used in the experiments on the effect of temperature on the virulence of fungal isolates against *T. evansi* with the only difference that mites were maintained at various temperatures (20, 25, 30 and 35°C) and the treatment was replicated four times.

Effect of temperature on germination of fungi

Fungal isolates (11) that caused mortalities >70% within 10 days (LT_{90}) were selected for this study. Conidial suspension (0.1 ml) of 3×10^6 conidia ml^{-1} was spread on SDA plates. A sterile microscope cover slip was randomly placed on each plate. Plates were sealed with Parafilm M and incubated at 20, 25, 30 and 35°C in complete darkness. At 24 h post-inoculation, 1 ml formaldehyde (0.5%) was transferred onto each plate to halt germination. Percentage germination was then determined from 100-spores for each plate at 400× magnification. Treatments were arranged in complete randomized blocks and replicated four times.

Effect of temperature on radial growth

This study was also carried out on the 11 selected fungal isolates during the virulence bioassay. Conidial suspension of 1×10^7 conidia ml^{-1} was spread-plated on SDA plates, which were then incubated at 26 ± 2°C for 3 days in order to obtain mycelial mats. The

unsporulated mycelial mats were cut from culture plates into round agar plugs using an 8-mm diameter cork borer (Rapilly 1968). Each agar plug was then transferred singly onto the centre of a fresh SDA plate. Plates were sealed with Parafilm M and incubated in complete darkness at 20, 25, 30 and 35°C. Radial growth was then recorded daily for 10 days by measuring colony cardinal diameters, through two orthogonal axes previously drawn on the bottom of each Petri dish to serve as a reference, using a simple plastic ruler. The experiment was replicated four times.

Statistical analysis

Mortality data were corrected for natural mortality in the controls (Abbott 1925) and arcsine-transformed to normalize the data before analysis of variance (ANOVA) (SAS Institute 1999–2001). Means were separated by Student-Newman-Keuls test at $P = 0.05$. Lethal time to 50% mortality (LT_{50}) and the lethal time to 90% mortality (LT_{90}) values were estimated with repeated measures logistic regression using generalised estimating equations (GEE) (Stokes et al. 2000). All analyses were carried out using the GENMOD procedure of SAS (SAS Institute 1999–2001). Data on germination were also arcsine-transformed before ANOVA. Radial growth data were also subjected to analysis for a completely randomised design using the ANOVA procedure of SAS (SAS Institute 1999–2001).

Results

Virulence of fungal isolates

In the viability tests, 86.9 ± 1.0 to 96.3 ± 0.7% of spores germinated. Mean mortality in the control was 12.9 ± 0.9% ten days after treatment. All the 26 fungal isolates tested were pathogenic to adult females of *T. evansi*. Mortality caused by *B. bassiana* was not significantly different between the isolates. However, there was a significant difference in mortality between isolates of *M. anisopliae* ($F_{26,135} = 20.49$; $P < 0.0001$) (Table 1). The LT_{50} values ranged from 4.2 to 8.1 days and the LT_{90} values from 5.6 to 15.1 days, with *B. bassiana* isolate ICIPE279 having the shortest lethal time values of 4.2 (LT_{50}) and 5.6 days (LT_{90}) (Table 1). Based on these results, 11 fungal isolates were selected for further studies.

Effect of temperature on germination of fungal isolates

The germination for all isolates was above 65% at the four temperatures, except at 35°C where germination was low in *B. bassiana* isolates ICIPE279 and ICIPE278. Germination values ranged from 65.8% to 86.3%, from 74.9% to 95.0%, from 81.7% to 96.8% and from 15.1% to 85.6% at 20, 25, 30 and 35°C, respectively. Significant differences in germination between fungal isolates were observed at 20°C ($F_{10,33} = 9.18$; $P < 0.0001$), 25°C ($F_{10,33} = 28.16$; $P < 0.0001$), 30°C ($F_{10,33} = 7.92$; $P < 0.0001$) and 35°C ($F_{10,33} = 284.49$; $P < 0.0001$).

Effect of temperature on radial growth

As in the case of germination, there were significant differences in radial growth between fungal isolates at 20°C ($F_{10,33} = 31.28$; $P < 0.0001$), 25°C ($F_{10,33} = 14.22$; $P < 0.0001$),

Table 1 Pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* isolates against *Tetranychus evansi*

Species/ Isolates	Year of isolation, Host/ Substrate	Percent mortality ± SE	LT_{50} (days) (95% fiducial limits)	LT_{90} (days) (95% fiducial limits)
Beauveria bassiana				
ICIPE279	1996, Soil	95.2 ± 2.3a	4.2 (3.6–4.9)	5.6 (4.8–6.4)
ICIPE273	2004, Soil	83.3 ± 5.6abcd	6.0 (5.3–7.9)	7.5 (6.4–8.7)
ICIPE278	2005, *Cyclocephala* sp.	83.0 ± 7.7abcd	5.0 (3.3–7.7)	7.1 (4.6–10.9)
Metarhizium anisopliae				
ICIPE24	1999, Soil	90.5 ± 3.8ab	6.3 (5.8–6.8)	7.3 (6.6–8.1)
ICIPE84	2003, *Onitacris turbidda cavroisi*	89.4 ± 3.7abc	6.5 (6.0–6.9)	7.5 (7.0–8.0)
ICIPE78	1990, *Temnoschoita nigroplagiata*	86.8 ± 8.8abc	5.7 (4.6–7.0)	7.6 (6.1–9.5)
ICIPE43	2005, Soil	84.8 ± 5.7abcd	6.0 (4.9–7.4)	8.0 (6.7–9.6)
ICIPE55	2005, Soil	81.7 ± 9.2abcd	5.6 (4.5–6.9)	7.4 (5.7–9.7)
ICIPE59	2005, Caterpillar	79.9 ± 7.1abcd	6.0 (6.4–7.6)	8.3 (7.4–9.2)
ICIPE8	1990, *Galleria mellonella*	79.3 ± 8.8abcd	6.5 (4.6–7.0)	7.9 (6.5–9.5)
ICIPE51	2005, Soil	73.0 ± 7.2abcde	7.5 (6.6–8.4)	9.4 (8.3–10.6)
ICIPE7	1996, *Amblyomma variegatum*	70.9 ± 6.3abcdef	7.7 (6.6–9.0)	9.8 (8.2–11.7)
ICIPE25	1999, Sandy Soil	68.4 ± 8.0abcdef	7.2 (6.6–7.9)	8.9 (7.7–10.1)
ICIPE48	2005, Unknown	60.1 ± 7.9bcdefg	7.6 (6.6–8.8)	10.7 (9.1–12.6)
ICIPE49	2005, Soil	57.9 ± 9.0cdefg	7.9 (4.0–15.5)	12.9 (7.0–23.8)
ICIPE315	2005, *Tetranychus urticae*	54.6 ± 6.3defg	7.7 (6.3–9.4)	15.1 (10.8–21.0)
ICIPE316	2005, *Tetranychus* spp.	54.0 ± 10.5defg	8.1 (6.6–9.9)	11.2 (8.7–14.3)
ICIPE95	2005, Soil	44.3 ± 11.5efg	–	–
ICIPE62	1990, Soil	43.8 ± 10.4efg	–	–
ICIPE21	1999, *Lacusta gregaria*	43.7 ± 8.2efg	–	–
ICIPE30	1989, *Busseola fusca*	42.4 ± 12.5efg	–	–
ICIPE41	1990, Soil	40.6 ± 3.5fg	–	–
ICIPE18	1989, Soil	37.0 ± 9.4gh	–	–
ICIPE97	2005, Unknown	35.8 ± 7.3gh	–	–
ICIPE69	1990, Soil	35.0 ± 4.3gh	–	–
ICIPE20	1989, Soil	30.4 ± 4.5gh	–	–
Control	–	12.9 ± 0.9h	–	–

Percent mortality, LT_{50} and LT_{90} values at 25 ± 2°C

Means followed by the same letter are not significantly different (Student-Newman-Keuls test, $P > 0.05$)

30°C ($F_{10,33} = 21.76$; $P < 0.0001$) and 35°C ($F_{10,33} = 39.69$; $P < 0.0001$). Fungal isolates grew at all temperatures but for most isolates, the growth was slower at 20 and 35°C, than at 25 and 30°C. The two isolates of *B. bassiana* recorded the least radial growth at all temperatures. Fungal radial growth varied from 0.6 to 2.1, from 1.2 to 2.4, from 1.2 to 4.4 and from 0.7 to 2.3 mm day^{-1} at 20, 25, 30 and 35°C, respectively.

Effect of temperature on virulence

Mortality in the controls did not exceed 7.5%, except at 35°C where 16.3% mortality was recorded. The 11 fungal isolates tested were pathogenic to the tomato spider mite at all temperatures; however, mortality varied with fungal isolate and temperature (Table 2). For instance, significant differences in mortalities were observed between fungal isolates at 20 ($F_{10,33} = 2.43$; $P = 0.0267$) and 25°C ($F_{10,33} = 2.62$; $P = 0.0181$). There was no significant difference in mortality between fungal isolates at 30 ($F_{10,33} = 0.98$; $P = 0.4762$) and 35°C ($F_{10,33} = 1.15$; $P = 0.3607$) (Table 2). Among the fungal isolates, only *B. bassiana* isolate ICIPE279 was virulent across temperatures followed by *B. bassiana* isolate ICIPE278 and *M. anisopliae* isolate ICIPE7 (Table 2). The LT_{50} values ranged from 6.8 to 24.6 days at 20°C, from 4.8 to 9.7 days at 25°C, from 2.6 to 5.8 days at 30°C and from 1.9 to 3.4 days at 35°C (Table 2). The LT_{90} values ranged from 9.0 to 35.3, from 6.7 to 11.6, from 3.3 to 7.2 and from 2.8 to 4.9 days at 20, 25, 30 and 35°C, respectively (Table 2).

Discussion

Out of 26 fungal isolates tested in the present study, 13 isolates were previously tested against *T. evansi* (Wekesa et al. 2005) and were all pathogenic to the mite; but there was significant variation between the isolates. Variation in the pathogenic activity between the 26 fungal isolates against *T. evansi* was also observed in the present study. Intraspecific differences in the pathogenicity of the mitosporic fungi *B. bassiana, M. anisopliae, Hirsutella thompsonii* Fisher and *Lecanicillium lecanii* complex have also been reported in other mite species (Tamai et al. 2002; Barreto et al. 2004; Alves et al. 2005; Brooks and Wall 2005; Chandler et al. 2005).

It is generally admitted that the most virulent fungal isolates are the ones isolated from the host. This is true in some cases as the one reported by Pěna et al. (1996) where fungal isolates that originated from the mite *Polyphagotarsonemus latus* Banks were more virulent to this species than those isolated from other hosts. However, this was not the case in our study where the virulent isolates did not originate from spider mite species. The two fungal isolates isolated from *Tetranychus* species (ICIPE315 and ICIPE316) were not virulent to *T. evansi.* Wekesa et al. (2005) reported similar results with the same mite species. The virulence of fungal isolates from non-Acari hosts to Acari hosts have also been reported elsewhere (Kaaya et al. 1996; Samish et al. 2001; Shaw et al. 2002).

The best temperature for germination of the 11 selected fungal isolates was between 25 and 30°C, which is in agreement with other published reports (Ekesi et al. 1999; Tefera and Pringle 2003; Dimbi et al. 2004; Kiewnick 2006). However, germination of *B. bassiana* isolates was low at 35°C, compared to those of *M. anisopliae* isolates. Although all the fungal isolates grew at all the temperatures, it appeared that the favourable temperature for most isolates was 30°C. Similar results were reported by Tefera and Pringle (2003). However, Ekesi et al. (1999) and Dimbi et al. (2004) reported that the optimum temperature for radial growth of most isolates of *B. bassiana* and *M. anisopliae* was 25 and 30°C. Ouedraogo et al. (1997) reported that the optimum temperature for vegetative growth of *M. anisopliae* isolates ranged between 25 and 32°C, with 25°C being the optimum for most isolates. The growth rate of fungal isolates did not necessarily relate to virulence. For example, the *B. bassiana* isolate ICIPE279 that had a radial growth of 0.6 mm/day at 20°C

Table 2 Effect of temperature on virulence of *Beauveria bassiana* and *Metarhizium anisopliae* to the tomato spider mite, *Tetranychus evansi*: Lethal time to 50% and 90% mortality

Species/isolates	20°C			25°C		
	% Mortality ± SE	LT_{50} (95% fiducial limits)	LT_{90} (95% fiducial limits)	% Mortality SE	LT_{50} (95% fiducial limits)	LT_{90} (95% fiducial limits)
B. bassiana						
ICIPE279	72.5 ± 15.1aA	6.8 (5.3–8.6)	9.0 (6.6–12.3)	88.5 ± 4.3abA	7.5 (6.4–8.8)	8.7 (7.6–10.1)
ICIPE278	50.5 ± 15.8abB	9.2 (6.4–13.2)	13.4 (8.9–20.0)	90.4 ± 4.1abA	4.8 (3.0–7.6)	6.7 (4.4–10.2)
M. anisopliae						
ICIPE55	26.3 ± 10.1abC	11.3 (10.5–12.3)	12.7 (11.8–13.7)	54.4 ± 9.8bB	9.7 (8.6–10.9)	12.5 (10.5–14.8)
ICIPE59	8.8 ± 2.4bB	24.6 (15.2–40.0)	35.3 (17.0–73.4)	75.0 ± 10.2bA	7.1 (6.0–8.4)	9.2 (7.5–11.2)
ICIPE78	50.9 ± 12.1abB	8.9 (6.1–13.0)	13.4 (8.4–21.3)	72.7 ± 7.8abAB	9.3 (8.9–9.7)	11.6 (10.3–13.0)
ICIPE84	37.8 ± 12.1abB	10.8 (8.7–13.4)	13.4 (10.3–17.5)	97.1 ± 1.7aA	6.2 (5.9–6.5)	7.7 (7.5–8.0)
ICIPE24	43.8 ± 6.6abB	11.1 (8.1–15.3)	18.8 (14.5–24.4)	77.3 ± 13.3abA	7.4 (6.4–8.6)	9.4 (7.5–11.8)
ICIPE25	21.3 ± 8.8abB	13.0 (10.2–16.4)	15.6 (11.6–20.8)	90.3 ± 6.2abA	6.5 (5.1–8.2)	8.1 (6.5–10.2)
ICIPE8	42.5 ± 10.6abC	10.5 (6.8–16.1)	15.9 (11.6–21.9)	66.5 ± 6.5abB	8.1 (7.1–9.2)	11.0 (9.3–13.1)
ICIPE43	30.4 ± 12.3abB	13.5 (11.4–15.9)	16.1 (13.1–19.7)	65.4 ± 12.3abA	8.8 (7.9–9.8)	10.5 (9.2–11.9)
ICIPE7	64.8 ± 13.4abB	7.4 (5.8–9.5)	10.0 (7.5–13.3)	83.7 ± 2.8abAB	6.8 (6.4–7.3)	8.3 (7.8–8.8)
	30°C			35°C		
	% Mortality ± SE	LT_{50} (95% fiducial limits)	LT_{90} (95% fiducial limits)	% Mortality SE	LT_{50} (95% fiducial limits)	LT_{90} (95% fiducial limits)
B. bassiana						
ICIPE279	100aA	4.1 (3.8–4.6)	5.0 (4.8–5.2)	98.5 ± 1.5aA	1.9 (1.0–3.5)	2.9 (1.6–5.1)
ICIPE278	98.5 ± 4.4aA	2.6 (2.1–3.2)	3.3 (2.7–4.1)	97.1 ± 1.7aA	2.0 (1.5–2.6)	2.8 (2.4–3.2)
M. anisopliae						
ICIPE55	100aA	3.7 (3.0–4.6)	4.4 (3.4–5.8)	97.2 ± 1.6aA	2.6 (2.5–2.8)	3.0 (2.8–5.1)
ICIPE59	93.8 ± 4.4aA	5.8 (5.0–6.7)	7.2 (6.3–8.3)	91.5 ± 3.7aA	3.4 (3.0–3.9)	4.4 (3.8–5.1)
ICIPE78	100aA	4.4 (3.9–5.0)	5.2 (4.5–6.0)	100aA	2.7 (2.2–3.3)	3.3 (2.7–3.9)

Table 2 continued

	30°C			35°C		
	% Mortality ± SE	LT_{50} (95% fiducial limits)	LT_{90} (95% fiducial limits)	% Mortality SE	LT_{50} (95% fiducial limits)	LT_{90} (95% fiducial limits)
ICIPE84	100aA	4.3 (3.8–4.8)	5.3 (4.6–6.0)	86.8 ± 11.4aA	2.9 (2.4–3.3)	3.4 (2.9–4.1)
ICIPE24	100aA	4.4 (3.9–4.9)	5.4 (4.8–6.0)	93.8 ± 4.4aA	2.8 (2.4–3.2)	3.3 (2.7–3.9)
ICIPE25	93.8 ± 6.3aA	5.2 (4.6–5.8)	6.5 (5.5–7.6)	100aA	3.0 (2.6–3.5)	3.6 (3.0–4.3)
ICIPE8	100aA	4.7 (4.0–5.5)	5.8 (5.3–6.4)	100aA	3.0 (2.3–3.8)	3.9 (3.1–5.0)
ICIPE43	98.6 ± 1.4aA	3.5 (3.0–4.0)	4.4 (3.8–5.1)	100aA	2.9 (2.8–3.1)	3.2 (3.1–3.3)
ICIPE7	98.6 ± 1.4aA	3.5 (2.5–4.9)	4.3 (3.0–6.2)	91.4 ± 5.0aAB	3.2 (1.9–5.5)	4.9 (3.4–7.0)

Means (± SE) within within one temperature (comparing 11 isolates) followed by the same lower case letter, and within one isolate (comparing 4 temperatures) followed by the same upper case letter are not significantly different (Student-Newman-Keuls test, $P > 0.05$)

caused 72.5% mortality in *T. evansi* while the *M. anisopliae* isolate ICIPE55 with a radial growth of 2.1 mm/day induced only 26.3% mortality at the same temperature.

The infection of *B. bassiana* and *M. anisopliae* in *T. evansi* increased as temperature increased. Most fungal isolates were more (highly) virulent at 25, 30 and 35°C than at 20°C, which is in agreement with other published reports (Thomas and Jenkins 1997; Ekesi et al. 1999; Dimbi et al. 2004). For instance, Dimbi et al. (2004) reported that *M. anisopliae* isolates were more virulent to the three African tephritid fruit flies, *Ceratitis capitata* (Wiedemann), *C. fasciventris* (Bezzi) and *C. cosyra* (Walker) at 25, 30 and 35, than at 20°C. However, Ekesi et al. (1999) showed that some isolates of *B. bassiana* and *M. anisopliae* were highly virulent at 20°C when infecting the legume flower thrips, *Megalurothrips sjostedti* (Trybom).

The findings of this study highlight the importance of strain selection as stressed by Soper and Ward (1981). *Beauveria bassiana* isolates ICIPE279 and ICIPE278, and *M. anisopliae* isolates ICIPE78 and ICIPE7 were selected as candidates for control of *T. evansi* because of their ability to infect and cause a high rate of mortality between 20 and 35°C, which is the range of temperature where the pest is found. However, further studies-such as the effect of these isolates on the various developmental stages of *T. evansi*, the effect of host plant on the virulence of the fungal isolate(s), the efficacy of these isolates in reducing *T. evansi* populations in screenhouse and field-need to be carried out in order to develop the isolates as biological control agents of *T. evansi* and as component of integrated spider mite management.

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Side-effects of pesticides on the life cycle of the mite pathogenic fungus *Neozygites floridana*

V. W. Wekesa · M. Knapp · I. Delalibera Jr.

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Abstract The tomato red spider mite, *Tetranychus evansi* Baker and Pritchard, is an invasive species in Africa causing considerable damage to Solanaceous crops. The fungal pathogen *Neozygites floridana* Weiser and Muma from Brazil has been considered a potential candidate for introduction into Africa for the control of *T. evansi*. To be incorporated in the tomato production system, *N. floridana* has to be compatible with the pesticides used for the control of other pests and diseases. Pesticides used in tomatoes that might affect the fungus were therefore studied by the use of different methods. Two insecticides (Lambda-cyhalothrin and Methomyl), two acaricides (Propargite and Abamectin), and two fungicides (Captan and Mancozeb) were tested in two concentrations: the mean commercial rate (CR) and 50% of the mean commercial rate (CR/2). Fungus-killed mite cadavers or the substrates used for sporulation (leaf discs and coverslips) were either immersed or sprayed with the pesticides before testing their effects on sporulation, germination of primary conidia and infectivity of *N. floridana*. Direct immersion of cadavers, coverslips or leaf discs into pesticides affected sporulation and germination stronger than the spray tower method, although infectivity of capilliconidia was neither affected by the method of application nor the concentration of the pesticides. The fungicides Captan and Mancozeb resulted in a high reduction in sporulation and germination at both concentrations. Propargite did not inhibit sporulation but affected germination of primary conidia. Methomyl and Abamectin resulted in less effects on *N. floridana*.

Keywords *Neozygites floridana* · Toxicity · Tomato · *Tetranychus evansi* · Side effects

V. W. Wekesa · I. Delalibera Jr. (✉)
Departamento de Entomologia, Fitopatologia e Zoologia Agrícola, Escola Superior de Agricultura 'Luiz de Queiroz', ESALQ/USP, 13418-900 Piracicaba, SP, Brazil
e-mail: italo@esalq.usp.br

M. Knapp
ICIPE-African Insect Science for Food and Health, PO Box 30772, 00100 Nairobi, Kenya

Introduction

The tomato red spider mite, *Tetranychus evansi* Baker and Pritchard, is an invasive species in Africa causing considerable damage to solanaceous crops (Saunyama and Knapp 2003). In tomato, *T. evansi* is frequently controlled by intensive application of acaricides (Blair 1989). Lepidopteran insect pests and fungal diseases such as tomato late blight, *Phytophthora infestans* (Mont.), are controlled by the use of insecticides and fungicides, respectively.

In several countries, epizootics of *Neozygites floridana* Weiser and Muma (Zygomycetes: Entomophthorales) have been associated with rapid decline in populations of spider mites (Carner and Canerday 1970; Smith and Furr 1975; Boykin et al. 1984), including *T. evansi* on tomato (Humber et al. 1981). The apparent importance of this fungus as a natural control agent in agro-ecosystems suggests that it can be used in conjunction with other strategies in Integrated Pest Management programs for sustainable pest control. *Neozygites floridana* collected in Brazil has been considered as a potential candidate for classical biological control of *T. evansi* in Africa. To maximize the potential of *N. floridana* in controlling *T. evansi* on tomato, it is necessary to incorporate the use of selective pesticides for management of other tomato pests to reduce the negative effect this might have on *N. floridana*.

The direct impact fungicides have on natural epizootics of entomopathogenic fungi has been demonstrated for different species. For example, application of fungicides has been implicated in the reduction of *Neozygites* spp. incidence in the field resulting in host population increases of mainly aphids and mites (Brandenburg and Kennedy 1983; Boykin et al. 1984; Bower et al. 1995; Klingen and Westrum 2007).

Although it might be desirable to conduct field experiments to determine the effect of pesticides on the disease dynamics of the entomopathogenic fungi, field experiments are expensive, time consuming, and often not appropriate to identify specific factors that affect the entomopathogenic fungi. To identify compatibility of pesticides on entomopathogens, laboratory bioassays are therefore usually the first steps in selecting pesticides for use in integrated pest management programs (Morjan et al. 2002).

Studies conducted to determine the inhibitory effects of pesticides on other species of entomophthoralean fungi usually focus on the impact of pesticides on germination of conidia and hyphal growth in culture media containing each pesticide (Hall and Dunn 1959; Jaques and Patterson 1962; Yendol 1968; Boykin et al. 1984). Studies on the effects of pesticides on *N. floridana* have received little attention, mostly because of difficulties associated with the establishment of in vitro cultures of this pathogen (Morjan et al. 2002).

Neozygites floridana produces three types of spores and has a more complex life cycle than the anamorphs of the Ascomycota within the order Hypocreales ("imperfect fungi" formerly in the Deuteromycota). Primary conidia of *N. floridana* are actively discharged from the conidiophores of the mummified host mites, referred to as cadavers. A primary conidium lands on the leaf surface and germinates to form a secondary type of conidium, the infective capilliconidium (Smitley et al. 1986; Oduor et al. 1996; Delalibera et al. 2006). The host needs to come in contact with the capilliconidium to become infected. *Neozygites floridana* also produces resting spores for long-term survival probably when conditions are unfavorable. Therefore, there are many stages of the life cycle that can be affected by application of pesticides.

The aim of this study was to test the effect of fungicides, acaricides and insecticides used in commercial tomato production on sporulation, germination, infectivity and mortality of

T. evansi by *N. floridana* and to describe laboratory methods which can be used for toxicity tests without necessarily growing the fungus on artificial media.

Materials and methods

Fungal production

The *N. floridana* isolate (LQ2) used in this study was initially collected as mummified *T. evansi* mites at a greenhouse of the University of São Paulo in Piracicaba, São Paulo, Brazil, during an epizootic in September 2004. It was stored for 1 year in vials containing silica gel at −10°C, before use in this study. New cadavers were produced by exposing healthy *T. evansi* females to sporulating cadavers from the stock culture. Sporulation was obtained by keeping cadavers at 25°C in darkness on tomato leaf discs (1.2 cm diameter) placed on top of a moist sponge in closed Petri dishes (9 cm diameter) at 100% RH for 16 h. Mites exposed to the sporulating cadavers were then maintained in an incubator at 25°C and 50% RH under natural light–dark regime (12D:12L) and cadavers were collected 3–7 days later for use in the bioassays. The effects of the pesticides were tested by direct application on only newly formed cadavers (not stored ones) and on conidia discharged from them.

Pesticides used

Information on trade names, active ingredients, and types of pesticide, formulations, chemical groups and recommended concentrations of the pesticides are shown in Table 1. Two insecticides, Karate Zeon® 50 CS (Syngenta) and Lannate® BR (Dupont), two acaricides, Omite® 720 EC (Crompton) and Abamex® (Bequisa), and two fungicides, Orthocide® 500 (Arysta Lifescience) and Dithane NT (Dow Agrosciences), were chosen for their frequent use by farmers in tomato crop. Pesticides were used in two concentrations: commercial rate (CR), which is the mean recommended concentration for application in tomato, and 50% of the mean commercial rate (CR/2). All the pesticides were diluted with distilled water amended with 0.05% Tween 80 as a surfactant.

General experimental set up

The effect of pesticides on *N. floridana* was tested using two contamination methods: dipping and spraying. Cadavers, leaf discs or coverslips used in the bioassays were either dipped into, or sprayed with, the pesticides. Different bioassays were conducted to

Table 1 Pesticides used in selectivity studies with *Neozygites floridana*

Active ingredient	Trade name	Type of pesticide	Formulation	Chemical group	Concentration (CR)
Methomyl	Lannate	Insecticide	SC	Carbamate	100 ml/100 l
Lambda-cyhalothrin	Karate	Insecticide	CS	Pyrethroid	40 ml/100 l
Propargite	Omite	Acaricide	EC	Alkyl sulfate	50 ml/100 l
Abamectin	Abamex	Acaricide	EC	Avermectin	75 ml/100 l
Captan	Orthocide	Fungicide	WP	Dicarboximide	240 g/100 l
Mancozeb	Dithane	Fungicide/Acaricide	WP	Dithiocarbamate	300 g/100 l

CR = Mean recommended concentration of the formulated product for application in 100 l of water per ha, EC = Emusifiable concentrate, WP = Wettable powder, SC = Soluble concentrate, CS = Capsulated suspension

determine the effect of pesticides on sporulation, germination of primary conidia, and development of the fungus inside the mite. All experiments were repeated three times.

Effect of pesticides on sporulation

The effect of pesticides on sporulation was evaluated by measuring direct and indirect effects. The direct effects were measured by immersing cadavers into the pesticides (cadaver treatment). The indirect effect was measured by immersing leaf discs into pesticides (leaf treatment) before transferring the cadavers onto these discs. Similarly, direct and indirect effects were tested by spraying cadavers on leaf discs or spraying leaf discs before transferring the cadavers onto them.

Cadaver treatment

Ten cadavers were introduced into microcentrifuge tubes and then 0.5 ml of each pesticide at either concentration (CR or CR/2) amended with 0.05% Tween 80 was added into each tube and agitated for 2 min. The content of each tube was then poured onto filter papers to drain the excess pesticides. Control cadavers were given the same treatments as described above except that they were introduced into distilled water amended with 0.05% Tween 80. After 2 h, the treated cadavers were individually placed on untreated discs of tomato leaves (1.2 cm in diameter) resting on a wet sponge inside a Petri dish (9 cm diameter). The dishes were closed to reach about 100% RH and incubated at 25°C in darkness for 16 h. The number of conidia discharged per mummy was estimated by observing the leaf disc directly under a compound microscope and scoring conidia numbers according to a categorical scale (0: no sporulation, 1: 1–100, 2: 101–500, and 3: >501 conidia).

In a parallel experiment, ten cadavers were placed on a filter paper and sprayed with 2 ml of each pesticide at either concentration using a Potter Precision Laboratory Spray Tower (Burkard Manufacturing, Rickmansworth, Herts, UK), calibrated at 68.95 kPa with a mean deposition of 1.5 mg of residue/cm^2. Sprayed cadavers were air-dried for 2 h and were then transferred individually onto unsprayed tomato leaf discs and processed as described above.

Leaf treatment

Ten leaf discs were individually dipped in each pesticide at either concentrations (CR and CR/2) for 2 min and were then air-dried for 2 h. Similarly, ten leaf discs were sprayed with 2 ml of each pesticide solution. A cadaver taken from the stock culture was then placed in the center of each disc, transferred onto moist sponge in a closed Petri dish and incubated at 25°C in darkness for 16 h, before sporulation was evaluated as described above. Control leaf discs were dipped in or sprayed with distilled water amended with 0.05% Tween 80 and all other experimental procedures were similar to those described above.

Effect of pesticides on germination of primary conidia

Three square photo-etched coverslips (23 × 23 mm) (Electron Microscopy Sciences, Hatfield, PA, USA) with alphanumeric coded squares were immersed into or sprayed with the mean concentration (CR) of each pesticide and air-dried for 2 h before two cadavers were put at the center of each coverslip. The control slides were immersed into distilled water amended in 0.05% Tween 80. The coverslips with cadavers were then transferred onto a sponge soaked in distilled water in a closed Petri dish at 25°C in darkness for 16 h.

Germination of conidia was observed using a compound microscope and the number of germinated and un-germinated conidia in five arbitrarily selected squares within the field of view was recorded using an enumeration counter. Total conidial germination included conidia that were in the process of forming or had already formed secondary conidia or capilliconidia. Percent germination was computed by dividing the number of germinated conidia with the total number of conidia counted in a specified field and multiplying by 100.

Effect of pesticides on infectivity of capilliconidia

Leaf discs with sporulating cadavers—from the study on the effect of pesticides on sporulation by immersion and spraying—were used to test the infectivity of the produced capilliconidia. Only leaf discs with the highest spore numbers (category 3) were selected for the test. Fifteen *T. evansi* females were introduced onto each of the ten leaf discs containing the spores and placed at 25°C in the incubation chamber. Mortality of these mites was checked daily for 7 days.

Effect of pesticides on mortality of *N. floridana* inoculated *T. evansi*

Only Captan and Methomyl were used in this study because they were the only pesticides that did not kill the mites exposed to leaf discs containing these pesticides. Ten leaf discs were immersed into each pesticide in a single concentration (CR) and allowed to dry for 2 h. Fifteen *T. evansi* females were transferred to the treated leaf discs. After 48 h of feeding on the treated leaf discs, the mites were transferred to new leaf discs each with a sporulating *N. floridana* cadaver. These mites stayed for 24 h on these leaf discs for contamination and were then transferred to new and larger leaf discs and observed daily for infection and mortality for 7 days. Control leaf discs were immersed in distilled water amended with 0.05% Tween 80 and all other experimental procedures were as described for the pesticide treatments. The leaf discs were changed after the fourth day. Dead mites were mounted and observed under the microscope for hyphal bodies to confirm that the cause of death was *N. floridana*.

Data analysis

The effects of pesticides on sporulation, germination and infectivity were compared by using a two-way analysis of variance (ANOVA) with treatment, concentration and application method as factors (PROC GLM, SAS Institute 1998). Percentages germination and mortality were arcsine transformed before analysis to homogenize variances. Means were compared using Duncan Multiple Range Test (DMRT) ($P < 0.05$). A pre-planned comparison between treatments was performed separately for each group of pesticide to determine within group treatment effects.

Results

Effect of pesticides on sporulation of *N. floridana*

Cadaver treatment

The negative effect of pesticides on *N. floridana* sporulation was higher when the cadavers were immersed into pesticides than when sprayed ($F_{35,324} = 11.66$, $P = 0.0001$) (Table 2).

Table 2 Sporulation of *Neozygites floridana* from mummified *Tetranychus evansi* females after immersion or spraying with mean recommended concentration (CR) or half that concentration (CR/2) of pesticides

Pesticide	Immersed cadavers			Sprayed cadavers		
	CR/2	CR	Control	CR/2	CR	Control
Methomyl	1.7 ± 0.2bc	1.4 ± 0.2c	2.3 ± 0.2ab	2.6 ± 0.2a	2.3 ± 0.3ab	2.3 ± 0.1ab
Lambda-cyhalothrin	1.8 ± 0.2bc	1.5 ± 0.2c	2.2 ± 0.2ab	2.3 ± 0.3ab	2.3 ± 0.3ab	2.7 ± 0.2a
Propargite	2.5 ± 0.2b	0.3 ± 0.2d	2.5 ± 0.2ab	1.9 ± 0.4bc	1.9 ± 0.4bc	2.5 ± 0.2ab
Abamectin	3.0 ± 0a	1.3 ± 0.4c	2.8 ± 0.1a	1.8 ± 0.4bc	1.6 ± 0.4bc	2.4 ± 0.2ab
Captan	0.6 ± 0.2c	0.3 ± 0.2cd	2.1 ± 0.2ab	0.5 ± 0.2c	0.4 ± 0.2cd	2.4 ± 0.3a
Mancozeb	0.0 ± 0e	0.0 ± 0.0e	1.6 ± 0.2b	0.3 ± 0.2cd	0.1 ± 0.1cd	2.4 ± 0.2a

Numbers of conidia (Mean ± SE) were estimated based on a categorical scale of 0 = 0, 1 = 1–100, 2 = 101–500, and 3 = >501 conidia/mummified mite

Means within columns and rows followed by the same letters are not significantly different (DMRT, $P > 0.05$)

When cadavers were immersed into the pesticides, Lambda-cyhalothrin, Methomyl, Abamectin and Propargite had no effect on sporulation at CR/2 concentration but sporulation was lower at CR. Captan and Mancozeb significantly reduced sporulation both at CR/2 and CR. When cadavers were sprayed with the pesticides, Methomyl, Lambda-Cyhalothrin, Propargite and Abamectin had no effect on sporulation at neither of the concentrations. Cadavers sprayed with Mancozeb and Captan sporulated less both at CR/2 and CR.

Leaf treatment

Cadavers placed on leaf discs that were immersed into Lambda-Cyhalothrin sporulated and produced as many conidia as the control (Table 3). Methomyl, Propargite and Abamectin at CR/2 did not affect sporulation, but at CR these pesticides affected sporulation. Mancozeb and Captan significantly affected sporulation at both CR/2 and CR. When Propargite was sprayed on leaf discs, sporulation was unaffected at the lower concentration, but at CR sporulation was affected. Leaf discs sprayed with CR/2 or CR of Methomyl, Lambda-Cyhalothrin, Abamectin and Captan did not result in differences in sporulation. Mancozeb, however, inhibited sporulation at both CR/2 and CR when leaf discs were sprayed. In general, sporulation was significantly higher when leaf discs were sprayed than when immersed in both Mancozeb and Captan ($F_{11,108} = 11.78$, $P = 0.0001$).

Effect of pesticides on germination of primary conidia

Germination of primary conidia was significantly affected by the pesticides and also by the method of pesticides application: immersion and spray of coverslips (Table 4). Propargite and Mancozeb totally inhibited germination of conidia after immersion of coverslips. When coverslips were sprayed, germination was totally inhibited by Mancozeb and only 7.0 ± 2.0% of primary conidia germinated when sprayed with Propargite. Lambda-Cyhalothrin and Captan also reduced germination in both application methods. Methomyl was the only pesticide that did not affect germination when coverslips were either immersed or sprayed. Overall, germination of primary conidia was significantly higher on coverslips that were sprayed than immersed ($F_{29,60} = 22.51$, $P = 0.0001$).

Table 3 Sporulation of *Neozygites floridana* from mummified *Tetranychus evansi* females on leaf discs immersed or sprayed with mean recommended concentration (CR) or half the mean recommended concentration (CR/2) of pesticides

Pesticide	Immersed leaf discs			Sprayed leaf discs		
	CR/2	CR	Control	CR/2	CR	Control
Methomyl	2.0 ± 0.2b	1.4 ± 0.2d	2.2 ± 0.2b	2.9 ± 0.1a	2.9 ± 0.1a	2.9 ± 0.1a
Lambda-cyhalothrin	1.9 ± 0.2bc	1.7 ± 0.2bc	2.0 ± 0.3bc	2.5 ± 0.3abc	2.1 ± 0.4bcd	2.7 ± 0.2ab
Propargite	3.0 ± 0.0a	1.8 ± 0.4bc	3.0 ± 0.0a	2.1 ± 0.4bc	1.9 ± 0.3cd	2.7 ± 0.2ab
Abamectin	2.9 ± 0.1a	1.9 ± 0.4bc	3.0 ± 0.0a	2.5 ± 0.3abc	2.3 ± 0.3abc	2.3 ± 0.2abc
Captan	0.7 ± 0.3e	0.4 ± 0.2ef	2.2 ± 0.2b	1.7 ± 0.5abc	1.4 ± 0.4cde	2.5 ± 0.2abc
Mancozeb	0.0 ± 0.0g	0.0 ± 0.0g	1.9 ± 0.2bcd	1.0 ± 0.3de	0.7 ± 0.4de	2.5 ± 0.2abc

Numbers of conidia (Mean ± SE) were estimated based on a categorical scale of 0 = 0, 1 = 1–100, 2 = 101–500, and 3 = >501 conidia/mummified mite

Means within columns and rows followed by same letters are not significantly different (DMRT, $P > 0.05$)

Table 4 Effect of pesticides on germination of *Neozygites floridana* primary conidia when cadavers were placed to sporulate on coverslips immersed into or sprayed with mean recommended concentration (CR) of pesticides

Pesticide	Immersed coverslips		Sprayed coverslips	
	CR	Control	CR	Control
Methomyl	78.2 ± 5.8abc	70.2 ± 5.8abc	84.7 ± 2.0a	78.1 ± 7.6a
Lambda-cyhalothrin	42.8 ± 21.7d	70.2 ± 5.8abc	61.3 ± 4.8bc	78.1 ± 7.6a
Abamectin	54.9 ± 11.2 cd	56.6 ± 9.0 cd	57.1 ± 4.2 cd	82.5 ± 4.0a
Propargite	0.0 ± 0.0e	56.6 ± 9.0 cd	7.0 ± 2.0e	82.5 ± 4.0a
Captan	21.4 ± 1.4e	59.4 ± 9.5bcd	66.8 ± 4.1bc	77.7 ± 0.7a
Mancozeb	0.0 ± 0.0e	59.4 ± 9.5bcd	0.0 ± 0.0e	77.7 ± 0.7a

Data are mean percent germination ± SE

Means within columns and rows followed by the same letters are not significantly different (DMRT, $P > 0.05$)

Effect of pesticides on infectivity of capilliconidia

Infectivity of conidia after exposure to pesticides was measured as a function of mortality of the exposed mites. More than half of the mites transferred to leaf discs treated with Propargite, Abamectin, Mancozeb or Lambda-Cyhalothrin died after 1 day indicating a direct effect of the products on the mites and therefore these products were not used in the infectivity test (Table 5). Methomyl and Captan were the only pesticides used to test infectivity when leaf discs were either immersed or sprayed. Lower mortality of fungus-inoculated mites was observed when leaf discs were immersed rather than sprayed with Methomyl and Captan ($F_{9,39} = 10.22$, $P = 0.0001$) regardless of the concentration used, indicating an effect of the pesticide application method. Neither Methomyl nor Captan affected infectivity when leaf discs were sprayed with the pesticides. However, reduced infectivity was seen for leaf discs immersed into pesticides at CR. When cadavers were either immersed or sprayed, all pesticides except Mancozeb (not tested due to insufficient sporulation) were used to test pathogenicity of the sporulating fungus. None of the pesticides reduced the percent of mites killed by *N. floridana* compared to the controls. However, when cadavers were sprayed, mite mortality was higher than when immersed

Table 5 Effect of pesticides on infectivity of *Neozygites floridana* capilliconidia to *Tetranychus evansi* when leaf discs were immersed or sprayed with mean concentration (CR) or half the mean concentration (CR/2) of pesticides

Pesticide	Immersed leaf discs			Sprayed leaf discs		
	CR/2	CR	Control	CR/2	CR	Control
Methomyl	47.3 ± 7.4cd	41.8 ± 4.4d	61.3 ± 6.1bc	76.9 ± 6.4ab	82.6 ± 1.4a	76.9 ± 2.4ab
Captan	48.2 ± 4.9cd	39.5 ± 3.0d	61.3 ± 6.1bc	77.5 ± 6.7ab	77.1 ± 6.0b	76.9 ± 2.4ab

Data represent the percent mortality (mean ± SE) of *T. evansi* 7 days post-inoculation

Means within columns and rows followed by the same letters are not significantly different (DMRT, $P > 0.05$)

Table 6 Effect of pesticides on infectivity of *Neozygites floridana* capilliconidia to *Tetranychus evansi* when mummifeid mites were immersed in or sprayed with mean recommended concentration (CR) or half the mean recommended concentration (CR/2) of pesticides

Pesticide	Immersed cadavers			Sprayed cadavers		
	CR/2	CR	Control	CR/2	CR	Control
Methomyl	56.0 ± 4.0cdef	53.3 ± 3.4cdef	61.3 ± 6.1bcde	70.5 ± 5.8abcd	82.8 ± 5.1a	72.9 ± 3.4abc
Lambda-cyhalothrin	55.6 ± 4.8cdef	53.0 ± 4.6def	61.3 ± 6.1bcde	73.7 ± 7.1abc	77.7 ± 6.1ab	72.9 ± 3.4abc
Captan	40.4 ± 4.7f	46.7 ± 6.7ef	52.0 ± 5.6def	70.4 ± 4.4abcd	77.8 ± 6.2ab	79.0 ± 4.7ab
Abamectin	54.9 ± 6.7cdef	51.3 ± 5.7def	60.3 ± 3.8bcde	76.0 ± 3.4ab	75.3 ± 6.7ab	78.0 ± 5.4ab
Propargite	63.3 ± 4.9bcde	53.3 ± 3.9def	60.3 ± 3.8bcde	68.6 ± 8.9abcd	75.1 ± 7.2ab	78.0 ± 5.4ab

Data represents the percent mortality (mean ± SE) of *T. evansi* 7 days post-inoculation

Means within columns and rows followed by the same letters are not significantly different (DMRT, $P > 0.05$)

into the pesticides ($F_{25,97} = 4.30$, $P = 0.0001$) (Table 6). For all pesticides, mortality did not differ between concentrations CR/2 and CR when cadavers were immersed ($F_{1,8} = 0.16$, $P = 0.70$) or sprayed ($F_{1,8} = 0.20$, $P = 0.67$). Immersion of cadavers into Methomyl at CR resulted in lower mite mortality than sprayed cadavers ($F_{1,8} = 18.17$, $P = 0.0028$).

Effect of pesticides on mortality of *N. floridana* inoculated *T. evansi*

Neither Methomyl nor Captan affected mortality by *N. floridana* on *T. evansi* ($F_{2,27} = 0.18$, $P = 0.84$). Mean mortality of mites that were placed onto leaf discs contaminated with Methomyl, Captan or water (=control) before transfer to leaf discs with sporulating cadavers of *N. floridana* was 73.2 ± 4.1, 76.5 ± 5.0 and 73.2 ± 4.4%, respectively.

Discussion

The detrimental effects of pesticides used to control insects, mites and fungal diseases in commercial tomato production on sporulation, germination and infectivity of *N. floridana* varied as a function of the methods of contamination, chemical nature and concentration. The fungicides Mancozeb and Captan that resulted in the most negative effects on sporulation and germination of *N. floridana* may reduce disease transmission and development of epizootics. Boykin et al. (1984) demonstrated that application of Benomyl and Mancozeb (Dithane) reduced the incidence and efficiency of *N. floridana* in *Tetranychus urticae* Koch

in peanut fields. Brandenburg and Kennedy (1983) also reported a reduced incidence of *N. floridana* in *T. urticae* after application of the fungicides Benomyl and Chlorothalonil and associated this effect to inhibition of conidial germination by these fungicides.

Acaricides such as Propargite, which do not inhibit sporulation but affect primary conidia germination, may have a moderate effect on the fungus in the field compared to those pesticides that inhibit sporulation because the life span of a primary conidium is much shorter than the life span of a mummified mite. However, any pesticide that inhibits the formation of capilliconidia, the only infective spores of *N. floridana*, may have an impact on the overall mite control. The impact of Propargite on *N. floridana* can only be determined conclusively with results from a field experiment.

No effects on infectivity of the capilliconidia was observed from the pesticides after exposure. Seemingly, some pesticides inhibit sporulation or germination of primary conidia, but the capilliconidia produced under the exposure of these pesticides mantain the potential to infect their hosts. Viability of conidia is very important because the power of the fungus to kill its hosts depends on this factor as only viable conidia have the capacity to germinate and adhere to healthy hosts.

It was expected that once the mites feed on pesticide contaminated leaves, they could ingest and accumulate the pesticides that may inhibit vegetative growth of the fungus and reduce mite mortality due to infection. Since the control mites were not subjected to pesticide contaminated leaf discs, higher mortality due to the fungus was anticipated. However, mortality in treatments with the insecticide Methomyl and the fungicide Captan was similar to the mortality in the controls suggesting that the pesticides did not affect fungal development.

The effect of pesticides on *N. floridana* was higher when immersed than sprayed and this is probably associated with the amount of the product that the fungus is exposed to, despite being of equal concentration. Differences between the controls observed in the germination study were attributed to independent incubation of control lots together with each pesticide group. It is also possible that Tween 80, the surfactant used in the two controls, could have been the cause of differential germination because more of the products could be retained on the coverslips when they were immersed than sprayed.

Although the spray tower method may give comparable results to field application of pesticides, the equipment may not be readily available in many laboratories, as a result, its use in pesticide testing may be limited. However, the effect of direct immersion of leaf discs or cadavers into pesticide solutions is stronger and may not reflect a field situation, but it represents a rapid method to assess both direct and indirect effects of these pesticides on the fungus and may assist in making quick decisions on the pesticides to be applied during pest attack. Also, if a product is considered compatible with the pathogen in this laboratory method (worst scenario) it may warrant selectivity in the field. The same line of thought applies to differences observed between maximum concentration and half the concentrations recommended for field application. A higher concentration in the laboratory that does not affect the fungi has higher chances of being non-toxic in the field than a low concentration that is toxic under laboratory conditions.

An important consideration in the use of laboratory methods is the determination of how accurately they represent field conditions. However, it is unlikely that pesticides which affect the fungus at low concentrations in in vitro tests will fail to produce effects under recommended field concentrations. Given that high toxicity of chemical products in laboratory experiments does not always reveal high toxicity in the field, the laboratory tests are useful and indicate the possibility of the effects that may occur in the field (Alves et al. 1998). Field applications of pesticides usually achieve less-than-perfect coverage, perhaps providing spatial refugia for entomopathogenic fungi. Apart from the presence of refuges,

spatial heterogeneity, photodegradation and removal by rain could be possible reasons for reduced exposure of the fungus to toxic compounds and this could be the reason why in vitro studies and field studies may not always produce similar results (Jaros-Su et al. 1999).

The effect of pesticides on *Neozygites* spp. has been studied mostly in the field (Boykin et al. 1984; Wells et al. 2000). Field studies are usually limited to a small number of products and it takes a long time to reveal any differences in the infection levels or the density of propagules in the soil. For this reason, there is need for the generation of laboratory data on the effect of pesticides on specific aspects of the fungus such as sporulation, germination and viability. However, this has been hampered by lack of a defined protocol to test this fungus without growing it on artificial media. The laboratory tests described here simulate an in vivo situation and allow the flexibility of dosing a pesticide under controlled conditions. These tests also bypass the process of growing *N. floridana* on artificial media. The results obtained using these methods indicate that the insecticide Methomyl, and the acaricide Abamectin produced varied effects on *N. floridana* with both inhibiting sporulation at CR where leaf discs were immersed but not when sprayed. Methomyl also reduces infectivity when leaf discs are immersed and not when sprayed. Thus, these pesticides may not affect the inoculum potential of the *N. floridana* in the field and may be compatible with conservation strategies of pest control. Lambda-Cyhalothrin has a mild effect on conidia germination of *N. floridana* when the coverslips are immersed and this effect substantially reduces when they are sprayed. The acaricide Propargite strongly affects germination just like the fungicides Mancozeb and Captan both of which affect sporulation and may not be compatible with *N. floridana*.

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Natural enemies of mass-reared predatory mites (family Phytoseiidae) used for biological pest control

Susan Bjørnson

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Abstract Predatory mites of the family Phytoseiidae are valued natural enemies that provide effective pest control in greenhouses and on agricultural crops. Mass-reared phytoseiids are occasionally associated with microorganisms and although their effects are not always apparent, some are pathogenic and reduce host fitness. Invertebrate pathogens are encountered more frequently in mass production systems than in nature because rearing environments often cause overcrowding and other stresses that favour pathogen transmission and increase an individual's susceptibility to disease. Although unidentified microorganisms have been reported in phytoseiids, bacteria and microsporidia have been detected with considerable frequency. The bacterium *Acaricomes phytoseiuli* is associated with an accumulation of birefringent crystals in the legs of *Phytoseiulus persimilis* and infection reduces the fitness of this spider mite predator. *Wolbachia*, detected in *Metaseiulus occidentalis* and other phytoseiids, may cause cytoplasmic incompatibilities that affect fecundity. However, the effects of *Rickettsiella phytoseiuli* on *P. persimilis* are unknown. Microsporidia are spore-forming pathogens that infect *Neoseiulus cucumeris*, *N. barkeri*, *M. occidentalis* and *P. persimilis*. Microsporidia cause chronic, debilitating disease and these pathogens often remain undetected in mass-rearings until a decrease in productivity is noticed. Routine screening of individuals is important to prevent diseased mites from being introduced into existing mass-rearings and to ensure that mite populations remain free from pathogens. The means by which bacteria and microsporidia are detected and strategies for their management in phytoseiid mass-rearings are discussed.

Keywords *Amblyseius* · *Metaseiulus* · *Neoseiulus* · *Phytoseiulus* · Phytoseiids · Microorganisms · Bacteria · Microsporidia · Disease

S. Bjørnson (✉)
Department of Biology, Saint Mary's University, 923 Robie Street, Halifax, NS, Canada B3H 3C3
e-mail: susan.bjornson@smu.ca

Introduction

The spider mite predator, *Phytoseiulus persimilis* Athias-Henriot, was the first mass-produced natural enemy to be made commercially available for biological pest control in Europe (van Lenteren et al. 1997). Since its introduction almost 40 years ago, phytoseiids have gained recognition for their importance as natural enemies of thrips, whiteflies and spider mites. Several phytoseiids are now available for pest control.

Phytoseiids, like other mass-produced and field-collected arthropods, are occasionally associated with microorganisms. Although some microorganisms are known to affect host fitness, the role of others has yet to be determined. Diseases, and the microorganisms that cause them, are encountered more frequently in mass production systems than in nature because rearing environments often cause overcrowding and this favours pathogen transmission (Goodwin 1984). Overcrowding may also lead to temporary starvation or other stresses, which are thought to increase disease susceptibility (Goodwin 1984; Kluge and Caldwell 1992).

Once detected, the identification of a particular microorganism is essential if one is to determine its significance. Not all microorganisms are capable of causing disease; therefore, the mere presence of a particular microbe is often insufficient for determining a cause and effect relationship. Depending on the microorganism that is detected, a conclusive diagnosis may involve simple or complex laboratory procedures and, in many cases, the satisfaction of Koch's Postulates.

This summary will focus on the types of natural enemies associated with phytoseiids, their effects on host fitness and efficacy, the means by which disease-causing microbes are detected, and strategies for their management in mass production systems. Further information may be found in comprehensive reviews regarding the parasites, pathogens and diseases of mites (Poinar and Poinar 1998; van der Geest et al. 2000) and pathogens of mass-produced natural enemies and pollinators (Bjørnson and Schütte 2003).

Unidentified microorganisms

Hess and Hoy (1982) reported two unidentified microorganisms in *Metaseiulus occidentalis* (Nesbitt) that are associated with two, distinct pathologies. Some adult females have extruding rectal plugs that often stick to the substrate and prevent the affected mites from moving. Other mites become thin and translucent and high mortality is observed among immature mites. In both cases, affected females fail to oviposit or produce few eggs. One type of microorganism was found in all mites examined but it is not considered to be detrimental to *M. occidentalis*. However, a second, rickettsia-like microorganism found in the ovaries of some females is associated with rectal plug formation. Both pathologies are observed when mites are reared under crowded conditions.

Viruses

Šuťáková and Rüttgen (1978) observed virus-like particles in *P. persimilis* but these are present only in the cytoplasm of cells infected with *Rickettsiella phytoseiuli*. The authors conclude that the virus inadvertently infects *P. persimilis* when virus-contaminated food is ingested. Mites infected with *R. phytoseiuli* and the virus show no external signs associated with infection and host mortality is not affected (Šuťáková and Rüttgen 1978; Šuťáková

1988). In other studies, non-occluded viruses were observed in *Neoseiulus* (formerly *Amblyseius*) *cucumeris* (Oudemans) and *P. persimilis* (Steiner 1993; Bjørnson et al. 1997) but their effects are unknown.

Bacteria

Hoy and Jeyaprakash (2005) sequenced four bacterial genomes from *M. occidentalis* and three additional genomes from the spider mite *Tetranychus urticae* Koch, which is often prey for *M. occidentalis* and other phytoseiids. Although *Wolbachia* are the only microorganisms detected in both *M. occidentalis* and *T. urticae*, there are no genetic differences in the rRNA sequences of these microorganisms. *Wolbachia* are thought to cause cytoplasmic incompatibility in *M. occidentalis* (Johanowicz and Hoy 1996) and although other microorganisms have been detected in this predator (Hess and Hoy 1982; Jeyaprakash and Hoy 2004; Hoy and Jeyaprakash 2005), they may or may not affect host fitness. *Wolbachia* are also detected in other phytoseiids (Steiner 1993) but their effects on host fitness have not been established.

Rickettsiella phytoseiuli was detected in *P. persimilis* from a laboratory mass-rearing in the USSR (Šuťáková and Rüttgen 1978) but not in predators that were examined from other sources (Šuťáková and Arutunyan 1990). *R. phytoseiuli* is a pleomorphic (able to assume different forms) bacterium that was detected in all mites examined. It is present in most tissues of adult mites and is particularly abundant in the dorsal body region. However, this bacterium does not infect immature *P. persimilis*.

The bacterium *Acaricomes phytoseiuli* causes specific disease symptoms in adult female *P. persimilis* known as 'non-responding syndrome' (Pukall et al. 2006; Schütte et al. 2006a). Although molecular screening revealed that *A. phytoseiuli* is a rather common pathogen in European populations of *P. persimilis*, the bacterium has not been detected in mites from other continents (Gols et al. 2007). Mites with non-responding syndrome do not react as strongly to herbivore-induced plant volatiles as do uninfected mites (Schütte et al. 2006a). This aberrant behaviour may develop in unaffected mites when they are exposed to live, non-responsive females or their faeces (Schütte et al. 2006b).

Although normal in size after mating, the majority of female predators (76%) from the non-responding population becomes dorso-ventrally flattened, has reduced oviposition rates and dies prematurely. Affected mites consume few prey and tend to disperse from areas where prey is located. Mortality is higher for non-responsive mites than for *P. persimilis* that respond to herbivore-induced plant volatiles and some affected females have an accumulation of birefringent, dumbbell-shaped crystals in their legs. Unaffected (responsive) females may accumulate similar crystals in their bodies but these are restricted to the Malpighian tubules and rectum.

Arutunjan (1985) described dumbbell-shaped entities in the gut of *P. persimilis* as bacteria. These entities are similar in morphology to dumbbell-shaped crystals that were reported by Bjørnson et al. (1997) and Schütte et al. (2006a). The accumulation of crystals in the digestive tract is associated with white coloration of the opisthosoma and is observed when live mites are examined by stereomicroscopy. Mites may have a white dot in the distal opisthosoma (when crystals accumulate in the rectum) or white stripes along the sides of the body in the region of the Malpighian tubules. Occasionally predators with both symptoms are observed (Bjørnson and Raworth 2003) and in some cases, crystals accumulate in the legs (Schütte et al. 2006a). The proportion of white opisthosomal coloration in *P. persimilis* increases when predators are fed prey mites (*T. urticae*) that fed on plants

treated with high concentrations of 20–20–20 (N–P–K) fertilizer. Furthermore, some symptomatic mites become asymptomatic after wastes are egested from the anus (Bjørnson and Raworth 2003). Birefringent crystals are thought to be excreted under normal circumstances but in some cases, crystal accumulation is linked to reduced fecundity and poor performance (Bjørnson et al. 2000; Schütte et al. 2006a), particularly when crystals accumulate in the legs (Schütte et al. 2006a).

Discoloration of the distal opisthosoma has been observed in *Euseius hibisci* (Chant) when fed a diet consisting only of citrus red mites, *Panonychus citri* (McGregor) (see Tanagoshi et al. 1981). The guts of affected female mites are dark red and this discoloration is attributed to incomplete digestion of prey. Predators become less robust at successive moults and adult females are dorso-ventrally flattened and produce few or no eggs. The authors conclude that *E. hibisci* is not an obligate predator of *P. citri* and requires pollen as well as prey mites for food.

In many cases, bacteria are readily detected by light microscopy. Simple and differential staining provides some information regarding bacterial shape, size and morphology but further tests are required for taxonomic identification (see Pukall et al. 2006). Transmission electron microscopy and molecular techniques can be used to detect and identify bacteria (*Wolbachia* and *Rickettsia*) that are too small to be observed by light microscopy (see Jeyaprakash and Hoy 2004; Hoy and Jeyaprakash 2005). Antibiotics are used to eliminate *Wolbachia* from insect parasitoids (Dedeine et al. 2001; Lundgren and Heimpel 2003) but the efficacy of such remedies in mass-reared phytoseiids has not been investigated.

Microsporidia

Microsporidia are spore-forming, intracellular pathogens that cause sub-lethal and debilitating disease. Microsporidian spores may be transmitted both horizontally (from one individual to another) or vertically (from parent to offspring) and are somewhat resistant to harsh environmental conditions (Maddox 1973). Mass-reared arthropods are often confined to small areas and high host population densities favour pathogen transmission. Microsporidia may remain undetected in mite colonies because symptoms are not usually associated with infection. These pathogens may be detected once predatory mites fail to thrive and a decrease in their productivity is noticed.

Microsporidia reduce the productivity of mass-reared *N. cucumeris* and *N. barkeri* (Hughes) (Beerling and van der Geest 1991). These predators are commercially available for controlling western flower and onion thrips [*Frankliniella occidentalis* (Pergande) and *Thrips tabaci* Lindeman], respectively. Symptoms of infection (sluggishness, swollen and whitish bodies) are observed only in heavily infected individuals when spores are abundant. The organs of these individuals are occluded when whole mounts of mites are examined by light microscopy. Prey mites (*Acarus siro* L. and *Tyrophagus putrescentiae* Schrank) are also infected. Symptoms are similar to those observed for *N. cucumeris* and *N. barkeri* but less pronounced. Based on spore dimensions and the hosts infected, three microsporidia are thought to infect both predatory and prey mites of this production system (Beerling and van der Geest 1991; Beerling et al. 1993).

Beerling and van der Geest (1991) conclude that vertical transmission plays an important role in pathogen transmission in mass-rearings of *N. cucumeris* and *N. barkeri* and suggest several possible means for successful horizontal transmission. These include: direct contact with spores that are liberated into the environment, direct contact between healthy and diseased individuals, and transmission through cannibalism or grooming.

Host fitness of the spider mite predator, *M. occidentalis*, is reduced when laboratory colonies are infected with the microsporidium, *Oligosporidium occidentalis*. Microsporidia-infected mites do not live as long or produce as many eggs as uninfected mites and microsporidiosis results in male-biased sex ratios (Olsen and Hoy 2002). *O. occidentalis* produces two types of spores: one is common in immature and young adult mites whereas the other is found in eggs and in older adults. The first spore type is slightly smaller than the latter and is thought to be important for autoinfection (re-infection of the same host) and transovarial (vertical) transmission of the pathogen. The second spore type is thought to be transmitted horizontally (Becnel et al. 2002). Cannibalism of eggs and immatures by *M. occidentalis* adult females provides a route for pathogen transmission (Olsen and Hoy 2002). Microsporidian spores and other developmental stages infect several host tissues but there are no external signs associated with infection (Becnel et al. 2002). Spider mites (*T. urticae*) are not infected with the pathogen.

Three microsporidia were found in the spider mite predator *P. persimilis* from three commercial sources (Bjørnson and Keddie 2000). Microsporidia reduce the fecundity, longevity and prey consumption of infected *P. persimilis* females (Bjørnson and Keddie 1999). As is the case for *M. occidentalis*, sex ratios of microsporidia-infected *P. persimilis* are male-biased and although several host tissues are infected, there are no overt external signs or symptoms associated with infection (Bjørnson and Keddie 2001). In some cases, microsporidia may reduce the performance of predators (Bjørnson and Keddie 1999; Olsen and Hoy 2002) and may ultimately prevent predator populations from becoming established in new environments.

One of the microsporidian pathogens in *P. persimilis* is transmitted vertically and may become prevalent within mass-rearings over a short time (Bjørnson and Keddie 2001). Horizontal transmission occurs through direct contact but this is not observed frequently under laboratory conditions. Prey mites (*T. urticae*) are not infected with microsporidia and are unlikely to play a role in pathogen transmission (Bjørnson and Keddie 2001).

Microsporidia are also known to infect *A. siro* and *T. putrescentiae* in phytoseiid mass-rearings (Beerling et al. 1993; Larsson et al. 1997) but horizontal transmission of microsporidia from prey to predatory mites has not been demonstrated. Even if microsporidia from infected prey mites prove to be host specific and are not transmitted to predators, it is important to ensure that prey mite colonies remain free from these pathogens. Microsporidiosis may affect the vigour of prey mites and the sustainability of phytoseiid colonies that depend on prey mites for food.

Routine examination of infected phytoseiids by light microscopy can be labour-intensive and require some expertise but it is a reliable and relatively inexpensive means of detecting microsporidian spores. Microsporidia may be present in only a few individuals when pathogen prevalence is low; therefore, the examination of many individuals from a particular colony may be necessary to detect the pathogen. Smear preparations are typically made from whole mites that are air-dried, fixed in methanol and stained in buffered Giemsa prior to their examination by light microscopy.

Screening may be used as a means to isolate healthy individuals and establish microsporidia-free colonies. First, parent females are isolated and allowed to produce eggs and progeny, which are also isolated. When vertical transmission of a microsporidium is high, as may be the case in *M. occidentalis* and *P. persimilis* mass-rearings (Bjørnson and Keddie 2001; Olsen and Hoy 2002), random examination of some of these progeny over a prolonged period will help determine if the parent female is infected. As a final measure, each parent female is examined for microsporidian spores to verify that her remaining progeny are pathogen-free. In this way, uninfected mites may be isolated from infected ones and

microsporidia-free colonies may be established from the uninfected progeny. This technique of separating uninfected individuals from infected ones is referred to as Pasteur's method (Tanada and Kaya 1993). Although other methods for removing microsporidia from arthropods have proven successful (Olsen and Hoy 2002), the methodology introduced by Pasteur remains the only means for definitively removing microsporidia from arthropod colonies with low levels of infection.

Another means of detecting microsporidia in phytoseiids involves the use of monoclonal antibodies for ELISA testing (Beerling et al. 1993). Although immunoassays may be efficient for detecting pathogens in mass-reared arthropods, the use of ELISA testing requires specialized equipment and may be costly to develop.

In some cases, microsporidia may be reduced or eliminated by treating infected arthropods with chemicals or heat treatments (Hsiao and Hsiao 1973; Geden et al. 1995; Olsen and Hoy 2002). Although antimicrosporidial agents (benzimidazole) have been used for controlling microsporidia in insects with variable success, chemical compounds do not provide effective control of microsporidia in *P. persimilis* (Bjørnson 1998). Further studies may prove fruitful; however, chemical compounds may not be well suited for controlling microsporidia in phytoseiids. Chemicals are usually added to artificial diets or sugar solutions but some arthropods (particularly phytoseiids) cannot be reared successfully on artificial diets. Furthermore, it is difficult to determine how much of the chemical agent is consumed when chemicals are added to food that is eaten.

Heat treatments are successful for reducing infection in *M. occidentalis* (Olsen and Hoy 2002). The number of viable microsporidian spores is reduced when microsporidia-infected mites are reared at high temperatures (32–35°C) for several days. Under these conditions, spores that remain in the host tissues are thought to become non-viable because all subsequent eggs deposited by heat-treated females are microsporidia-free. Although heat treatments are successful for eliminating microsporidia from *M. occidentalis*, this technique may be of limited value when used to control microsporidia in other phytoseiids. The mortality of *M. occidentalis* was low (~20%; Olsen and Hoy 2002) when individuals were reared at high temperatures; however, *P. persimilis* does not survive well at high temperatures and heat treatments are not effective for controlling microsporidia in this predator (Bjørnson 1998). Spore viability is dependent on environmental factors, including temperature, humidity, and exposure to ultraviolet light (Maddox 1973). The use of heat treatments as a means to reduce microsporidiosis in *M. occidentalis* (Olsen and Hoy 2002) provides evidence that pathogen development and spore viability may be reduced when rearing conditions are altered. Sanitation of rearing facilities and equipment also helps reduce pathogen transmission.

Conclusion

Although many factors influence the outcome of a particular biological control program, the use of pathogen and parasitoid-free natural enemies is the foundation for success. Invertebrate pathogens are often overlooked in scientific studies and in mass-production systems when things go awry. It is essential to use pathogen-free beneficial arthropods in scientific studies if quality control testing is to have meaning and to avoid the misinterpretation of data (Goodwin 1984).

Not all microorganisms are pathogenic; therefore, it is important to correctly identify all microorganisms and determine their impact on host fitness. Both bacteria and microsporidia have been reported from mass-reared phytoseiids and some of these cause subtle

symptoms that may be overlooked. Quarantine of introduced or newly-acquired arthropods, in combination with routine microscopic examination of field-collected specimens (or specimens otherwise introduced into a mass rearing), is recommended so that invertebrate pathogens are not inadvertently introduced into existing arthropod colonies (Goodwin 1984; Bjørnson and Keddie 1999).

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Verified and potential pathogens of predatory mites (Acari: Phytoseiidae)

Conny Schütte · Marcel Dicke

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Abstract Several species of phytoseiid mites (Acari: Phytoseiidae), including species of the genera *Amblyseius*, *Galendromus*, *Metaseiulus*, *Neoseiulus*, *Phytoseiulus* and *Typhlodromus*, are currently reared for biological control of various crop pests and/or as model organisms for the study of predator–prey interactions. Pathogen-free phytoseiid mites are important to obtain high efficacy in biological pest control and to get reliable data in mite research, as pathogens may affect the performance of their host or alter their reproduction and behaviour. Potential and verified pathogens have been reported for phytoseiid mites during the past 25 years. The present review provides an overview, including potential pathogens with unknown host effects (17 reports), endosymbiotic *Wolbachia* (seven reports), other bacteria (including *Cardinium* and *Spiroplasma*) (four reports), cases of unidentified diseases (three reports) and cases of verified pathogens (six reports). From the latter group four reports refer to Microsporidia, one to a fungus and one to a bacterium. Only five entities have been studied in detail, including *Wolbachia* infecting seven predatory mite species, other endosymbiotic bacteria infecting *Metaseiulus* (*Galendromus*, *Typhlodromus*) *occidentalis* (Nesbitt), the bacterium *Acaricomes phytoseiuli* infecting *Phytoseiulus persimilis* Athias-Henriot, the microsporidium *Microsporidium phytoseiuli* infecting *P. persimilis* and the microsporidium *Oligosproridium occidentalis* infecting *M. occidentalis*. In four cases (*Wolbachia*, *A. phytoseiuli*, *M. phytoseiuli* and *O. occidentalis*) an infection may be connected with fitness costs of the host. Moreover, infection is not always readily visible as no obvious gross symptoms are present. Monitoring of these entities on a routine and continuous basis should therefore get more attention, especially in commercial mass-production. Special attention should be paid to field-collected mites before introduction into the laboratory or mass rearing, and to mites that are exchanged among rearing facilities. However, at present general pathogen monitoring is not yet practical as effects of many entities are unknown. More research effort is needed concerning verified and potential pathogens of commercially reared arthropods and those used as model organisms in research.

C. Schütte (✉) · M. Dicke
Laboratory of Entomology, Wageningen University, P.O. Box 8031,
6700 EH Wageningen, The Netherlands
e-mail: conny.schuette@telfort.nl

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Introduction

Several species of phytoseiid mites, including species of the genera *Amblyseius*, *Galendromus*, *Metaseiulus*, *Neoseiulus*, *Phytoseiulus* and *Typhlodromus*, are currently reared for biological control of pests, including spider mites (*Tetranychus* spp.) and thrips (*Thrips tabaci* Lindeman and *Frankliniella occidentalis* Pergande) in protected crops, outdoor vegetables, fruit and other horticultural crops (van Lenteren 2003a, b). Phytoseiid predatory mites include specialists such as *Phytoseiulus persimilis* Athias-Henriot, which attack spider mites (*Tetranychus* spp.), selective predators such as *Neoseiulus* (*Amblyseius*) *californicus* (McGregor) and generalists such as *Neoseiulus* (*Amblyseius*) *cucumeris* (Oudemans), that prey on microarthropods but can reproduce on a pollen diet and utilise plant exudates, honeydew and fungi as food supplements (McMurtry and Croft 1997). Among the 30 species that, by the beginning of this century, are being produced in commercial insectaries on a large scale are four phytoseiid species (van Lenteren 2003a, b).

The success of biological control programmes is, among other factors, dependent on the health of the beneficials that are used. In several cases reports of poor performance in mass-reared phytoseiid mites have raised questions regarding their quality and efficacy in biological control (Steiner 1993a, b; Steiner and Bjørnson 1996; Bjørnson et al. 2000; Raworth and Bjørnson 2002; Blümel and Hausdorf 2002) and have stimulated research in mite pathology (Poinar and Poinar 1998; van der Geest et al. 2000; Bjørnson and Schütte 2003). Moreover, phytoseiid mites are used in several research groups for the study of predator–prey interactions and foraging behaviour (Yao and Chant 1990; Margolies et al. 1997; Dicke et al. 1998; Zemek and Nachman 1999; Janssen 1999; Schausberger and Croft 2000; Maeda et al. 2001; Skirvin and Fenlon 2003a, b). Pathogens may also alter the behaviour of their host (Horton and Moore 1993), thereby influencing outcomes of behavioural research. Hence, care should be taken to maintain healthy laboratory stocks.

Verified and potential pathogens have been reported in phytoseiid mites collected from the field (Furtado et al. 1996), from those currently mass-produced for biological pest control (Beerling and van der Geest 1991a, b; Bjørnson and Keddie 2000; Gols et al. 2007) and from laboratory populations (Hess and Hoy 1982; Becnel et al. 2002; Schütte et al. 2008b; Pukall et al. 2006; Gols et al. 2007). For the latter two cases it could not be determined whether the entities originated from field-collected natural enemies or arose in mass-rearing systems as a result of intense and continuous rearing under laboratory conditions. Mass-reared host populations may be more susceptible to diseases than field populations, as genetic variation is lower and immune responses may be compromised by stress factors including sub-optimal climatic conditions, starvation and overcrowding (Lighthart et al. 1988; Sikorowski and Lawrence 1994). Moreover, in mass-production of arthropods climatic conditions may be better suited for pathogens and horizontal pathogen transmission may be more effective than in natural situations (Sikorowski and Lawrence 1994). These factors may thus enhance disease incidence and the development of novel diseases and/or virulent pathotypes in mass-reared populations. The following review of verified and potential pathogens in phytoseiid mites includes cases with unknown host effects, cases of infection with endosymbiotic bacteria, cases of unidentified diseases and cases of identified diseases, with known pathologies and transmission modes.

Viruses

General characteristics of viruses in insects and mites

Viruses may be defined as biological macromolecules that have the ability to multiply within living cells. They are reported from mites and virtually every insect order and are the smallest of all entomopathogens. These pathogens, comprised of genomic RNA or DNA bound to a protein coat (capsid), are considered the simplest entities capable of replication (Boucias and Pendland 1998). Viral diseases are one of the most widely investigated infections in insects (Tanada and Kaya 1993). Some viruses are occluded at random in proteinaceous occlusion bodies that can be detected under the light microscope, whereas most non-occluded viruses can be detected only with the aid of the electron microscope (Lacey 1997).

In general, infection occurs after viruses have been ingested, but transmission may occur via the host egg (=transovarially), through natural body openings (for example spiracles) or through wounds (Tanada and Kaya 1993). Diagnostic features considered as general characteristics of viral infection in insects include: coloration (white, yellow, light blue, iridescent blue, green, purple or orange) of the gut, the fat body or the entire body, blackening of the body after death, weakening of the outer skin leading to rupturing and release of liquefied body contents (Evans and Shapiro 1997). Infected individuals may show reduced feeding, poor reproduction performance, extended development, extremely extended longevity, body paralysis or lethal sensitivity to CO_2 (Evans and Shapiro 1997). Behavioural changes of insects infected by viruses include: changes in level of activity (wandering behaviour) and changes of microhabitat preference, such as elevation seeking behaviour (="tree-top" diseases), movement to exposed locations and diurnal behaviour of nocturnal insects (Horton and Moore 1993).

Viruses of phytoseiid mites

Six reports exist on unidentified viruses of phytoseiid mites (Table 1). In all cases virus-like particles were detected in electron microscopic studies, but host effects have not been studied. Unidentified, non-occluded virus-like particles were observed in the yolk of developing eggs inside *N. cucumeris* females (Bjørnson et al. 1997). Also gravid *P. persimilis* females carried unidentified, non-occluded virus-like particles in the yolk of developing eggs (Steiner 1993a; Bjørnson et al. 1997).

Table 1 Viruses recorded in phytoseiid mites

Pathogen	Phytoseiid host/origin[a]	Symptoms	References
Non-occluded virus	*Neoseiulus cucumeris*/c	Unknown	Bjørnson et al. (1997)
Non-occluded virus	*Phytoseiulus persimilis*/c	Unknown	Steiner (1993a), Bjørnson et al. (1997)
Virus-like particles	*Phytoseiulus persimilis*/l	Unknown	Šut'áková and Rüttgen (1978)
Virus-like particles (type 1)	*Metaseiulus occidentalis*/l	Unknown	Poinar and Poinar (1998)
Virus-like particles (type 2)	*Metaseiulus occidentalis*/l	Known	Poinar and Poinar (1998)
Virus-like particles (type 3)	*Metaseiulus occidentalis*/l	Known	Poinar and Poinar (1998)

[a] Origin: c, commercial population; l, laboratory population

Phytoseiulus persimilis infected with *Rickettsiella phytoseiuli* contained non-occluded virus-like particles, which both were abundant and visible in the dorsal part of the body, immediately below the cuticle (Šut'áková and Rüttgen 1978). The authors report an interaction of both entities: viruses were only present in the cytoplasm of cells infected with *R. phytoseiuli* and morphological and structural changes were induced in *R. phytoseiuli* when the host was also carrying the virus-like particles (Šut'áková and Rüttgen 1978).

Three separate types of non-occluded icosahedral virus-like particles were detected in ultrastructural micrographs of *M. occidentalis*. The predatory mites originated from populations showing the following disease symptoms: adult female predators had a lower egg production, reduced longevity, died suddenly and had a paralyzed appearance after death (Poinar and Poinar 1998). The authors clearly state that particles located in the epithelial cells (diameter = 47 nm, electron dense core 35 nm) were not associated with any particular disease symptom. These particles were similar to particles found in epithelial cells of healthy as well as diseased prey mites *Tetranychus urticae* Koch (Poinar and Poinar 1998). A second type of particles (diameter = 38 nm, electron dense core 30 nm) was present in the gut cells. These particles were found in large numbers in the midgut nuclei and as free virions in the cytoplasm of the gut cells and the midgut lumen (Poinar and Poinar 1998). The third type of particles (diameter = 45 nm, electron dense core 35 nm) was only present in the gut tissue. The authors do not give any detailed information whether the latter two types of viruses were only found in predatory mites showing disease symptoms. They also did not perform experiments to show whether these viruses are the primary source of infection or rather secondary invaders. However they suggest that viruses may be important disease agents in mites and that they may be present as latent as well as overt infections (Poinar and Poinar 1998).

Bacteria

General characteristics of bacteria in insects and mites

Bacteria are unicellular prokaryotes, their genetic information being contained within a single, double-stranded DNA molecule and small self-replicating DNA molecules termed plasmids or prophages (Boucias and Pendland 1998). Many bacteria are opportunistic pathogens that may exist in nature as saprophytes and may become pathogenic if conditions are favourable. Others are more fastidious and can grow only in the appropriate host (Boucias and Pendland 1998).

Bacterial pathogens invade their hosts mostly through the mouth and digestive tract. Less often, they are transmitted through the egg, trachea or wounds in the integument (Tanada and Kaya 1993). Upon invasion, bacterial pathogens may develop as intracellular pathogens (Rickettsiaceae) or extracellular pathogens (many opportunistic bacteria). Bacterial infections may be classified as (1) bacteremia, when bacteria multiply in the hemolymph of the host without producing toxins; (2) septicaemia, when bacteria multiply in the hemocoel and may produce toxins and kill the host; or (3) toxaemia, when bacteria stay confined to the gut lumen where they produce toxins (Tanada and Kaya 1993).

Diagnostic features considered as general characteristics of bacterial infection include: distinct colour changes (white, red, amber, black or brown), reduced feeding, stopping of feeding, excretion of diarrhoea-like feces, vomiting, weakening of the outer skin, degeneration of internal tissues, cadavers becoming black, odiferous, shrivelled, dry and hard (Tanada and Kaya 1993; Lacey 1997).

The vast majority of research on bacterial insect pathogens over the past 30 years has focused on the toxin-producing *Bacillus* species (Boucias and Pendland 1998). However, studies on the effects of *ß*-exotoxin from *Bacillus thuringiensis* on phytyoseiid mites are not included in the present review as they do not represent a pathogen in the true sense of the word (for a review see van der Geest et al. 2000). Only very little work has been done on other bacterial pathogens. This is mainly due to the fact that bacteria isolated from insects that have been described as opportunistic pathogens belong to genera containing species that may infect plants and vertebrates, which makes them less interesting for the development as microbial control agents (Boucias and Pendland 1998). Several entomopathogenic species have been identified in the genus *Serratia* including *S. marcescens*. Various entomopathogenic strains of *S. marcescens* are characterised by the production of enzymes and exocellular toxins. However, it is still unclear whether this pathogen is able to actively invade its host. In many cases diseases have been associated with poor sanitation and crowded rearing conditions (Boucias and Pendland 1998).

Bacteria belonging to the family Rickettsiaceae are obligately intracellular and multiply in eukaryotic cells. Entomopathogens of this group belong to the genera *Rickettsia*, *Rickettsiella* and *Wolbachia* (Boucias and Pendland 1998). Members of the genus *Rickettsiella* are common pathogens, whereas those of the genus *Wolbachia* are seldom pathogenic in the true sense but have evolved various means to manipulate their hosts in order to enhance their own transmission (see Stouthamer et al. 1999).

The genus *Rickettsiella* is comprised of a heterogeneous group of bacteria, all members being highly fastidious arthropod pathogens. A lack of homology has been demonstrated for certain members of this genus, suggesting the eventual revision of this group (Boucias and Pendland 1998). *Rickettsiella* have developmental cycles involving the production of various cell phenotypes. The infectious particle is a small, dense rod or disc-shaped cell. All species are transmitted by feeding or through wounds. Many *Rickettsiella* undergo extensive replication in the fat body following ingestion and penetration of the alimentary tract. At present relatively few species associated to insects have been found (Boucias and Pendland 1998). Rickettsial infections may induce prominent behavioural changes in the host, including elevation-seeking behaviour and changes in temperature preference (Horton and Moore 1993).

Wolbachia are common cytoplasmic symbionts of insects, crustaceans, mites and filarial nematodes (see Stouthamer et al. 1999). They are rarely pathogenic but may manipulate the host biology by inducing parthenogenesis (whereby infected females exclusively produce daughters), feminisation (whereby infected genetic males reproduce as females), male-killing (whereby infected male embryos die, while female embryos develop into infected females), cytoplasmic incompatibility (unidirectional in its simplest form: whereby the crossing of an uninfected female and an infected male may result in embryo mortality), or by enhancing host fecundity (Stouthamer et al. 1999).

Wolbachia may be present in various tissues but are predominately present in gonadal tissue (Stouthamer et al. 1999). The symbionts are transmitted vertically through the egg. Therefore, infected mothers give rise to infected offspring. Phylogenetic studies of *Wolbachia* indicate that horizontal transmission must have taken place rather frequently. An intraspecific horizontal transfer of *Wolbachia* has recently been reported (Huigens et al. 2000). Because culturing of *Wolbachia* outside hosts has been successful in only one case, molecular techniques such as the polymerase chain reaction (PCR) are used in detecting *Wolbachia* infections (Stouthamer et al. 1993).

Recently a novel lineage of intracellular bacteria has been shown to be associated with several reproductive disorders, including (1) parthenogenesis in a number of parasitoid

wasps in the genus *Encarsia* (Zchori-Fein et al. 2001, 2004), (2) feminization in several *Brevipalpus* mite species (Weeks et al. 2001; Groot and Breeuwer 2006), and (3) cytoplasmic incompatibility in *Encarsia pergandiella* Howard (Hunter et al. 2003). Phylogenetic analysis of the 16S rRNA gene placed this bacterium in the *Bacteroidetes* group (=*Cytophaga-Flexibacter-Bacteroides* or CFB group). This bacterium has been called the *Encarsia* bacterium (Zchori-Fein et al. 2001), the CFB-BP (Weeks and Breeuwer 2003), and the *Cytophaga*-like organism (CLO) (Hunter et al. 2003; Weeks et al. 2003; Weeks and Stouthamer 2004). Recently it has been suggested to classify this symbiont from *Encarsia* as "*Candidatus Cardinium hertigii*" (Zchori-Fein et al. 2004). A large screening study has shown that the bacterium is prevalent among arthropods, and that double infection with *Wolbachia* may occur (Weeks et al. 2003).

Members of the bacterial genus *Spiroplasma* have also been shown to interfere with reproduction in their arthropod hosts. They are referred to as sex ratio organisms or SRO's and they cause the total elimination of the male progeny in several species. The genus *Spiroplasma* is very diverse, containing species that may infect plants, insects and vertebrates (Boucias and Pendland 1998). Several species are well known insect pathogens whereas others are known as SRO's and for others no effects have been recorded (Boucias and Pendland 1998).

Bacteria of phytoseiid mites

The majority of the identified bacteria recorded in phytoseiid mites are intracellular bacteria of the genera *Rickettsiella*, *Wolbachia*, *Cardinium* and *Spiroplasma* (Table 2). *Wolbachia* seem to be widespread among phytoseiid mites, as they are found by several authors in numerous populations of seven phytoseiid species.

Rickettsiella phytoseiuli

Intracellular, rickettsia-like entities named *Rickettsiella phytoseiuli* have been observed during microscopic studies of *P. persimilis* (see for a review Šut'áková 1994). Predators originated from a laboratory population of the Ukraine (Šut'áková and Rüttgen 1978) and did not show developmental abnormalities, morphological changes or increased mortality. However, all investigated mites contained polymorphous entities that were considered to represent six different stages of the reproduction cycle: dense, intermediate, bacterial, giant, crystal-forming and small dark particles (Šut'áková and Rüttgen 1978). In adult mites, infection was detected in all organs except the nervous tissue, whereas larvae and nymphs and prey spider mites (*T. urticae*) were never infected with *R. phytoseiuli* (Šut'áková 1988, 1991). A *P. persimilis* population from Slovakia exhibited the same infection, whereas a population from the Armenian Republic did not. However, other apparently symbiotic microorganisms were present in the ovaries of predators from the latter population (Šut'áková and Arutunyan 1990). *Rickettsiella phytoseiuli* isolated from *P. persimilis* could be cultivated in adult female *Dermacentor reticulatus* Fabricius ticks, where it formed all six known developmental stages (Šut'áková and Rehácek 1989). Pathological effects were never recorded, though some individuals carried the microbes in high densities (Šut'áková 1991).

Endosymbiotic bacteria including *Wolbachia*, *Cardinium* and *Spiroplasma*

By using molecular methods (PCR with *Wolbachia*-specific primers), *Wolbachia* endosymbionts were detected in eight of nine laboratory populations of *M. occidentalis* and in four

Table 2 Bacteria recorded in phytoseiid mites

Pathogen	Phytoseiid host/origin[a]	Symptoms	References
Rickettsiella phytoseiuli	*Phytoseiulus persimilis*/l	Unknown	Šut'áková and Rüttgen (1978)
Wolbachia	*Galendromus annectens*/?	Unknown	Weeks et al. (2003)
	Phytoseiulus longipes/?	Unknown	Weeks et al. (2003)
	Metaseiulus occidentalis/l	Known[b]	Johanowicz and Hoy (1996), Breeuwer and Jacobs (1996), Weeks et al. (2003)
	Neoseiulus barkeri/f	Unknown	Breeuwer and Jacobs (1996)
	Neoseiulus bibens/l	Unknown	Breeuwer and Jacobs (1996)
	Phytoseiulus persimilis/c	Unknown	Steiner (1993b), Breeuwer and Jacobs (1996), Bjørnson et al. (1997), Weeks et al. (2003)
	Proprioseiopsis lenis/l	Unknown	Corpuz-Raros (2005)
Cardinium	*Metaseiulus occidentalis*/l, f	Known[b]	Weeks et al. (2003), Weeks and Stouthamer (2004), Hoy and Jeyaprakash (2005)
	Euseius finlandicus/f	Unknown	Enigl and Schausberger (2007)
Spiroplasma	*Neoseiulus californicus*/l	Unknown	Enigl and Schausberger (2007)
Bacteroidetes & *Enterobacter*	*Metaseiulus occidentalis*/l, f	Unknown	Hoy and Jeyaprakash (2005)
Acaricomes phytoseiuli	*Phytoseiulus persimilis*/l, c	Known[b]	Schütte (2006), Schütte et al. (1995, 1996, 1998, 2006a, b, 2008a, b), Dicke et al. (2000), Pukall et al. (2006), Gols et al. (2007)
Unidentified bacteria	*Metaseiulus occidentalis*/l	Known	Hess and Hoy (1982)
	Phytoseiulus persimilis/c	Unknown	Steiner (1993b), cited in Schütte et al. (2005)
	Neoseiulus cucumeris/?	Unknown	Cited in Schütte et al. (2005)
	Neoseiulus barkeri/?	Unknown	Cited in Schütte et al. (2005)

[a] Origin: c, commercial population; l, laboratory population; f, field population; ?, unknown

[b] Symptom induction established by experiments

laboratory populations of *T. urticae* that served as food for *M. occidentalis* (Johanowicz and Hoy 1996). In *M. occidentalis*, *Wolbachia* caused non-reciprocal reproductive incompatibilities between infected males and uninfected females. Uninfected females crossed with infected males produced few eggs and no female progeny. Many of the produced eggs were shrivelled (Johanowicz and Hoy 1998b). The mechanisms by which *Wolbachia* cause reproductive incompatibilities in *M. occidentalis* are unknown. *Wolbachia* infection seems to be associated with fitness costs as the number of female progeny was lower in infected control crosses than in uninfected control crosses. These fitness costs may have prevented the rapid spread of *Wolbachia* in three laboratory populations of *M. occidentalis* (Johanowicz and Hoy 1999). *Wolbachia* were eliminated from *M. occidentalis* when the predators were reared at an elevated temperature (33°C) (Johanowicz and Hoy 1998a, b).

Moreover, Breeuwer and Jacobs (1996) detected *Wolbachia* in a population of *M. occidentalis* from the USA, in a commercial population of *P. persimilis* from the

Netherlands, in a population of *Neoseiulus* (*Amblyseius*) *barkeri* Hughes collected in the Netherlands and a population of *Neoseiulus* (*Amblyseius*) *bibens* Blommers from Madagascar. *Wolbachia*-infection has also been found in *Galendromus annectens* (De Leon) and *Phytoseiulus longipes* Evans (Weeks et al. 2003) and in a laboratory stock of *Proprioseiopsis* (*Amblyseius*) *lenis* (Corpuz-Raros & Rimando) from the Philippines (Corpuz-Raros 2005). The effects of *Wolbachia* on the species other than *M. occidentalis* have not yet been investigated, but it is likely that *Wolbachia* are associated with non-reciprocal reproductive incompatibilities (for a discussion, see Breeuwer and Jacobs 1996). Rickettsia-like particles, belonging to the genus *Wolbachia* were also reported by Steiner (1993b); Bjørnson et al. (1997). The latter author detected with molecular methods that *Wolbachia* was present in commercial *P. persimilis* populations from seven sources. It even has been suggested that the intracellular bacteria of *P. persimilis* named *Rickettsiella phytoseiuli* (Šut'áková and Rüttgen 1978) and the rickettsia-like microorganisms observed in *M. occidentalis* (Hess and Hoy 1982) may in fact be *Wolbachia* (for a discussion see van der Geest et al. 2000).

However, recently Enigl et al. (2005) screened several strains of *P. persimilis* (seven strains obtained from Europe, Africa and the USA and alcohol samples of 10 other strains) for the occurrence of *Wolbachia* and no sample tested positive. They therefore suggested that infection of *P. persimilis* with *Wolbachia* seems to be rare and of minor importance (Enigl et al. 2005). During this study *T. urticae* used as food for the different strains of *P. persimilis* was infected with *Wolbachia*. The authors prevented false positive results from undigested prey by starving *P. persimilis* or feeding them *Wolbachia*-free *T. urticae* before the PCR tests. After a period of 16 h at 25°C and 48 h at 20°C *Wolbachia* was no longer detected in the predators (Enigl et al. 2005). Moreover, the same authors could not detect *Wolbachia* in six other phytoseiid species, including *N. cucumeris*, *N. barkeri*, *Euseius finlandicus* (Oudemans), *Kampimodromus aberrans* (Oudemans), *N. californicus* and *Typhlodromus pyri* Scheuten (Enigl and Schausberger 2007).

In a large-scale survey of arthropod hosts infection with the endosymbiotic bacterium *Candidatus Cardinium hertigii* was detected by sensitive hemi-nested PCR in *M. occidentalis* (Weeks et al. 2003). Test results were negative for *P. persimilis*, *Phytoseiulus macropilis* (Banks), *Neoseiulus* (*Amblyseius*) *fallacis* (Garman), *M. longipes*, *Galendromus helveolus* (Chant) and *G. annectens*. Interestingly *M. occidentalis* showed double infection of *Wolbachia* and *Cardinium*. In another study Weeks and Stouthamer (2004) reported that three inbred lines of *M. occidentalis* showed a clear and significant increase in fecundity associated with infection by *Cardinium*. Fecundity advantage of infected females versus non-infected females was approximately 1.6 times over a 6-day oviposition period. As the endosymbiont described by Hess and Hoy (1982) has recently been identified as *Cardinium* (Weeks and Breeuwer 2003) and as *M. occidentalis* may harbour both *Wolbachia* and *Cardinium* at the same time, the authors suggest that the results of the studies of Johanowicz and Hoy (1998a) on cytoplasmic incompatibility in *M. occidentalis* may have been influenced by the presence of *Cardinium* (Weeks et al. 2003). In a molecular screening, using a high-fidelity PCR protocol (allowing the detection of as few as 100 copies of *Wolbachia* DNA, Jeyaprakash and Hoy 2004), several bacterial species were detected in *M. occidentalis* after the clones were sequenced: one each was closely related to species in the genera *Enterobacter*, *Wolbachia* and *Cardinium*, and one was related to an unnamed microorganism in the phylum *Bacteroidetes* (Hoy and Jeyaprakash 2005). PCR tests with newly designed primers for the sequences of the detected bacteria were positive for several laboratory and field-collected *M. occidentalis* populations suggesting that all bacteria are important in the biology of this phytoseiid species (Hoy and Jeyaprakash 2005).

In a recent survey Enigl and Schausberger (2007) screened several predatory mite species with a PCR technique for infection with *Cardinium* and *Spiroplasma*. *Cardinium* was detected in two populations of *E. finlandicus*. Test results were negative for *N. cucumeris*, *N. barkeri*, *K. aberrans*, *N. californicus* and *T. pyri*. *Spiroplasma* was found in *N. californicus*. Test results were negative for *N. cucumeris*, *N. barkeri*, *E. finlandicus*, *K. aberrans* and *T. pyri*. However, it is not very probable that *Spiroplasma* is a pathogen or SRO in this case, as in an earlier study *Spiroplasma* did not have any effect on the number of eggs produced and the percentage of female offspring of *N. californicus* (Zchori-Fein et al., unpublished data, cited in Enigl and Schausberger 2007).

Acaricomes phytoseiuli

Lighthart et al. (1988) were the first to show susceptibility of a predatory mite to a bacterial pathogen. The authors tested the effect of several stress factors on the susceptibility of *M. occidentalis* to the weak bacterial pathogen *Serratia marcescens*. A high pre-inoculation temperature pulse under relatively uncrowded conditions was most effective in enhancing susceptibility, higher mortality being the only disease symptom. Remarkably, starvation did not have such an effect. However, the bacterial isolate did not originate from mites.

Thus, the only well documented case of a bacterial disease in phytoseiid mites represents the infection of *P. persimilis* with *Acaricomes phytoseiuli* (Schütte 2006; Schütte et al. 1995, 1996, 1998, 2006a, b, 2008a, b; Dicke et al. 2000; Pukall et al. 2006; Gols et al. 2007). During the early 1990s the first conspicuous disease symptom that became obvious was a behavioural change (Schütte et al. 1995; Dicke et al. 2000). An important behavioural characteristic of healthy adult female *P. persimilis* is their attraction to plant odours, currently called "herbivore-induced plant volatiles" (HIPV), which are released in response to feeding damage by their prey *T. urticae* (Sabelis and van de Baan 1983; Dicke and Sabelis 1988). Since 1983 this behavioural response has been reported in numerous laboratories (see reviews by Dicke et al. 1998, Sabelis et al., 1999) and it has been shown that it plays an important role in successful host location in the field (Zemek and Nachman 1999; Janssen 1999). However, since mid 1992 a laboratory population of *P. persimilis* showed a lower degree of attraction to herbivore-induced plant volatiles than in the first part of 1992 and the previous year. This so-called non-responding (=NR) population originated from the normally responding population from a Dutch natural enemy producer and had been reared in the laboratory for many years prior to 1992. The behavioural change occurred suddenly and was of a permanent nature. Moreover at the beginning of 1994 the same behavioural change occurred in a population of *P. persimilis* from a commercial source (Dicke et al. 2000). A similar phenomenon had occurred earlier in two other species of phytoseiid mites reared at the same laboratory. Between July 1985 and November 1987 the attraction to herbivore-induced plant volatiles fluctuated widely in three laboratory populations of *Amblyseius potentillae* (Garman) (=*Amblyseius andersoni* (Chant)) and one laboratory population of *T. pyri* (Dicke et al. 1991). Several possible causes for this variation were investigated, but no definite conclusions could be drawn (Dicke et al. 1991).

As several experiments on the behavioural change in *P. persimilis* indicated that it was most probably caused by an infectious agent (Schütte et al., unpublished; Dicke et al. 2000), follow-up studies aimed at verifying this hypothesis. A crucial first step towards verification of this hypothesis was evidence of the infectious character of the behavioural change. Mated female *P. persimilis* that had been exposed to dead conspecifics of the NR-population and their products showed a lower degree of attraction and a higher

mortality than predators that had been exposed to the products of live conspecifics of the NR-population (Schütte et al. 1998). In a diseased population early dying individuals are likely candidates to carry and release pathogens and common routes of disease transmission consist of pathogen release prior to or after death and cannibalism on dead conspecifics (Andreadis 1987).

In a comparative study other characteristics of the NR-population were investigated in order to describe a distinct disease syndrome, designated the 'non-responding (=NR) syndrome' (Schütte et al. 2006a). The following set of symptoms was described for adult females from the NR-population: (1) size change by shrinkage to dorso-ventrally flattened form, (2) reduced fecundity caused by oviposition stop after shrinkage, (3) high mortality several days after shrinkage, (4) presence of excretory crystals in the legs, (5) low predation and/or feeding rate, (6) low excretion rate, (7) low degree of attraction to prey-induced plant volatiles, (8) short choice time during behavioural test, (9) early dispersal from prey-patches (Schütte et al. 1995, 2006a). Interestingly there are several publications in which remarkable peculiarities of *P. persimilis* have been stated, that are similar to the NR-syndrome among which (1) poor performance in terms of fecundity and survival (Steiner 1993a, b; Steiner and Bjørnson 1996; Raworth and Bjørnson 2002; Blümel and Hausdorf 2002); (2) poor performance in terms of life span (De Courcy Williams et al. 2004a); (3) size change by shrinkage to dorso-ventrally flattened form (Bjørnson et al. 2000); (4) remarkable effect of rearing condition on olfactory response (Maeda et al. 2000); (5) remarkable differences in dispersal behaviour (van de Vrie and Price 1997; Skirvin and Fenlon 2003a); (6) unusual results concerning the predation rate (Skirvin and Fenlon 2003b). However, most of these studies did not consider or test the possibility of pathogen infection (for a detailed discussion see Schütte 2006).

Several routes of transmission were investigated for six of the nine symptoms of the NR-syndrome. There was no evidence for (1) vertical transmission, i.e. transmission from parent to offspring directly via the egg, (2) interspecific horizontal transmission between the prey *T. urticae* and adult female *P. persimilis*, (3) horizontal transmission via body fluids, i.e. from squashed female predators to female *P. persimilis* (Schütte et al. 2006b). Instead there was clear evidence for horizontal transmission between and among generations via feces and debris released by diseased adult female *P. persimilis* (Schütte et al. 2006b). After contact with feces and debris deposited by diseased females during only 1 day, the majority of healthy female *P. persimilis* (65%) became dorso-ventrally flattened after only 3 days. From eggs laid by diseased mothers a minority of the offspring became dorso-ventrally flattened (17%) when adult. This was only the case, when the eggs were left on the place where the mother had laid them (Schütte et al. 2006b).

With knowledge about the main reservoir of the infectious agent it could be determined to which group the pathogen in question belongs (Schütte et al. 2008a). A reliable bioassay for testing the infectiousness of predator feces and debris fractions was developed, by keeping healthy adult female predators during a period of 3 days on prey-infested bean leaves, which had previously been sprayed with an aqueous suspension of feces and debris. After exposure six of the nine symptoms as listed above were assessed. A vast majority of healthy female *P. persimilis* (88–100%) became dorso-ventrally flattened after contact with a feces and debris suspension collected from symptomatic females. This effect vanished totally when the suspension was treated with the antibiotic tetracycline. Moreover did the bacterial fraction of feces and debris suspension collected from symptomatic predators induce the NR-syndrome whereas the viral fraction of the same suspension did not (Schütte et al. 2008a). These findings proved that bacteria are involved in the induction of the NR-syndrome.

Numerous bacterial isolates from mated female predators from the NR-population and their feces and debris were tested for their effects on healthy adult female *P. persimilis* (Schütte et al., unpublished data). The final aim, namely satisfying the Koch's postulates of pathogenicity was achieved with only one isolate, representing a new bacterial species in a new genus, described as *Acaricomes phytoseiuli* (Pukall et al. 2006). The NR-syndrome was clearly induced in those predators that had been exposed to the bacterial inoculum (=treatment predators), whereas predators exposed to water (=control predators) did not show the NR-syndrome. Moreover, *A. phytoseiuli* was never isolated from control predators whereas it could be re-isolated from 60% of the treatment predators and from feces of 41% of treatment predators (Schütte et al. 2008b). Light and electron microscopic studies of predators exposed to *A. phytoseiuli* revealed striking bacterial accumulations in the lumen of the alimentary tract together with extreme degeneration of its epithelium. In addition, bacterial foci also occurred in the fat body. These phenomena were not observed in control predators that had been exposed to sterile water (Schütte et al. 2008b). Thus *A. phytoseiuli* may infect the predatory mite *P. persimilis* and induce the occurrence of the NR-syndrome in adult female *P. persimilis*.

Acaricomes phytoseiuli is a gram-positive, rod-shaped, none-spore-forming bacterium. Comparative analysis of the 16S rDNA sequence revealed that the strain was a new member of the family of the Micrococcaceae. Nearest phylogenetic neighbours were determined as *Renibacterium salmoninarum* (94.0%), *Arthrobacter globiformis* (94.8%) and *Arthrobacter russicus* (94.6%) (Pukall et al. 2006). It appears that the new genus *Acaricomes* is closely related to the genus *Arthrobacter*. Recently a specific and reliable PCR-test has been developed for the detection of *A. phytoseiuli* (Gols et al. 2007). In two validation tests healthy female *P. persimilis* were previously infected with *A. phytoseiuli*. In one test 36% of the predators had become symptomatic and 38% of the predators tested positive; in the second test 70% of the predators had become symptomatic and 61% of the predators tested positive. Moreover a significant negative correlation was found between the proportion of predators being attracted to herbivore induced plant volatiles and the proportion of PCR-positive samples (Gols et al. 2007). By using this molecular detection method it was demonstrated that *A. phytoseiuli* is rather widespread among European populations of *P. persimilis*. All but one of the seven European populations of *P. persimilis* tested were *A. phytoseiuli*-positive, whereas two populations from outside Europe turned out to be negative. The prey mite *T. urticae* and other commercially used predatory mite species including *A. andersoni* (=*A. potentillae*), *N. cucumeris*, *Iphiseius* (*Amblyseius*) *degenerans* (Berlese), *N. californicus*, *Hypoaspis aculeifer* Canestrini and *Hypoaspis miles* Berlese, were not infected (Gols et al. 2007). It can thus be concluded, that *A. phytoseiuli* is currently infecting several populations of commercial and laboratory populations of *P. persimilis*, and that it has detrimental effects on them. Screening of populations on a regular basis for an infection with *A. phytoseiuli* should therefore be executed, as in this case a reliable detection method has been developed. Possible methods of curing infected populations consist of antibiotic treatment (Schütte et al. 2008a) and washing of eggs with bleech (Schütte et al. 2006b).

Unidentified bacteria

Hess and Hoy (1982) observed two different pathological manifestations in several laboratory populations of *M. occidentalis*. (1) Some adult females were plump and had a cream-coloured to pink rectal "plug" that extruded from their posterior end and occasionally caused mites to become glued to the substrate. The rectal plug was associated with

motor dysfunction, reduced oviposition and eventually death, and was most common in older females. Immatures and males rarely had rectal plugs. (2) Mites became very pale and so thin that they became translucent. Females failed to oviposit, immatures exhibited high mortality and colonies died out. According to the authors both pathologies were associated with overcrowding (Hess and Hoy 1982). The authors described two morphologically distinct unidentified micro-organisms in symptomatic and non-symptomatic *M. occidentalis*. Whether these forms represent one or two species was not established. One form (which they called type A) was exclusively intracellular. This type was present in all mites in varying numbers and in all tissues examined, except ovarian and nervous tissues. According to the authors this micro-organism did not appear to be detrimental. The second rickettsia-like form (type B) occurred both intra- and extracellularly. This type was present in two-thirds of symptomatic and asymptomatic mites. In some cases it completely dominated the internal organs and the hemocoel and was associated with the rectal plug. Thin and pale mites also contained predominantly the second type, but tissues of these mites appeared more damaged, perhaps accounting for their lucidity. When present in moderate numbers, these micro-organisms were observed in the hemocoel, the Malpighian tubules and within the ovarian tissue, which may suggest transovarial transmission (Hess and Hoy 1982). The authors did not determine whether the increase of the second bacterial type was the primary cause of the disease or a secondary effect. Later it has been suggested that the rickettsia-like microorganisms (type B) may in fact be *Wolbachia* (for a discussion see van der Geest et al. 2000) and that the endosymbiont (type A) is likely to be *Cardinium* (Weeks and Breeuwer 2003).

In a microscopic study of the digestive tract of *P. persimilis*, bacteria-like entities detected in the gut lumen were thought to have entered the digestive tract during feeding (Arutunyan 1985). However, these bacteria bear a marked similarity to birefringent dumbbell-shaped crystals that are frequently observed in the Malpighian tubules, the digestive tract and rectum of phytoseiid mites (Steiner 1993b; Schütte et al. 1995; Di Palma 1996; Bjørnson et al. 1997, 2000; R. G. Kleespies, pers. comm.).

Bacterial micro-organisms other than rickettsia have been recorded for dead and moribund *P. persimilis* (Steiner 1993b). However, the author stated that these bacteria are secondary opportunistic invaders rather than a primary infection source. Moreover unidentified bacteria were reported in microscopic investigations of several diseased mite populations of *P. persimilis*, *N. cucumeris* and *N. barkeri* (cited in Schütte et al. 2005).

Protozoa

General characteristics of protozoa in insects and mites

All protozoa recorded for phytoseiid mites belong to the phylum Microspora. Microsporidia are small, spore-forming protozoa. However, recent molecular studies indicate that they are related to fungi, which may in part explain the sensitivity of microsporidia to selected anti-fungal drugs (Boucias and Pendland 1998). Microsporidia infect a wide range of hosts from all major animal phyla, fish and arthropods being their most common hosts (Tanada and Kaya 1993). They are obligate intracellular parasites that lack typical mitochondria, a classical Golgi apparatus, centrioles and peroxisomes (Boucias and Pendland 1998). Many species cause severe and acute infections in insects, but some produce only inapparent and chronic infections, that nonetheless may play an important role in host regulation (Tanada and Kaya 1993).

The microsporidia have a complex biology that may involve two obligate hosts, vertical or horizontal transmission and/or multiple cell-types (Boucias and Pendland 1998). The life cycle consists of two phases, the vegetative phase and the sporulation phase, which results in the production of transmissible spores. In most cases the spore-to-spore cycle takes place in one cell (Tanada and Kaya 1993). Microsporidia may invade the host tissues when spores are ingested, when the pathogen is transmitted from parent to progeny, or occasionally through wounds in the integument (Tanada and Kaya 1993). Microsporidian spores are structurally unique and contain a characteristic tube-like polar filament through which an infective stage (sporoplasm) is injected into an adjacent host cell. This starts the infective cycle of the pathogen.

Diagnostic features considered as general characteristics of microsporidian infection are variable and may include: retardation of development and growth, reduced activity, abnormal coloration, diapause alterations, reduction of longevity and reproductive performance (Boucias and Pendland 1998). Microsporidia-infected insects may also exhibit behavioural changes including changes in temperature preference (Horton and Moore 1993).

Protozoa of phytoseiid mites

Microsporidia seem to be rather common among phytoseiid mites. Microsporidiosis has been observed in four phytoseiid species of varying origin (Table 3).

Oligosporidium occidentalis

A new microsporidian pathogen has recently been isolated from a laboratory population of *M. occidentalis* (Becnel et al. 2002). Immature stages and mature spores were found in the cytoplasm of ceacal cells, lyrate organ cells, ganglia, epithelial cells, muscle, ovary and mature eggs (Becnel et al. 2002). Microsporidia were never detected in the spider mite (*T. urticae*) prey of *M. occidentalis* (Olsen and Hoy 2002). Two classes of uninucleate spores were produced, differing primarily in the length of the polar filaments and the presence of a large posterior vacuole in one spore type (Becnel et al. 2002). The authors suspect that spores with long filaments are involved in horizontal disease transmission, which may take place by cannibalism of infected eggs (Olsen and Hoy 2002), whereas

Table 3 Protozoa recorded in phytoseiid mites

Pathogen	Phytoseiid host/origin[a]	Symptoms	References
Microsporidium phytoseiuli	*Phytoseiulus persimilis*/c	Known[b]	Bjørnson et al. (1996)
Oligosporidium occidentalis	*Metaseiulus occidentalis*/l	Known[b]	Becnel et al. (2002)
Nosema steinhausi	*Neoseiulus cucumeris*/c	Unknown	Huger (1988)
	Neoseiulus barkeri/c	Unknown	Huger (1988)
Unidentified microsporidia	*Neoseiulus barkeri*/c	Known	Beerling and van der Geest (1991a, b)
	Neoseiulus cucumeris/c	Known	Beerling and van der Geest (1991a, b)
	Phytoseiulus persimilis/c	Unknown	Bjørnson and Keddie (2000)
	Phytoseiulus persimilis/c	Unknown	Bjørnson and Keddie (2000)

[a] Origin: c, commercial population; l, laboratory population

[b] Symptom induction established by experiments

spores with the short polar filament may play a role in autoinfection and vertical transovarial transmission, that is highly efficient (99% infected offspring is produced by infected parents) (Olsen and Hoy 2002).

Molecular data (analysis of small subunit ribosomal DNA) indicated that this microsporidium is a new species, which is most closely related to the *Nosemal Variomorpha* clade of microsporidia, whereas developmental and morphological data suggest a placement into the genus *Unikaryon* or *Oligosporidium*. The authors discuss this conflict of morphological and molecular data and assign the new species the name *Oligosporidium occidentalis*.

Predators infected by *O. occidentalis* did not exhibit any external signs of infections. However, *O. occidentalis* has clear negative effects on its host. Infected female predators had a shorter life span, a lower oviposition rate and a lower number of female offspring, as infected mites have a male-biased sex ratio (Olsen and Hoy 2002). Heat treatment was effective to cure infected populations of *M. occidentalis* and did induce relatively low mortality ($\sim$20%). Predator colonies initiated from mites that were reared from egg to adult at 33°C showed an initial reduction in infection. However, disease incidence raised to 98% after 10 weeks. Colonies initiated from progeny of the heat-treated mites remained healthy during the observation period of 10 weeks (Olsen and Hoy 2002).

Microsporidium phytoseiuli and unidentified microsporidia in *Phytoseiulus persimilis*

Three distinct species of microsporidia have been reported from *P. persimilis* from three commercial sources. The species assigned as *Microsporidium phytoseiuli* was isolated from a European population (Bjørnson et al. 1996), one unnamed species (A) was found in a population from North America and another unnamed species (B) in a population from Israel (Bjørnson and Keddie 2000). Becnel et al. (2002) suggested that *M. phytoseiuli* may also be a member of the genus *Oligosporidium*, because of a number of biological and morphological similarities with *O. occidentalis*.

The microsporidia of *P. persimilis* were not restricted to specific tissues and spores were found in muscle fibres, the super- and suboesophageal ganglia, ovaries, eggs, cells underlying the cuticle, and cells lining the caecal lumen and Malpighian tubules. Early development of all three microsporidia occurred in cells of the lyrate organ. The lyrate organ occupies a significant portion of the body and is thought to be involved in oogenesis or embryogenesis. Each microsporidium occupied a specific site within these cells. Infection of the lyrate organ may be necessary for the efficient vertical transmission of microsporidia in *P. persimilis* (Bjørnson et al. 1996; Bjørnson and Keddie 2000).

Microsporidium phytoseiuli was not present in the prey mites, *T. urticae*. Therefore, prey mites did not contribute to pathogen transmission among *P. persimilis* mites. Maternal-mediated vertical transmission of *M. phytoseiuli* was 100%. Males did not contribute to infection of the progeny. Horizontal transmission of *M. phytoseiuli* did not occur when uninfected adult predators were kept together with infected *P. persimilis* females or on leaves carrying solutions of microsporidian spores. Horizontal transmission was low (about 15%) when uninfected immatures were kept together with infected adult and immature mites (Bjørnson and Keddie 2001). At present little is known regarding the mechanisms of transmission.

Microsporidia-infected *P. persimilis* did not exhibit any obvious external symptoms. Therefore, routine monitoring is necessary to detect microsporidia when disease prevalence is low (Bjørnson and Keddie 1999). *P. persimilis* infected by *M. phytoseiuli* produced fewer eggs, had a reduced longevity and lower prey consumption rate than

healthy predators. Moreover, infected females produced fewer female progeny than uninfected females, as the sex ratio of offspring of infected females is male biased (Bjørnson and Keddie 1999).

Several methods to cure an infection with microsporidia were tested by Bjørnson (1998). The antimicrobial compounds albendazole, fumagillin, metronidazole and nifedipine were ineffective for control of microsporidia in *P. persimilis*, regardless of their dose. The author doubted whether the chemical compounds were able to penetrate the egg chorion. Rearing predators at 30°C did not eliminate microsporidian infections either. The Pasteur method, whereby progeny of healthy mothers is selected for the rearing, was the only effective means to eliminate microsporidia from *P. persimilis* populations (Bjørnson 1998).

Unidentified microsporidia and *Nosema steinhausi* in *Neoseiulus cucumeris* and *N. barkeri*

Unidentified microsporidia were reported in commercial mass-rearings of *N. cucumeris* and *N. barkeri* (Beerling and van der Geest 1991a, b). This was the first report of microsporidia in mass-reared predatory mites. Predators of the commercial populations showed a low reproduction rate and unsatisfactory predation capacity. Moreover, mites were sluggish and had a swollen and whitish appearance (Beerling and van der Geest 1991a). Squash preparations of symptomatic mites revealed the presence of numerous microsporidian spores and heavily infected predators released spores after death (Beerling and van der Geest 1991a). Microsporidia were also present in the prey mites but the mechanisms of pathogen transmission have not been determined for this system. Three types of microsporidian spores have been found in *N. cucumeris* and *N. barkeri* (Beerling et al. 1993), but it is unclear whether they represent one species of microsporidia with three different spore types or three distinct species. Oblong spores were detected in both predator and prey species, small and more oval spores were exclusively found in prey mites. Beerling et al. (1993) developed a monoclonal antibody ELISA as a bioassay for the detection of microsporidia in mass-reared *N. cucumeris* and *N. barkeri*. Monoclonal antibodies were produced for one spore type that was present in both predator and prey species. Further work is needed to determine the sensitivity of this test as a suitable screening method for microsporidia in mites. Interestingly, Huger (1988) detected the microsporidium *Nosema steinhausi* in diseased mass-reared populations of the same phytoseiid species (*N. cucumeris* and *N. barkeri*).

Fungi

General characteristics of fungi in insects and mites

Fungi are eukaryotic heterotrophes that obtain nutrients either from dead organic matter (saprobes) or from living organisms (parasites). Some parasitic fungi are obligate pathogens, but the majority are facultative pathogens capable of growing without their host (Tanada and Kaya 1993). Entomopathogenic fungi are characterized by their ability to attach to and penetrate host cuticle or spiracles; however, some penetrate through the gut. They replicate inside the host, usually in the hemocoel, where they compete for soluble nutrients and may release mycotoxins, which interfere with normal host development and metamorphosis and in some cases with the immune defense mechanisms (Boucias and Pendland 1998). Fungi then invade and digest tissues and cause premature death of the

Table 4 Fungi recorded in phytoseiid mites

Pathogen	Phytoseiid host/origin[a]	Symptoms	References
Neozygites sp.	*Euseius citrifolius*/f	Known	Furtado et al. (1996)
Neozygites acaricida	*Euseius citrifolius*/f	Unknown	Keller (1997)
N. cf. *acaridis*	*Euseius citrifolius*/f	Unknown	Keller (1997)
Unidentified fungi	*Phytoseiulus persimilis*/?	Unknown	Cited in Schütte et al. (2005)

[a] Origin: f, field population; ?, unknown

host. Thereafter the fungus lives as a saprophyte on the cadaver, producing spores. Under unfavorable conditions resting forms are produced (Tanada and Kaya 1993). Adhesion and germination of fungal spores on the host cuticle are highly dependent on relative humidity and temperature, but light conditions and nutritional requirements are also important factors (Tanada and Kaya 1993).

Diagnostic features considered as general characteristics of fungal infection may include: blackening surfaces at sites where fungi have penetrated, coloration (white, yellow, black), reduced feeding, the presence of filamentous hyphae, the presence of characteristically coloured reproductive structures (fruiting structures, spores) on the external surface of the dead host, weakness and partial paralysis, bodies may be hard (Boucias and Pendland 1998).

In some cases, behavioural changes occur prior to death. Symptoms in insects may include restlessness, loss of coordination and body tremors, reproductive behaviour by castrated hosts and changes in microhabitat preference (Horton and Moore 1993; Boucias and Pendland 1998). The latter include elevation-seeking behaviour (fungal "summit disease"), movement to exposed locations, change in oviposition or foraging sites and change in temperature preference (Horton and Moore 1993).

Fungi in phytoseiid mites

Pathogenic fungi have been recorded for only two phytoseiid species up to now (Table 4). Field-collected *Euseius citrifolius* Denmark and Muma were heavily infected by the fungus *Neozygites* sp. (Furtado et al. 1996) and showed a high rate of mortality. Some cadavers carried near-white hyphae that produced pear-shaped conidia. However, *Neoseiulus* (*Amblyseius*) *idaeus* Denmark and Muma and *Typhlodromalus* (*Amblyseius*) *limonicus* (Garman and McGregor) were not infected by *Neozygites* sp. isolated from the cassava green mite in laboratory tests (De Moraes and Delalibera 1992). *Euseius citrifolius* collected in Brazil on two subsequent occasions contained viable resting spores and hyphal bodies of two distinct fungal species identified as *Neozygites acaricida* and *N.* cf. *acaridis* (Keller 1997). Moreover unidentified fungi were reported in microscopic investigations of a diseased population of *P. persimilis* (cited in Schütte et al. 2005).

Unidentified diseases

General characteristics in insects and mites

Insect diseases may be broadly categorised as either infectious or non-infectious, based on the respective presence or absence of a transmissible living organism. Diseases classified

as non-infectious may be caused by mechanical injury, adverse physical environmental factors, chemical agents, injuries made by predators and parasitoids, genetic factors, nutritional deficiencies and hormonal disruption (Tanada and Kaya 1993). Traditionally, insect pathologists have focused their research on infectious diseases that might be caused by a variety of pathogens. However, non-infectious diseases may play an important role in insect populations (Tanada and Kaya 1993). Thus, in cases of obvious detrimental symptoms where the involvement of pathogens cannot be proved, pathogens may not (yet) be detectable or other factors may cause the disease.

Unidentified diseases of phytoseiid mites

For phytoseiid mites several reports exist on poor performance, anatomical peculiarities and peculiar colorations (Tanigoshi et al. 1981; Tanigoshi 1982; Hess and Hoy 1982; Bjørnson et al. 1997, 2000). However, in these cases it was not unambiguously shown that pathogens may have been involved (Table 5).

Tanigoshi et al. (1981) observed the formation of a dark-red occlusion within the alimentary tract near the distal end of the opisthosoma for *Euseius* (*Amblyseius*) *hibisci* (Chant) of both sexes when fed exclusively on *Panonychus citri* (McGregor). Newly eclosed *E. hibisci* larvae acquired a red coloration of the gut directly after feeding and became less robust and vigorous after each moult. Complete immature mortality occurred at 32 and 35°C. Immediately after the last moult female predators became dorso-ventrally flattened, more concave in profile, lethargic, did not lay eggs and exhibited the characteristic dark-red gut occlusion prior to their death. The pigmented mass inside the mite was thought to be associated with the incomplete digestion of the prey mites, as symptoms were not observed in mites fed a diet of pollen from the ice plant, *Malephora crocea* Jacq. (Tanigoshi et al. 1981).

Birefringent, dumbbell-shaped crystals have been observed in *P. persimilis* from several sources (Bjørnson et al. 1997, 2000). Excessive crystal formation was associated with white discoloration of the opisthosoma. Discoloration may include (1) a white dorsal spot at the distal end of the opisthosoma, (2) two white stripes along the dorsal lateral sides of the body in the region of the Malpighian tubules, or (3) a combination of both forms (Bjørnson et al. 2000). Mites carrying discoloration(s) appeared lethargic and provided poor pest control (Steiner 1993b; Bjørnson et al. 1997). Rectal plugs, which were observed when symptoms were more pronounced, often disrupted normal excretion and might cause the affected individual to become stuck to the leaf surface (Bjørnson et al. 1997). The frequent occurrence of a prominent white dot in the opisthosoma of *P. persimilis* was correlated with reduced fecundity and predation rate in mites examined following shipment from commercial producers (Bjørnson et al. 2000). Crystals were observed in immature and adult *P. persimilis* (Bjørnson et al. 1997); therefore, non-excessive crystal formation is likely a normal physiological process (Bjørnson et al. 1997). An examination of *P. persimilis* from 14 commercial and academic sources revealed no correlation between the occurrence of crystals and the presence of microsporidia, rickettsia or virus-like particles in *P. persimilis*

Table 5 Unidentified diseases recorded in phytoseiid mites

Pathogen	Phytoseiid host/origin[a]	Symptoms	References
Unidentified	*Euseius hibisci*/l	Known	Tanigoshi et al. (1981)
Unidentified	*Phytoseiulus persimilis*/c	Known	Bjørnson et al. (1997, 2000)

[a] Origin: c, commercial population; l, laboratory population

(Bjørnson et al. 1997). However, in a follow-up study Bjørnson and Raworth (2003) found that the expression of white opisthosomal discolorations in *P. persimilis* does not necessarily affect predator performance and concluded that the opisthosomal discolorations are an expression of normal excretory function in *P. persimilis* related to plant nutrition (Bjørnson and Raworth 2003).

Conclusions

Several potential pathogens—pathogens in the true sense and unidentified diseases—have been reported for phytoseiid mites. However, the status and impact of many described entities on their host is unclear. Fourteen reports are descriptive with unknown host effects; three reports mention pathological manifestations without proving the final cause of the symptoms and eleven reports describe endosymbiotic bacteria. Only six reports present pathogens in the true sense of the word. From the latter group four reports refer to Microsporidia, one to a bacterium and one to a fungus. Microsporidian infections often appear not to be readily visible as no obvious external symptoms are present and female predators infected by *A. phytoseiuli* may be mistaken for unmated females. Such infections may thus remain undetected for extended periods meanwhile spreading in the case of exchange of predator populations among producers and laboratories. Screening of these pathogens on a regular basis is therefore advisable for maintenance of healthy predator populations over long periods. However, as only few pathogens in the true sense are described up to now it is too early to plead for regular general pathogen screening in phytoseiid mite mass rearings. Yet, the reports on true pathogens, often made in response to unexpected phenomena in a mass rearing, show that pathogens of beneficial mites can be an important factor hampering the quality of the mass-reared mites. The final conclusion of this review therefore is that more research on diseases of beneficial mites that are applied in biological pest control is needed.

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Symbionts, including pathogens, of the predatory mite *Metaseiulus occidentalis*: current and future analysis methods

Marjorie A. Hoy · A. Jeyaprakash

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Abstract *Metaseiulus* (= *Typhlodromus* or *Galendromus*) *occidentalis* (Nesbitt) (Acari: Phytoseiidae) is an effective natural enemy of pest mites in a variety of crops around the world, although it is considered to be endemic in the western USA. A broad understanding of much of its biology, ecology, behavior, and genetics has been obtained over the past 60 years, but the role(s) symbionts play, which includes pathogens and other microorganisms, remains to be resolved fully. Until molecular tools became available, analysis methods were limited primarily to microscopic observations; some viruses and rickettsia-like organisms were observed infecting 'diseased' *M. occidentalis*, but it is not clear which one(s) was the causal agent(s) of the disease(s). Subsequent to the development of the polymerase chain reaction (PCR) and genome sequencing, we identified putative gut symbionts and reproductive tract symbionts in *M. occidentalis*, as well as a microsporidian pathogen. A new phylogenetic analysis of the *Bacteroidetes-Flavobacterium* group suggests the unnamed *Bacteroidetes* in *M. occidentalis* is associated with the digestive tract. However, much of our current information about the role these microorganisms play in the biology of *M. occidentalis* is based on correlation, lacking the strength of fulfilling Koch's postulates. We also currently lack any knowledge of the importance of these microorganisms under field conditions. In the future, it should be possible to learn what role(s) these organisms play in the biology of this important predator using metagenomics approaches to analyze the transcriptome and to determine their relative abundance within their hosts with the quantitative PCR. We have just begun to resolve these relationships.

Keywords Phytoseiidae · *Metaseiulus* (= *Typhlodromus* or *Galendromus*) *occidentalis* · Microbial symbionts · Pathogens · Assessment methods · Metagenomics · *Bacteroidetes* · *Wolbachia* · *Cardinium* · *Enterobacter* · *Oligosporidium* · Viruses · *Serratia*

M. A. Hoy (✉) · A. Jeyaprakash
Department of Entomology and Nematology, University of Florida, PO Box 110620,
Gainesville, FL 32611, USA
e-mail: mahoy@ifas.ufl.edu

Introduction

The western orchard predatory mite, *Metaseiulus* (= *Typhlodromus* or *Galendromus*) *occidentalis* (Nesbitt) (Acari: Phytoseiidae) is an effective natural enemy of a variety of pest mites. It can control a variety of tetranychid, eriophyid, and tarsonemid pests in apples, grapes, almonds, cotton, and strawberries in the western United States of America (Hoyt 1969; Flaherty and Huffaker 1970; McMurtry et al. 1970; McMurtry 1982; Hoy 1985a; Kostiainen and Hoy 1996). It has been imported and established in Australia and New Zealand in classical biological control programs for the control of mites in apple and peach orchards (Readshaw 1975; Field 1978). This natural enemy is especially effective in integrated pest management programs (IPM) in deciduous orchards, vineyards, and strawberries, where it can maintain pest mites under low densities over long periods of time, in part because it developed resistances to insecticides through field selection. Between 1970 and early 1981, at least 470 papers were published on the Phytoseiidae (Tanigoshi 1982) and between 1960 and 1994, more than 420 papers were published on *M. occidentalis* alone (Kostiainen and Hoy 1996).

Thus, because *M. occidentalis* is an economically important species in IPM programs, we know a fair amount about its basic biology, including developmental rates when reared on different prey, mating behavior, sex ratios, preoviposition and oviposition behavior, overwintering and diapause behavior, feeding habits, tolerance of or resistance to pesticides, prey range, prey consumption, dispersal behavior, production of sex pheromones and interactions with other phytoseiids or with insect predators (for a review see Tanigoshi 1982; for an indexed bibliography on all papers published on the Phytoseiidae between 1960 and 1994 see Kostiainen and Hoy 1996; also see McMurtry et al. 1970; McMurtry 1982; Hoy1985a, b, 1990 for key references).

A number of genetic studies have been conducted on *M. occidentalis* (Hoy 1985b; Hoy and Cave 1985). The number of chromosomes (3 and 6 in males and females, respectively) and the genetic system of *M. occidentalis* was shown to be parahaploidy instead of arrhenotoky, with haploid males being derived from fertilized eggs in which the paternally derived chromosomes are heterochromatinized and lost from the nuclear genome to result in a haploid adult with chromosomes derived from their mothers (Hoy 1979; Nelson-Rees et al. 1980). Whether this unusual genetic system is influenced by the microbial associates (*Wolbachia* or *Cardinium*) of *M. occidentalis* is unknown. This predator has undergone laboratory selection for an inability to diapause in an effort to enhance its ability to control *Tetranychus urticae* Koch in greenhouse roses during the winter (Field and Hoy 1985), as well as for its ability to survive synthetic pyrethroid, carbamate and sulfur pesticides for use in IPM programs in apple, pear and almond orchards and vineyards (Hoy et al. 1985; Hoy 1990). Whether pesticide-resistant microbial symbionts are associated with these resistances also remains unknown, but many soil microorganisms have been documented to degrade pesticides (Felsot 1989) and microbial gut symbionts of a tephritid have been implicated in the pesticide resistance of its host (Boush and Matsumura 1967). *Metaseiulus occidentalis* has been genetically modified using recombinant DNA methods and was the first transgenic arthropod containing a transgene (the *lac*Z marker gene) to be released into the field in a small-scale, short-term field trial (Presnail and Hoy 1992; Hoy 2000). Recently, the complete mitochondrial genome of *M. occidentalis* was sequenced, revealing some unique characteristics compared to the mitochondrial genomes of other chelicerate arthropods (Jeyaprakash and Hoy 2007). Because mitochondrial organelles are derived from endosymbiotic bacteria, these unusual features will be discussed briefly below.

Despite this extensive research conducted over the past 60 years, until recently we knew little about the microbial associates of *M. occidentalis*, including its endosymbionts. We adhere to the term 'symbiosis' in its original definition of "organisms living together, usually in close association with one another, to the benefit of at least one organism, with the partners referred to as symbionts." Within the general concept of symbiosis, there are subcategories, including parasitism and mutualism (Douglas 1994; Bourtzis et al. 2006). Symbionts may be obligatory, facultative, intracellular, extracellular, have neutral, positive, or negative effects on their host's overall health. Infection by a particular bacterium may be beneficial to a host under some circumstances but harmful in other hosts or environments. At one extreme are the ancient symbionts that live in specialized 'bacteriomes' (host organs or cells specialized for housing the symbionts) and are required by their hosts. Facultative symbionts may not reside exclusively in specialized organs and are not strictly necessary for host survival. Pathogenic symbionts may be pathogens at all times, or they may become pathogenic only when the host immune system allows the microorganism to increase in density.

The goal of this review is to discuss what we know about the microbial associates (whether they are called endosymbionts, mutualists, pathogens, or have an unknown relationship) of this important predator and provide a brief overview of what can be learned as new methods are applied to understanding these relationships. First, we will discuss the known pathogens of *M. occidentalis*, which includes viruses, microsporidia, and bacteria. Then we will discuss what we know (and don't) about the microbial endosymbionts of *M. occidentalis* and their interactions with their host. In addition, we will discuss some of the methodological issues restricting our understanding of these relationships.

Pathogens and putative pathogens of *Metaseiulus occidentalis*

Viruses

The only viruses reported to infect this predator appear to be those based on transmission electron microscope (TEM) studies of laboratory colony specimens provided by M.A. Hoy to R. Poinar (Poinar and Poinar 1998). The 'sick' *M. occidentalis* females in the laboratory colonies exhibited lowered egg production, reduced longevity and died suddenly with a paralyzed appearance. TEM analysis showed three separate types of icosahedral virus particles present. One was located in epithelial cells, and was 47 nm in diameter with a 35-nm electron dense core. Some particles appeared hexagonal in cross section and were sometimes associated with paracrystalline structures. Poinar and Poinar (1998) indicated these icosahedral particles were similar to those reported in *Panonychus citri* (McGregor), and also were similar to the icosahedral particles found in epithelial cells of infected and healthy *T. urticae*. It is possible this virus was obtained from their *T. urticae* prey.

The second type of virus observed in *M. occidentalis* was approximately 38 nm in diameter and was found in the gut cells, with a 30-nm electron dense core (Poinar and Poinar 1998). These particles occurred in large numbers in the midgut nuclei, but free virions also were found in the cytoplasm of the gut cells and the lumen of the midgut. The third virus observed in *M. occidentalis* was 45 nm in diameter with a 35-nm dense core and only found in gut tissue.

It is not clear if one or more of these viruses were the cause of symptoms observed in these laboratory colonies of *M. occidentalis*. Nor do we know whether the viruses occur in field populations because the specimens provided were all from crowded laboratory

colonies. Likewise, it is unknown whether *M. occidentalis* became infected by feeding on virus-infected prey. Much more needs to be done to resolve the effects, if any, of viruses on the biology of *M. occidentalis*.

Microsporidia

A new species of microsporidium, *Oligosporidium occidentalis*, was described by Becnel et al. (2002) from specimens of *M. occidentalis* obtained from laboratory colonies in Gainesville, Florida. Both ultrastructural and molecular data were used to describe the species, which is in the *Nosema/Vairimorpha* clade of microsporidia based on molecular data, although the morphological and molecular data are not congruent.

All stages of *O. occidentalis* are haplokaryotic and develop in direct contact with the host-cell cytoplasm (Becnel et al. 2002). Sporogony is disporoblastic and spores are formed in eggs, immatures, and adults of *M. occidentalis*. There are two types of spores, one with a short and one with a longer polar filament. Horizontal transmission occurs by cannibalism of eggs and other stages and perhaps involves the spores with the long polar filament. Spores with the short polar filament may play a role in autoinfection and vertical (transovarial) transmission, which is highly efficient in transferring the microsporidium from adults of *M. occidentalis* to progeny.

The morphological and molecular data were in conflict, with the morphological data placing the new species in either the *Oligosporidium* or the *Unikaryon* genus, but the molecular data (based on ssrRNA) placed this microsporidium within the *Nosema/Vairimorpha* clade (Becnel et al. 2002). A 1.2-kb DNA band was amplified from genomic DNA preparations of infected *M. occidentalis* by high-fidelity PCR using microsporidian ssrRNA primers. The sequence data were unique, but most closely related to sequences obtained from GenBank from *Nosema apis* (U26534), *N. oulemae* (U27359), *N. ceranae* (U26533), *N. vespula* (UU11047), *N. portugal* (AF033316), *Vairimorpha lymantriae* (AF033315), and *V. necatrix* (Y00266) (Becnel et al. 2002). There are other recent examples where morphological and molecular data conflict and future molecular data on microsporidia from other mites and arachnids, as well as members of the Unikaryonidae, may establish better relationships.

Mites infected with *O. occidentalis* did not exhibit any external or gross signs of infection (Becnel et al. 2002). However, based on TEM analysis, *O. occidentalis* developed in eggs, larvae, nymphs, and adults of *M. occidentalis* and immature stages and mature spores of *O. occidentalis* were found in the cytoplasm of cecal cells, lyrate organ cells, ganglia, epithelial cells, muscle, within the ovary, and in developing and mature eggs.

Olson and Hoy (2002) quantified the effects of infection by *O. occidentalis* and showed that infected *M. occidentalis* females had a shorter life span, produced fewer eggs and adults, and fewer female progeny. Infection status appeared to have no effect on male longevity or progeny survival to larval and adult stages.

Three different heat treatments were tested to determine if it was possible to heat-cure the colonies (Olson and Hoy 2002). When eggs were placed for 7 days into a growth chamber at 33°C and 50–80% RH, then held at 27°C, infection rates were reduced but the microsporidia were not eliminated; 79% of the eggs treated in this manner survived to the adult stage, but 85% were infected (had spores visible upon microscopic analysis) immediately after treatment. Most of these spores probably were inviable because after 2 weeks, only 2% of the mites appeared infected but after 4, 6, 8, and 10 weeks the infection frequency in the colony derived from these heat-treated eggs climbed to 27, 83, 94, and 98%, respectively, indicating this heat treatment was only partly effective. By contrast,

when G_o eggs were deposited within the growth chamber and they and their progeny (G_1) were reared to adulthood at 33°C, all the G_1 mites were disease free.

It was possible to produce infected lines of *M. occidentalis* by feeding adult females with infected *M. occidentalis* eggs; cannibalism resulted in a high level of infection of the progeny of these fed females, indicating that horizontal transmission could occur by cannibalism (Olson and Hoy 2002). We had no evidence that the microsporidia are transmitted in feces of infected mites.

We are unaware of any field populations that have been infected with *O. occidentalis*. However, mass rearing of *M. occidentalis* for augmentative releases could be negatively affected should colonies become infected with this pathogen. The crowding and stress associated with mass rearing would facilitate the spread of the disease, especially if prey densities were inadequate. Because *M. occidentalis* is an obligatory predator, cannibalism can become more prevalent when colonies are reared with inadequate prey (Hoy, unpublished).

Other species of microsporidia have been found infecting other species of predatory mites, including *Phytoseiulus persimilis* Athias-Henriot (Bjornson et al. 1996, 1997), *Neoseiulus* (*Amblyseius*) *cucumeris* Oudemans and *N.* (*A.*) *barkeri* (Hughes) (Beerling and van der Geest 1991). It is unknown whether *M. occidentalis* can be infected by more than one species of microsporidia.

Rickettsia or *Rickettsia*-like microorganisms as pathogens?

Two morphologically distinct microorganisms, described as *Rickettsia*-like based on TEM, were found in tissues of three laboratory colonies of *M. occidentalis* reared on *T. urticae* (Hess and Hoy 1982). These colonies were examined because they were producing few eggs and several colonies died out. The 'sick' mites had two different pathologies: some adult females were plump and had a cream to pink plug that extruded from the rectum. Occasionally these plugged females became glued to the substrate. Plugged females rarely produced eggs, even though they appeared plump and gravid. Immatures and males rarely showed this plug. The second pathology affected females and immatures; in this case the mites became very pale and thin. These females failed to oviposit and the immatures often died, especially during their molts.

The two forms were described based on cell wall structure and cytoplasmic inclusions, although the forms were also described as pleomorphic (Hess and Hoy 1982). Type A was observed in all mites examined, while type B was observed in approximately two-thirds of the sick and healthy mites examined. Type B microorganisms occurred in all ovaries and eggs, indicating transovarial transmission may take place. In some mites, large numbers of type B bacteria were observed in all the internal organs, within the hemocoel, and within Malpighian tubule lumens and in the rectal plug. Because *Rickettsia* are intracellular microorganisms, their presence outside of tissues suggests they were pathogenic to their host cells.

The type A organism was small, ovoid, and approximately 0.75 microns long and 0.5 microns wide (Hess and Hoy 1982). The type A organism had a trilaminar membrane 7 nm thick. Exterior to the plasma membrane was a clear zone of variable width (12–15 nm) inside of which an intermediate electron-dense layer was often seen. This layer sometimes was applied closely to the outer cell wall. The cell wall was approximately 15 nm thick and divided into three layers. Another diagnostic of this organism was an internal fascicle of parallel-arranged tubular structures approximately 11 nm in diameter extending transversely through the organism. These tubular structures were associated with an electron-dense plate through which they extended to connect to the plasma membrane. All were

intracellular, occurring singly or in groups of two and three in all tissues except ovarian and nervous tissue. These were most numerous in the midgut, Malpighian tubules, and epidermis; their numbers increased to the point of filling the cytoplasm of a cell when type B microorganisms were also present.

Type B microorganisms were both intra- and extra-cellular, were rod-shaped, measuring 0.5 microns in width and up to 2 microns in length (Hess and Hoy 1982). They often had an indistinct internal unit membrane 7 m wide and were bounded by an outer trilaminar membrane of about 9 nm. The outer membrane frequently had a scalloped or wavy appearance. These membranes were separated by a clear area varying from 10 to 18 nm in width. Type B organisms varied considerably in substructure, but all possessed the same bounding membranes. Microorganisms of type B, when present in moderate numbers, were found free in the hemocoel, which is considered atypical for *Rickettsia*, and in the cytoplasm of the Malpighian tubules, as well as the ovaries.

Group B organisms were also found within membrane-bound vacuoles of cells "similar in structure to the mycetomes of insects" (Hess and Hoy 1982). To our knowledge, mycetomes have not been identified in the Phytoseiidae, but should be looked for because their presence suggests a very longterm relationship between the mite and the microbial inhabitants of the mycetomes. The "substructure of the microorganisms within the membrane-bound vacuoles differed from that of the microorganisms found free in the hemocoel" and "within the ovary," suggesting there might have been a third type of microorganism present. Because Hoy and Jeyaprakash (2005) found two bacterial species (*Bacteroidetes* and *Enterobacter*) typically associated with the gut of arthropods within *M. occidentalis* using a high-fidelity PCR protocol, it is possible that these type B organisms in the 'mycetomes' are symbionts that have increased in density.

Recently, Hoy and Jeyaprakash (unpublished) isolated *M. occidentalis* females from a crowded laboratory colony that had large anal 'plugs,' used 16S PCR primers to amplify the bacterial DNA, then cloned and sequenced the products. Interestingly, only the bacteria previously discovered in 'healthy' females were found, including *Wolbachia*, *Cardinium*, an undescribed species of *Bacteroidetes*, and an *Enterobacter* species (Hoy and Jeyaprakash 2005). These data suggest, but do not prove, that titers of one or more endosymbiont could increase in stressed mites, leading to 'disease.' Dale and Moran (2006) stated that "as more cases of chronic bacterial infection are characterized, the distinction between pathogenesis and mutualism has become increasingly blurred. Infection by a particular bacterium may be beneficial to a host under some circumstances but harmful in other hosts or environments."

An alternative interpretation is that pathogenic *Rickettsia*-like organisms could be obtained by *M. occidentalis* from their prey and remain in the gut or move into other tissues, but it is not clear whether *M. occidentalis* picked up any *Rickettsia* through feeding or horizontal transfer in the study by Hess and Hoy (1982). Hoy and Jeyaprakash (2005) subsequently found a *Rickettsia*-like organism in some populations of *T. urticae*, a common prey species for *M. occidentalis*, although these *Rickettsia* DNA sequences were not found in the carbaryl-organophosphorus-sulfur resistant (COS) colony of *M. occidentalis* in Gainesville, where the subsequent PCR analysis of the plug was conducted, perhaps because the adults were fed on *T. urticae* that lacked the *Rickettsia*. However, we do not know the infection status of the *T. urticae* fed to the 'sick' mites examined in the earlier Hess and Hoy (1982) study and these also could have been the cause of the pathology. Without molecular data to identify the bacteria it is difficult to compare past and current observations.

The presence of *Rickettsia* in some *T. urticae* colonies by Hoy and Jeyaprakash (2005) was a puzzle because bacteria in this genus are best known as arthropod-vectored

pathogens of vertebrates. Recent surveys, however, have shown that *Rickettsia* are found fairly often in arthropods and other invertebrates, even though the arthropod hosts are not associated with vertebrates. Perlman et al. (2006) suggested that *Rickettsia* could be symbionts that are transmitted vertically in invertebrates and only secondarily serve as pathogens of some vertebrates. Clearly, we could continue to identify more microorganisms from *M. occidentalis* and *T. urticae* when other populations are evaluated.

Serratia marcescens

Lighthart et al. (1988) showed that, under laboratory conditions, experimentally applied *Serratia marcescens* could cause mortality and reduce fecundity under high relative humidity, and in crowded, food-stressed, and heat-stressed adult females of *M. occidentalis*. These results suggest that poor mass-rearing conditions could facilitate the infection of *M. occidentalis* with this pathogen (or others), but we are unaware of any colonies naturally infected with *S. marcescens*.

Symbionts of *Metaseiulus occidentalis*

The mitochondrion: the ultimate microbial endosymbiont

Mitochondria are descended from microbial endosymbionts and are now essential cellular organelles. The typical arthropod mitochondrial genome is circular, ranging in size from 15 to 17 kb in size and encodes 13 proteins, 22 transfer RNAs, two ribosomal RNAs and a D-loop control sequence for initiating replication, with the genes located on one or both strands (Wolstenholme 1992; Boore 1999). When the mitochondrion from *M. occidentalis* was sequenced completely, we were surprised to learn that it is nearly 25 kb in size, which makes it the largest known mitochondrion so far detected in the Chelicerata (Jeyaprakash and Hoy 2007).

The large size was due to the presence of a duplicated region (9.9 kb) and a small (345 bp) triplicated region, in addition to the expected 14.7-kb segment containing the expected protein-coding genes (Jeyaprakash and Hoy 2007). Surprisingly there were two copies of the D-loop control sequence and two genes (*ND3* and *ND6*) appeared to be missing. The gene order is completely different from the pattern in mitochondria from all other known Chelicerata. The transfer RNA genes all are atypical, degenerate and difficult to detect; all have lost their usual clover-leaf-shaped structure. Three transfer RNAs use anticodons different from those used by other Chelicerata and this is reflected in the codon usage by the 11 protein-coding genes. This mitochondrial genome has been exceptionally active, recombining, rearranging, and undergoing sequence losses, especially in the transfer RNAs. Whether these unusual features are unique to *M. occidentalis* will be resolved once other phytoseiid mitochondrial genomes are sequenced. These results suggest that the genomes of the symbionts of *M. occidentalis* could exhibit unusual features compared to related microorganisms in other arthropods.

Gut symbionts

Hoy and Jeyaprakash (2005) used high-fidelity PCR and primers designed to identify Eubacteria, Archaeabacteria, iridoviruses, *Helicosporidia*, *Cytophaga*-like microorganisms, *Wolbachia* and its bacteriophage WO, fungi, and yeast-like organisms to determine

the diversity of microorganisms in the COS laboratory colony and in field-collected colonies of *M. occidentalis*. No Archaeabacteria, iridoviruses, *Helicosporidia*, fungi, or yeast-like organisms were found in the colonies of *M. occidentalis* tested using the high-fidelity protocol, which detects as few as 100 copies of target DNA 100% of the time and 10 copies 50% of the time (Jeyaprakash and Hoy 2000).

One DNA sequence using 16S primers was obtained from *M. occidentalis* that was related to an unnamed bacterium belonging to the Phylum *Bacteroidetes* (GenBank accession AY753171) that is closely related to one isolated from the gut of the lepidopteran *Lymantria dispar* (L) (Hoy and Jeyaprakash 2005). In addition, two sequences were isolated that were related to *Enterobacter* species (GenBank accessions AY753172, AY753173), also related to an *Enterobacter* species isolated from the *L. dispar* gut. The two *Enterobacter* sequences displayed 8-bp differences and were 1.9 and 2.3% divergent from the gypsy moth *Enterobacter* and a *Psoroptes ovis* (Hering) *Enterobacter* sequence, respectively. Because *Enterobacter* strains are usually found associated with the digestive tract in arthropods, we concluded that it is likely that the two unidentified strains of *Enterobacter* in *M. occidentalis* are gut symbionts. Unfortunately, we have not confirmed their location(s) by in situ hybridization.

The *Bacteroidetes* species present in *M. occidentalis* was related to a bacterium isolated from the gut of *L. dispar*, and so could also be a gut symbiont or associated with mycetocytes (which have only been suggested as occurring in *M. occidentalis* by Hess and Hoy 1982). As noted by McCutcheon and Moran (2007), *Bacteroidetes* species are widely distributed in the environment and can be found in coastal marine waters, the human gut and dental plaques, and in insects. McCutcheon and Moran (2007) sequenced the genome of a *Bacteroidetes* species (*Sulcia*) that was isolated from the bacteriome of the sharpshooter *Homalodisca vitripennis* (German) and found that its genome was extremely reduced (to only 245 kb, encoding 228 protein genes), which is approximately one-tenth the size of the smallest known *Bacteroidetes* genome. Such a reduction indicates a very longterm relationship with its host. Based on their genome analysis, *Sulcia* is "largely devoted to essential amino acid synthesis," with the pathways for leucine, valine, threonine, isoleucine, phenylalanine, and tryptophan all complete. *Sulcia* has a minimal set of genes to transcribe RNA, to translate protein, and to replicate its genome, but its set of tRNA synthetases is incomplete. Genes involved in DNA repair are limited, and *Sulcia* contains few transporters that would allow molecules to cross the cell membrane. It appears that the metabolic capabilities of *Sulcia* and *Baumannia* (the other symbiont of the sharpshooter) are "broadly complementary in that *Sulcia* is primarily devoted to amino acid biosynthesis whereas *Baumannia* is primarily devoted to cofactor and vitamin synthesis" (McCutcheon and Moran 2007). We do not know whether the *Bacteroidetes* species in *M. occidentalis* also provides nutritional services for its host or its location in its host. However, because it is transovarially transmitted, it may be an important or, even, essential associate of *M. occidentalis*.

When the analysis of microbial associates of *M. occidentalis* was conducted by Hoy and Jeyaprakash (2005), few DNA sequences were available in GenBank for comparison in the *Bacteroidetes-Flavobacterium* group that were associated with arthropods. Figure 1 shows a new Bayesian phylogeny based on additional sequences. It is interesting to note that all *Cardinium* sequences cluster together, as expected. The unnamed species of *Bacteroidetes* from *M. occidentalis*, however, clusters with endosymbionts from the "fat body endosymbionts (*Blattabacterium* spp.)" from *Cryptocercus* woodroaches (Maekawa et al. 2005). It also clusters with the endosymbiotic bacteria associated with bacteriomes from armored scale insects (Gruwell et al. 2007), and to *Sulcia* isolated from bacteriomes from the

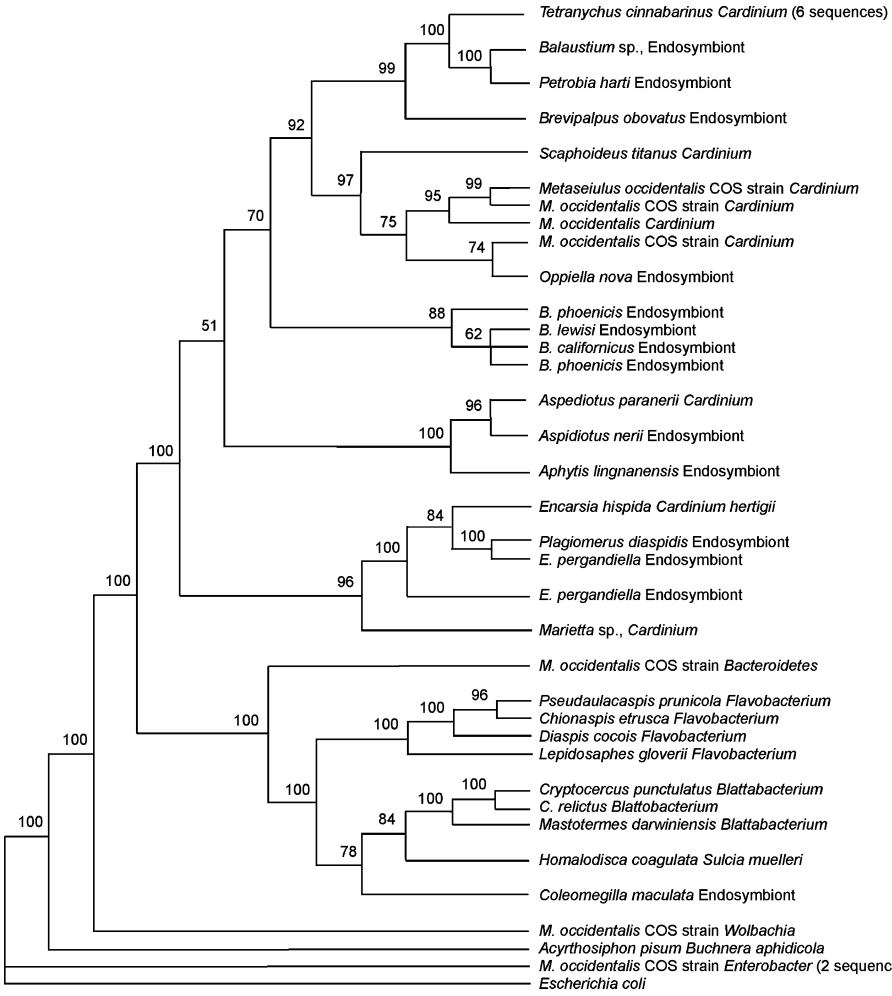

Fig. 1 Bacterial 16S rRNA sequences from the mites *M. occidentalis* (AY279413, AY635291, AY753169-AY753173 and AY754820), *Balaustium* sp., (AY279411), *Brevipalpus phoenicis* (AF279412 and AF350221), *B. lewisi* (AB116515), *B. californicus* (AB116514), *B. obovatus* (AY279401), *Oppiella nova* (AY279414), *Tetranychus cinnabarinus* (DQ369961-DQ369965 and DQ449047) and *Petrobia harti* (AY279410), the scale insects *Aspediotus paranerii* (AY327469), *Aspidiotus nerii* (AY279402), *Pseudaulacaspis prunicola* (DQ868853), *Chionaspis etrusca* (DQ868858), *Diaspis cocois* (DQ868816) and *Lepidosaphes gloverii* (DQ868832), the hymenopterans *Aphytis lingnanensis* (AY279404), *Encarsia hispida* (AY331187), *E. pergandiella* (AF319783 and AY026335), *Plagiomerus diaspidis* (AY327472) and *Marietta* sp. (AY327470), the leafhopper *Scaphoideus titanus* (AM042540), the cockroaches *Cryptocercus punctulatus* (AF322471) and *C. relictus* (AB211183), the termite *Mastotermes darwiniensis* (Z35665), the sharp-shooter *Homalodisca coagulata* (AY147399) and the ladybird beetle *Coleomegilla maculata* (Y13889) were obtained from GenBank. Sequences from the aphid *Acyrthosiphon pisum* (M27039) symbiont and from *Escherichia coli* (AE000474) were included as outgroups, all were aligned using CLUSTAL X (1618 aligned characters) to generate a tree using the bestfit model (GTR + G + I) in MrBayes (ngen = 1 million, printfreq = 10000, samplefreq = 100, nchains = 4, burinin = 2500). Posterior probability values are shown before the node

sharpshooter. These relationships suggest that the unnamed *Bacteroidetes* from *M. occidentalis* could be from a putative mycetocyte. The main anomaly in this clade is the endosymbiont from the ladybeetle *Coleomegilla maculata* (Mulsant), which Hurst et al. (1997) suggested is associated with male killing in *C. maculata*. However, Hurst et al. (1997) sequenced only four clones isolated from beetles with the sex-ratio trait and it is possible they could have found other microorganisms if more clones had been sequenced.

Alternatively, other *Bacteroidetes* species (especially *Cardinium*) have been implicated as causing reproductive incompatibility, parthenogenesis, or feminization in some arthropods (Hunter et al. 2003; Zchori-Fein and Perlman 2004; Zchori-Fein et al. 2004), so additional work must be conducted to resolve how diverse the phenotypes are that are associated with these microorganisms. Clearly, much more needs to be learned about the role of *Bacteroidetes* species in arthropod biology. Without obtaining colonies of *M. occidentalis* that have only one type of microorganism (e.g., either *Wolbachia* or *Cardinium* or the undescribed species of *Bacteroidetes*), it is difficult to assign a function to these microbial symbionts in *M. occidentalis*.

To determine if infections of *M. occidentalis* with the undescribed species of *Bacteroidetes* or *Enterobacter* were a laboratory artifact, species-specific primers were designed from the sequences obtained from the COS colony of *M. occidentalis* and used to amplify DNA from field-collected colonies (grapes from Washington, hops from Washington, almonds from California and cherries from California) (Hoy and Jeyaprakash 2005). The expected DNA bands were produced, indicating that these symbionts were found consistently in both laboratory-reared and field-collected populations of *M. occidentalis* sampled from diverse crops and geographic areas.

Reproductive tract symbionts

Using 16S rRNA primers and a high-fidelity PCR protocol, Hoy and Jeyaprakash (2005) found that the COS laboratory strain of *M. occidentalis* had one *Wolbachia* strain and one unnamed species related to *Candidatus* Cardinium hertigii (Fig. 1). When colonies of *M. occidentalis* isolated from grapes and hops in Washington or almonds and cherries in California were screened, all were positive for these sequences. As expected, both the *Wolbachia* and bacteria related to *Candidatus* Cardinium hertigii are transovarially transmitted (Jeyaprakash and Hoy 2004).

The role(s) that the *Enterobacter*, *Wolbachia*, *Cardinium* or unnamed species of *Bacteroidetes* symbionts play remains unclear due to several methodological difficulties (and due to a lack of resources to conduct the experiments). In fact, we don't know precisely where these microorganisms can be found in *M. occidentalis*. Without conducting in situ hybridization on different tissues using probes specific for these microorganisms, we can only speculate that they are restricted to specific tissues. Some *Wolbachia*, for example, can be found in tissues other than the reproductive tract in other arthropods.

Other potential endosymbionts, including pathogens

Enigl and Schausberger (2007) conducted a survey of seven species of phytoseiids using *Wolbachia*, *Cardinium*, and *Spiroplasma* primers. *Spiroplasma* was detected in the phytoseiid *Neoseiulus* (*Amblyseius*) *californicus* (McGregor), but none of the other phytoseiid species tested. Whether one or more populations of *M. occidentalis* carry *Spiroplasma* is unknown, because Hoy and Jeyaprakash (2005) did not survey their populations for

Spiroplasma and we are unaware of any other surveys of *M. occidentalis* populations for this organism.

Hoy and Jeyaprakash (2005) found that none of the six colonies of *M. occidentalis* tested contained the WO bacteriophage of *Wolbachia*, despite the fact that two of the *T. urticae* colonies did. It is always possible that a broader survey of *M. occidentalis* colonies would yield evidence of infection of *Wolbachia* with WO bacteriophage.

Gols et al. (2007) found a pathogenic bacterium, *Acaricomes phytoseiuli*, in six of seven populations of *P. persimilis* from several commercial sources. This bacterium reduced longevity and fecundity and altered behavior, reducing attraction to herbivore-induced plant volatiles, which could reduce efficacy of these important natural enemies in augmentative biological control programs. However, the other predators tested [*P. macropilis* (Banks), *A. andersoni* Chant, *N.* (*A.*) *cucumeris*, *Iphiseius* (*Amblyseius*) *degenerans* (Berlese), *N.* (*A.*) *californicus*, *Hypoaspis aculeifer* Canestrini, or *H. miles* Berlese] were not positive for *A. phytoseiuli*. Gols et al. (2007) questioned whether *A. phytoseiuli* is restricted to mass rearing programs where stress could enhance infection or whether the pathogen originates from infection of a field population of *P. persimilis* that was later introduced into the laboratory rearings. Infection with *A. phytoseiuli* is transmitted horizontally through contact with feces deposited by infected mites and the disease is considered to be "highly infectious." It would be interesting to learn whether this pathogen can be introduced into other phytoseiids, including *M. occidentalis*.

Methodology

High fidelity versus standard allele-specific PCR

When Johanowicz and Hoy (1996, 1998a, b, 1999) worked on *Wolbachia* using the 'standard' allele-specific PCR, we believed, based on a failure to amplify PCR products, that we had heat-cured *M. occidentalis* and removed the *Wolbachia*. At that time, we were not aware that *M. occidentalis* was infected with additional microorganisms such as the rickettsia-like microorganisms observed by Hess and Hoy (1982). Subsequently, when we began to use a more sensitive and specific PCR protocol [known as high-fidelity or long PCR (Barnes 1994) because it incorporates a second DNA polymerase that has proof-reading ability], we discovered that we were probably getting false negatives in our standard PCRs for *Wolbachia*. This is because the high-fidelity PCR is six to eight orders of magnitude more sensitive than standard allele-specific PCR (Jeyaprakash and Hoy 2000). It would be very helpful if, in the future, all authors reporting on the presence/absence of specific microorganisms reported the sensitivity of their PCR protocol.

Multiple displacement amplification

Because *M. occidentalis* is so small, it is difficult to obtain consistent PCR products using *Wolbachia*-specific primers from single eggs or adults, even when using high-fidelity PCR (Jeyaprakash and Hoy 2000). In order to determine whether individual adults or eggs were positive for *Wolbachia*, we used a multiple displacement amplification (MDA) protocol to amplify the DNA prior to conducting a high-fidelity PCR (Jeyaprakash and Hoy 2004). MDA amplifies genomic DNA from purified and unpurified lysates at 30°C in a few hours without a thermal cycler (Dean et al. 2002). It uses exonuclease-resistant thiophosphate-modified degenerate hexamers as primers and bacteriophage Phi29 DNA polymerase to

amplify the genomic DNA. It is assumed that the hexamers bind at random over the genome, allowing the Phi29 DNA polymerase to synthesize DNA strands up to 10 kb in length, thus enriching each amplified DNA stand by approximately 10,000-fold. DNA amplified by MDA can be used as templates for the PCR, as well as for use in other studies, such as Southern blot analysis.

We discovered that using MDA could produce a detectable DNA band after high-fidelity PCR from as little as 0.01 femtograms of plasmid containing the target DNA at least 50% of the time, which is equivalent to the ability to amplify a single copy (Jeyaprakash and Hoy 2004). Detectable PCR products using *wsp* primers for *Wolbachia* also could be produced from naturally infected single females and eggs of *M. occidentalis*, which allows detailed studies of infection frequency and transovarial transmission of several microorganisms associated with this predatory mite. MDA analysis of eggs of the COS colony of *M. occidentalis* indicated *Wolbachia* and *Cardinium* were transovarially transmitted. No PCR primers for the *Enterobacter* or unnamed species of *Bacteroidetes* were used, and it would be interesting to learn whether these species also are transovarially transmitted. A negative PCR after the use of the MDA protocol would provide more substance to claims that individuals are negative for a particular microorganism.

Wolbachia infection dynamics in experimental laboratory populations of *Metaseiulus occidentalis*

Wolbachia has been shown to increase the prevalence of *Wolbachia*-infected hosts in a population (reviewed by Johanowicz and Hoy 1999). Models using three parameters (the proportional failure of a mother to transmit *Wolbachia* to her offspring, the proportion of progeny produced by incompatible crosses relative to compatible crosses, and the relative fitness of infected matings relative to uninfected matings) have been used to predict whether *Wolbachia* would sweep through a population, which could be useful in driving useful genes into target populations in genetic manipulation projects.

To investigate whether *Wolbachia* would sweep through replicated populations of *M. occidentalis* under laboratory conditions over 12 generations after being initiated with an initial infection frequency of 10%, we monitored reproductive compatibility in populations 'with and without *Wolbachia*' and also used standard allele-specific PCR as a second indicator (Johanowicz and Hoy 1999). Unfortunately, at the time of this experiment, we were not aware that this population was also infected with *Cardinium*, *Enterobacter* or the unnamed species of *Bacteroidetes*. To date, we cannot determine whether the effects observed in the experiment were due to low titers of *Wolbachia* or to *Cardinium*, or, even, to the other bacterial symbionts. We have tried to eliminate *Wolbachia* and *Cardinium* in colonies of *M. occidentalis* using both heat and antibiotic treatments (Hoy and Jeyaprakash, unpublished), but have been unable to completely eliminate either based on high-fidelity PCR assays, perhaps due to the fact that high-fidelity PCR is so sensitive that we can detect as few as 100 copies of *Wolbachia* or *Cardinium* DNA consistently and as few as 10 copies approximately 50% of the time. Thus, although the titer of these microorganisms may have been reduced, they were not completely eliminated based on high-fidelity PCR data. Quantitative PCR could allow us to determine at what titer the reproductive incompatibility is expressed when heat-treated and untreated mites are crossed. Alternatively, the failure to eliminate both bacteria could be because the *Wolbachia* or *Cardinium* genomes have been incorporated into the nuclear genome of *M. occidentalis*, as it has with other arthropods.

In any case, Johanowicz and Hoy (1999) found that the proportion of compatible crosses did not change over time in the mixed populations containing individuals putatively with and without *Wolbachia*. Furthermore, unexpected compatibility occurred, which could be explained most easily by imperfect maternal transmission of *Wolbachia*, at a rate of 0.05. The relative numbers of viable eggs indicated that there were also fitness costs. Fewer daughters were produced in the incompatible crosses, which could be due to the effects of *Wolbachia* (or *Cardinium*) on the parahaploid genetic system of *M. occidentalis*. Thus, the initial infection frequency of 10% was apparently below an unstable equilibrium frequency (Hoffman et al. 1990).

When this study was designed, the choice of an initial infection frequency of 0.10 was influenced by earlier studies and by the belief that releasing more than 10% of the absolute population density in a practical pest management program is likely to be difficult and expensive. Estimates of population densities in the field suggest that there could be as many as 20 million *M. occidentalis* per acre (Hoy 1982). If so, then as many as 200,000 mites per acre might be needed to reach the 10% release rate; if releases were made in early spring, then as many as 2,000 mites per acre would be required, which is potentially cost effective. However, the fitness costs observed, whether due to *Wolbachia* or *Cardinium* or both, were serious; although egg production was not significantly different in infected versus uninfected control crosses, there were significantly fewer daughters produced in the infected crosses, resulting in a fitness cost of 0.60 for infected crosses relative to uninfected crosses. A variety of other assumptions were violated in the models. For example, remating by *M. occidentalis* females is common, which increases the chance that uninfected females will mate successfully with uninfected males within their reproductive lifespan. The normal sex ratio of *M. occidentalis* also violates the models' assumption of a 1:1 sex ratio and the assumption of panmixis also may be violated because non-random mating has been observed in this species (Hoy and Cave 1988).

This experiment, although flawed because we cannot resolve whether the results were due to infection with *Wolbachia* or *Cardinium* or other microbial endosymbionts, suggests that one or more of these microorganisms may be causing 'disease.' By contrast, Weeks and Stouthamer (2003) suggested that a *Cytophaga*-like organism, now considered *Cardinium*, was associated with a "significant increase in the fecundity of infected females." These authors used adults of *M. occidentalis* obtained from a commercial facility in California and reared them on *Tetranychus cinnabarinus* (Boisd.) (believed to not be infected with *Wolbachia* or *Cardinium*, although the authors do not indicate how they know the prey were negative nor do they indicate the sensitivity of their heminested PCR protocol used to conclude that the *M. occidentalis* were negative for these infections). A subsample of *M. occidentalis* was treated with tetracycline and inbred lines were developed that, putatively, were infected with *Cardinium* but not with *Wolbachia*. Crosses were made between three infected and uninfected lines and fitness was evaluated using the number of eggs, egg viability, F1 mortality, and adult sex ratio. All three crosses indicated the infected females deposited more eggs over 6 days compared to the uninfected females, although egg viability was not different. However, fecundity of infected females averaged only 10.0 eggs/female over 6 days for inbred line two, while fecundity for the uninfected lines averaged only 7.3 eggs/female/6 days. Because *M. occidentalis* females typically deposit 2 (or more) eggs per female per day if healthy and well fed during 6 days, the level of fecundity observed in their experiments is lower than expected for both categories. Whether this is due to their use of tetracycline to treat the colonies is unknown; we speculate that elimination of gut symbionts (such as *Enterobacter*, if present) could have reduced vigor in their colonies. Similar results are given for the second line. We agree with the conclusion of

Weeks and Stouthamer (2003) that "until these effects can be partitioned out, the actual phenotypic effects" of any of the symbionts cannot be resolved. We believe that any future studies on the symbionts of *M. occidentalis* should be conducted on populations that have had a complete characterization of their microbial fauna. Ideally, studies should be conducted on lines that lack all but the symbiont species of interest and the sensitivity of the PCR protocols used should be characterized.

Conclusions

Although we know a great deal about the biology, behavior, genetics, and effectiveness of *M. occidentalis* as a natural enemy in IPM systems, we clearly lack a comprehensive understanding of the role of pathogens, symbionts, and other microbial associates of this predatory mite. We know nothing about the role of pathogens in the population dynamics of this important predator under field conditions.

This gap in knowledge has occurred in part because there was little recognition that the microbes associated with *M. occidentalis* were important. In addition, the tools with which to study these interactions have been lacking until recently and still are underutilized. Until the invention of the PCR, it was difficult to resolve how many types of microorganisms were present within *M. occidentalis*. Furthermore, without the ability to work with strains of mites that have differing numbers of microbial endosymbionts, it is difficult to resolve the role(s) these organisms play in the biology of this predator. At present it is unclear whether any of these microorganisms are obligatory or whether they can be eliminated without harm, although the fact that *Wolbachia and Cardinium* are transovarially transmitted suggests these organisms have an especially intimate relationship with their host. Moreover, we do not know what tissues these microorganisms inhabit in *M. occidentalis*, nor do we understand whether their titer affects their host.

It is possible that the use of metagenomic tools may resolve some of these difficulties. If the genomes of these microorganisms were sequenced and the function(s) of the genes analyzed, the physiological roles that these organisms play may be resolved (Handelsman 2004; Woyke et al. 2006). As noted by McCutcheon and Moran (2007), obtaining symbiont DNA separately from insects (or mites) is difficult. McCutcheon and Moran (2007) attempted to enrich for the *Bacteroidetes* (*Sulcia*) DNA in their project by dissecting the appropriate bacteriome from the insect host, but they still got a complex sample containing DNA from the insect, as well as from *Sulcia* and *Baumannia* (another symbiont), with the insect DNA constituting the majority fraction of the sample by weight and the *Sulcia* DNA representing the majority of the bacterial fraction. However, the sequenced microbial DNA could be analyzed and a new software (GLIMMER) developed to discriminate between host and symbiont DNA was not needed (McCutcheon and Moran 2007). Certainly, if the nuclear genome of *M. occidentalis* is sequenced or EST (Expressed Sequence Tag) analysis is conducted, any 'bacterial' DNA sequences obtained should not be discarded as contaminants but, rather, assembled and analyzed for their possible function. It is likely that a considerable proportion of the microbial genomes in *M. occidentalis* can be obtained in this manner.

Because it has been difficult, at least using the colonies of *M. occidentalis* in our laboratory, to develop strains that lack *Wolbachia* or *Cardinium* (based on high-fidelity PCR data) using heat or antibiotic treatments, it also may be necessary to analyze these populations to determine whether *Wolbachia* or *Cardinium* genomes have been horizontally transferred into the nuclear genome, as has been shown for at least 11 other arthropod species

(Hotopp et al. 2007). If horizontal transfer into the nuclear genome of *M. occidentalis* has occurred, it will be interesting to determine whether any symbiont genes are transcribed, as was reported for some *Wolbachia* genes. Insertion of symbiont genes into the nuclear genome is reminiscent of the steps involved in the evolution of the bacterial symbiont that became the mitochondrial organelle. Clearly, many interesting questions remain to be answered about the relationships between the genomes of *Wolbachia*, *Bacteroidetes*, *Cardinium* and the nuclear and mitochondrial genomes of *M. occidentalis.*

Mass rearing or laboratory rearing of *M. occidentalis* typically occurs under conditions that are considerably more crowded than would occur under field conditions and these conditions cause stress. Whether the endosymbionts of *M. occidentalis* ever become pathogenic in the event that the immune system of *M. occidentalis* becomes compromised remains to be confirmed. The electron micrographs published by Poinar and Poinar (1998) suggest that pathogenesis might occur if these microbial symbionts were to increase in density because the phenotype of the electron micrographs suggest that *Cardinium*, the unnamed *Bacteroidetes*, and/or *Wolbachia* could have been the causative agents of pathogenesis. Quantitative PCR could allow rapid quantification of symbiont density under different environmental conditions and a correlation with 'disease' symptoms.

Acarologists wanting to understand the roles symbionts play in their acarine hosts have significant challenges to meet. However, we could use, in part, the information and methodology developed for The Human Microbiome Project, which has been organized to understand the diversity and evolution of microorganisms associated with the human skin, reproductive tract, digestive tract and mouth (Dethlefsen et al. 2007; Turnbaugh et al. 2007). It is becoming clear that the microbial communities of humans are characteristic and complex mixtures of many microorganisms that have co-evolved with humans. These microorganisms affect the nutrient or energetic value of food, the metabolism of xenobiotics, are involved in the renewal of gut epithelial cells, and the development and activity of the human immune system. In animal models, even the size of the heart and the behavior of the animal are affected by the lack of their normal gut fauna (Turnbaugh et al. 2007). The human microbial communities and their interactions with their human hosts have only begun to be described, as they have with arthropod-microbial consortia. It is recognized that "Only with an integrated approach will it be possible to comprehend the complex ecology of human health and the many ways in which interactions between humans and microorganisms can go awry" (Dethlefsen et al. 2007). The first step is to describe the microbial community composition, determine how this varies over time and among individuals, and with respect to changes in diet, host genotype, and health. This will require studies of model systems other than that of humans for technical and ethical reasons.

Experimental models using simple consortia, such as those seen in many invertebrate-microbe communities (including mites or ticks), will facilitate the molecular dissection of interactions in intact natural settings. The genetic tools available for some invertebrate model hosts will allow the identification of genes and proteins that control arthropod host responses and manage the consortia. The Human Microbiome project will attempt to "move beyond comparative genomics to an integrated 'systems metagenomics' approach that accounts for microbial community structure (the microbiota), gene content (the microbiome), gene expression (the 'meta-transcriptome' and 'metaproteome') and metabolism (the 'meta-metabolome')." Tools to achieve this will require the development of functional gene arrays to determine the relative abundance of specific genes or transcripts in microbiomes. The construction and sequencing of complementary DNA libraries, or expressed sequence tag analysis (EST) form an alternative approach (Nagaraj et al. 2006), but these require high-throughput methods for eliminating highly abundant transcripts

(Turnbaugh et al. 2007). It is possible that the relatively simple consortium of microbial associates of *M. occidentalis* could contribute to an understanding of the broader questions regarding the role(s) of microbiomes in the biology of their hosts.

It may also be true that arthropod-symbiont associations will have to be studied over time to understand that fine-scale evolutionary processes occur between the host and symbiont genomes (Riegler and O'Neill 2007). Recent papers reviewed in this article indicate that the host–symbiont relationship is more dynamic than appreciated, with some insect populations that formerly exhibited a fitness cost due to *Wolbachia* infection no longer doing so, perhaps due to adaptation in the *Wolbachia* genome over a period of about 15 years. In another example cited by Riegler and O'Neill (2007), a butterfly with a skewed sex ratio due to *Wolbachia* was shown to have evolved a resistance to the sex-ratio modifying ability of the *Wolbachia* over a period of a few years. This dynamism indicates that comparing symbiont effects on different populations of *M. occidentalis* at different times may yield different conclusions due either to the evolution of the host or of the symbiont.

Dillon and Dillon (2004) noted "A comprehensive understanding of the biology of insects requires that they be studied in an ecological context with microorganisms as an important component of the system." The same conclusion can be reached that a comprehensive understanding of the biology of *M. occidentalis* requires a full understanding of their microbial associates under both laboratory and field conditions.

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