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# Insect Pathogens and Insect Parasitic Nematodes

# Les Entomopathogènes et les Nématodes Parasites d'Insectes

editor:

Bernard Papierok

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IOBC / WPRS

Working group "Insect Pathogens and Insect Parasitic Nematodes"

OILB / SROP

Groupe de travail "Les Entomopathogènes et les Nématodes Parasites d'Insectes"

# 9th European Meeting

# "Growing Biocontrol Markets Challenge Research and Development"

at / à

Schloss Salzau, Kiel (Germany)
23-29 May 2003

Edited by Bernard Papierok

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## Preface

This bulletin contains the proceedings of the 9th European meeting of the IOBC/WPRS Working Group "Insect Pathogens and Insect Parasitic Nematodes". Entitled "Growing Biocontrol Markets Challenge Research and Development", this meeting was held in Salzau, Kiel, Germany, 23-29 May 2003, together with meetings of COST Action 842 "Biocontrol of Pest Insects and Mites, with Special Reference to Entomophthorales" and COST Action 850 "Biocontrol Symbiosis".

On behalf of the Working Group and all attendants, it is my pleasure to warmly express my gratitude towards the Local Organizing Committee and especially the Local Organizer, Dr Ralf-Udo Ehlers, from the Department of Biotechnology and Biological Control, Institute of Phytopathology, Christian-Albrechts-University Kiel, for the excellent organization of the meeting.

Thank you, Ralf, thank you also, Nicola, Heike, and all your colleagues for a scientifically and socially very fruitful meeting !

More than 140 people from European countries but also from Egypt, Indonesia, The Palestininan Authority, Turkey and USA attended the meeting, which offered oral presentations and poster sessions. Most of the contributions were dedicated to entomopathogenic fungi and insect parasitic nematodes, but there were sessions devoted to *Bacillus thuringiensis*, to other entomopathogens, to bacterial symbionts, to safety and risk assessment as well. Furthermore, a session was devoted to slug and snail control. This is due to the fact that the corresponding sub-group, as the sub-group "*Melolontha*", formerly from the Working Group "Soil Pests", were included in the Working Group "Insect Pathogens and Insect Parasitic Nematodes" when the Council of IOBC/WPRS closed the Working Group "Soil Pests" (Council Meeting of 25-26 January 2002).

Bernard Papierok
Convener of the Working Group
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# Insect Parasitic Nematodes and Slug Parasitic Nematodes

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# Potential of entomopathogenic nematodes for biological control of selected pest insects infesting urban trees

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**Abstract**: In the reported research infectivity and biocontrol potential of a series of *Steinernema feltiae* and *Heterorhabditis megidis* strains were tested against 12 species of the most common pest insects infesting urban trees. Eight of the examined species (i.e. *Operophtera brumata*, *O. fagata*, *Altica quercetorum*, *Agelastica alni*, *Phyllodecta laticollis*, *Phytodecta quinquepunctata*, *Acantholyda erythrocephala* and *Arge berberidis*) showed to be highly susceptible to nematode infection during pupation in the soil in both laboratory and semi-field conditions. For all, but two species (i.e. *P. laticollis* and *A. berberidis*) *H. megidis* was more effective than *S. feltiae*. However, great variation was observed between efficacy of particular strains in the latter species. Proper timing of application was essential to the nematode efficacy since in some species, such as *O. brumata* and *O. fagata* only mature larvae and prepupae were susceptible to infection, while pupae remained almost completely resistant. The potential of entomopathogenic nematodes for effective, environmentally safe, and still economic biological control of tree pests in urban parks and alleys is discussed.

**Key words**: biological control, entomopathogenic nematodes, *Heterorhabditis bacteriophora*, *Steinernema feltiae*, urban tree pests

## Introduction

High safety requirements set for the urban environment effectively limit the range of chemical insecticides available for control of pest insects within city green areas. The use of biological insecticides, such as *Bacillus thuringiensis* is only occasionally possible, due to relative selectivity of the agent, patchy distribution of trees and technical difficulty in spraying elevated tree canopy. However, during the period of pupation in the soil mature larvae and pupae of many tree pest species can be challenged with entomopathogenic nematodes, easily applied through a simple ground spraying. Up to the present only a few studies have been conducted to evaluate the nematode biocontrol efficacy against tree pests (Georgis & Hague, 1988; Mracek & David, 1986; Thurston, 1998.). However, the obtained results showed to be promising. Therefore, in 1998 we have undertaken a detailed laboratory and field research to examine the nematode potential in controlling urban tree pests in city parks and forests. The main objectives of the reported study were:

*i.* to investigate the susceptibility of selected pests of urban trees and shrubs to nematode infection,
*ii.* to examine effective methods of nematode application in urban parks and forests, and
*iii.* to search for potential new niches where entomopathogenic nematodes could be effectively used in the practice of tree protection.

## Material and methods

***Nematodes***

A series of strains and isolates of *Steinernema feltiae* and *Heterorhabditis megidis*, two species most commonly present in the soil of urban parks and forests were examined in detail. Occasionally, *H. bacteriophora*, *S. bicornutum*, *S. affinae*, and *S. carpocapsae* were also used in laboratory tests.

***Insects***

The nematode infectivity and biocontrol potential were examined against 12 species of the most important insect pests of urban trees. The life-cycle of all the selected species included at least a short period spent in, or on the soil. The insects represented three taxonomic orders, i.e. Lepidoptera (*Operophtera brumata*, *O. fagata*, *Cameraria ohridella*), Coleoptera (*Altica quercetorum*, *Agelastica alni*, *Phyllodecta laticollis*, *Phytodecta quinquepunctata*), and Hymenoptera (*Acantholyda erythrocephala*, *Arge berberidis*, *Arge ochropus*, *Pristiphora abietina*, *Caliroa annulipes*). Mature larvae of the insects were collected in the field and reared in the laboratory on appropriate plants until descending to the soil for pupation.

***Laboratory test***

The nematode infectivity was examined in the laboratory, in 12-well plates. The insect mature larvae, prepupae, pupae or young adults were individually placed into wells and covered with a moist peat-moss. The nematodes at the rate of 1 or 5 $x10^5$ IJ $m^{-2}$ were applied to the top of the substrate. After 3-day incubation at 20±1°C the insects were recovered, washed in distilled water and dissected. Both the number of infected insects, and the number of nematodes per individual insect were counted. The experiments were performed in 4 - 5 replicates per variant with a single, 12-well plate considered as a replicate.

***Semi-field tests***

The nematode biocontrol efficacy against selected insect species was examined in a series of semi-field tests. The experiments were set-up under the canopy of urban trees in city parks. The experimental variants included soil treatment with ***i.*** *S. feltiae*, ***ii.*** *H. megidis*, and ***iii.*** water control. In appropriate time for particular pest species plastic pots (16 cm in diameter and 13 cm in height) were partially inserted into the ground, and filled with a mixed type of sandy loam soil. Subsequently, a glass tube (16 cm in diameter and 30 cm in height) was fixed with masking tape atop each pot. Nematodes at a concentration of $10^5$ IJ $m^{-2}$ (i.e. 2000 IJ/pot) in 1 mL water were applied to the soil surface with an automatic pipette. After 1 hour a bundle of freshly cut twigs of host trees, in a 200 mL flask of water, was placed on the soil in each pot. Depending on the insect species 20-25 mature larvae were transferred to leaves of each bundle. The containers were then covered with a nylon screen affixed at the top with a rubber band. The pots were left under a mosaic shadow of tree canopy. Emergence of adult insects after pupation in the soil was examined and compared between experimental variants. Eight replicates (pots) were used per experimental variant.

## Results and discussion

***Lepidoptera***

The horse chestnut leaf miner (*C. ohridella*) is a major, and highly destructive pest of horse chestnuts in Europe. The insect larval and pupal development takes place inside the host tree leaves. The overwintering generation pupates inside fallen leaves on the soil surface. The nematodes applied to the soil can enter the mines and infect mature larvae late in the fall. The pest pupae were also occasionally infected by *S. bicornutum*. However, after the pupal cocoon

is span, the nematodes cannot reach the host. Therefore, nematode efficacy against this pest is rather negligible.

Winter moths (*Operophtera brumata* and O. *fagata*) are the most common lepidopterous pests of many tree species in urban parks. They significantly damage the foliage and frequently cause complete defoliation. Laboratory experiments revealed that pupae were completely resistant to *S. feltiae* and *H. megidis*, while mature larvae and prepupae were highly susceptible. Significant differences were, however, observed between the nematode species and strains (Tomalak, 2003). Results of the semi-field experiment clearly supported the laboratory findings and showed, that nematodes applied shortly before descending of winter moths larvae to the soil, effectively controlled populations of pupating insects. After 45-day experiment only 3.1, 42.5 and 58.7% individuals survived in the soil treated with *H. megidis* (wod-1), *S. feltiae* (ScP), and water (control), respectively (Tomalak, 2003).

***Coleoptera***

Several species of leaf beetles (Chrysomelidae) regularly invade urban trees and shrubs. Gregarious feeding of larvae, which graze the leaf surface, and biting out holes by adult insects can cause extensive damage to the foliage already in late spring and early summer. All the examined leaf beetle species pupate in the soil. Both prepupae and pupae showed to be highly susceptible to nematode infection. Fig. 1 shows results of the laboratory experiment on nematode infectivity to the insects' pupae. With exception of *P. laticollis* all species were more susceptible to *H. megidis* than to *S. feltiae*.

In the semi-field experiment only 56 and 78% of *A. quercetorum* and *A. alni*, respectively, completed their pupation and emerged from the soil in untreated control (Tomalak, in press). The presence of nematodes contributed to further reduction of the insect populations. None or only 0.7% of *A. alni* and *A. quercetorum*, respectively, emerged as adults after pupation in the soil pre-treated with *H. megidis*. *S. feltiae* was overall less effective. Distinct differences in mortality were also observed between the insect species. Respectively, 12 and 56% of *A. quercetorum* and *A. alni* emerged after pupation in the soil pre-treated with this nematode.

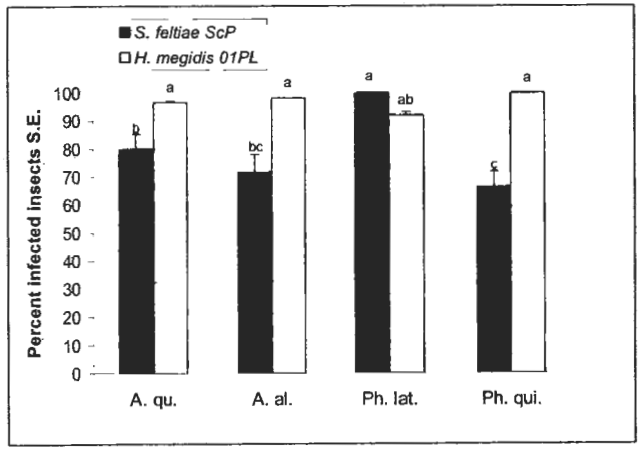


Figure 1. Infectivity of *Steinernema feltiae* and *Heterorhabditis bacteriophora* to leaf beetles pupae. (A. qu. – *Altica quercetorum*; A. al. – *Agelastica alni*; Ph. lat – *Phyllodecta laticollis*; Ph. qui.– *Phytodecta quinquepunctata*; nematodes were applied at $10^5$ IJ $m^{-2}$).

***Hymenoptera***

Many sawflies in the families Pamphilidae, Diprionidae and Tenthredinidae are important pests in the forestry. Some of them are also devastating to various ornamental trees and shrubs. Fig. 2 shows results of the experiment conducted in laboratory conditions. The results suggest that the nematode efficacy strongly depends on the insect species, its pupation habit, and nematode species.

Two most susceptible species (i.e. *A. berberidis* and *A. pagana*) build loose, double-layer pupation cocoons. The nematode infective juveniles can easily penetrate through the wall of such cocoons. In contrast, *P. abietina* and *C. annulipes* produce tight, waterproof cocoons which cannot be penetrated by the nematodes. Rare infections of these species were facilitated by accidental holes present in the cocoon wall. These species were, however, very susceptible as mature larvae before, and during spinning of the cocoon. *A. erythrocephala* does not built the cocoon and pupates directly in a small soil cavity. Surprisingly, prepupae (eonymphs and pronymphs) and pupae of this species showed to be highly susceptible to *H. megidis* and relatively resistant to infections caused by *S. feltiae*. Similar observations have been made in a related species *A. posticolis*.

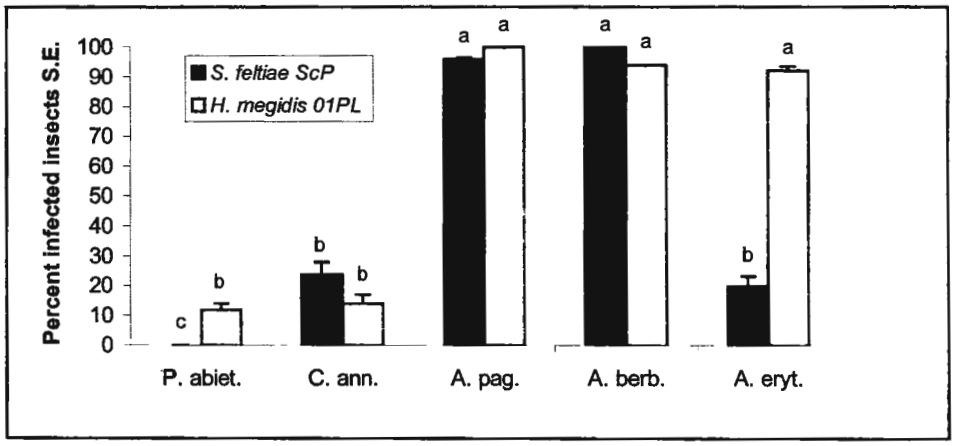


Figure 2. Infectivity of *Steinernema feltiae* and *Heterorhabditis megidis* to sawflies pupating in the soil. (P. abiet. - *Pristiphora abietina*, C. ann. - *Caliroa annulipes*, A. pag. - *Arge pagana*, A. berb. - *Arge berberidis*, Ac. eryt. - *Acantholyda erythrocephala;* nematodes were applied at $10^5$ IJ $m^{-2}$).

Our laboratory and field studies conducted since1998 revealed that many important pests of urban trees (e.g. winter moths, leaf beetles, sawflies) can be infested and killed by entomopathogenic nematodes during pupation and/or ovewintering in the soil. Susceptibility of insects depends on the nematode and insect species, and on the insect's pupation habit. Construction of a tough pupation cocoon by many sawfly species significantly limits the nematode ability to penetrate through the wall and infect the host. The obtained results suggest that such pests as winter moths, leaf beetles, and some of leaf-feeding sawflies can be effectively controlled by a local application of nematodes to the soil. Proper timing of the treatment is, however, essential.

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# Use of Italian EPNs in controlling *Rhytidoderes plicatus* Oliv. (Coleoptera, Curculionidae) in potted savoy cabbages

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**Abstract**: *Rhytidoderes plicatus* Oliv. (Coleoptera, Curculionidae) is an important and common insect pest of Cruciferae plants in Apulia Region. Most economic damage is caused by the larvae feeding on the roots and reducing plant growth while the adults feeding on foliage have small economic effect. Infestations on savoy cabbage (*Brassica oleracea* L. var. *sabauda*) plants are often so severe, that the cultivations are totally destroyed. The susceptibility of the last instar of *R. plicatus* larvae to 3 entomopathogenic nematode species, was assessed in Petri dishes and on potted savoy cabbage plants. Indigenous Southern Italian strains of *Steinernema feltiae* (ItS-G16), *S. carpocapsae* (ItS-MR7) and *Heterorhabditis bacteriophora* (strain ItH-CE1) were tested at about 20 °C in laboratory. *S. carpocapsae* (ItS-MR7) and *H. bacteriophora* (strain ItH-CE1) showed the best results, killing 100% of *R. plicatus* larvae in the Petri dishes after 48 hr. *S. feltiae* (strain ItS-G16) killed only 57% after 48 hr and 100% after 4 days. All the larvae of *R. plicatus,* on the roots of the potted plants, were killed by *H. bacteriophora* (strain ItH-CE1) after 10 days while *S. carpocapsae* (ItS-MR7) controlled 93% after 15 days. *S. feltiae* (strain ItS-G16) showed the worst performance reducing the larval populations by 30% after 15 days.

**Key words**: entomopathogenic nematodes, biological control, Southern Italy.

## Introduction

*Rhytidoderes plicatus* is one of the most destructive pest of cultivated and wild Brassicaceae plants in the Southern Europe, causing damage both above and below the ground. The adult insects emerge from the soil at the end of July until May and feed on leaves, but most economic damage is caused by the root-feeding larvae, which reduce plant growth and, in severe infestation, cause death of the plant. The number of grubs for each plants is extremely variable, even 200-300 larvae per plant of cauliflower were observed on 1971 in the Apulia Region (Southern Italy) during a severe infestation of the weevil *R. plicatus* (Monaco, 1971).

The control is based on chemical pesticides against the adults while they are ineffective against the grubs, since they spend their entire larval stage in the soil around the roots, so alternative strategies have to be studied. The results of numerous efforts to control different species of weevil larvae with nematodes have pointed out various degree of success (Berry *et al.*, 1997). In particular, some experiments performed in Italy have pointed out the real feasibility to apply *S. carpocapsae* (Weiser, 1955) and *Heterorhabditis* sp. (Deseö, 1987; Boselli *et al.*, 1997; Curto *et al.*, 1992) against larvae of sugar-beet weevil *Temnorrhinus* (= *Conorrhynchus*) *mendicus* Gyll., but no data are available on the use of EPNs nematodes in controlling *R. plicatus.*

Since numerous species and strains of *Steinernema* and *Heterorhabditis* have been collecting in Southern Italy by the authors and successfully applied against few insect pests (Triggiani, 1983; Ippolito & Triggiani, 1995; Tarasco & Triggiani, 1997; Triggiani &

Tarasco, 2000; Triggiani & Tarasco, 2002) we have tested the virulence of three Italian nematode species toward 5th instar larvae of *R. plicatus*.

## Material and methods

Ten samples of 1 kg soil were collected during January 2003, from a savoy cabbage irrigated cultivation on sandy loam soil of about 70 ha heavily infested by the insect, near Castellaneta town (Ionic Coast of Apulia Region), and searched for the presence of entomopathogenic nematodes using the technique of Bedding & Akhurst (1975).

Sixty savoy cabbage plants were deprived of the larvae around the roots and transplanted in plastic pots (20x10x10 cm). Each pot was filled up with the soil and protected in a transparent plastic bag to save the original humidity (about 70%). In the same time more than 2,000 larvae of *R. plicatus* were collected, assembled as groups of 50 in the laboratory, set in plastic containers (40x20x20 cm) with soil and let fed on pieces of savoy cabbage roots for 15 days. After that period only the most active last instar larvae were selected and used for the experiments. The virulence of Italian strains of *Steinernema feltiae* Filipjev, 1934 (ItS-G16), *S. carpocapsae* Weiser, 1955 (ItS-MR7) and *Heterorhabditis bacteriophora* Poinar, 1976 (ItS-CE1) were compared in Petri dishes and in pots tests in the laboratory at 20° C.

In the first experiment for each of the three isolates, 1 ml tap water suspension containing approximately 2,000 IJs was applied on 9 cm diam filter paper in a 9 cm Petri dish. In the control, only tap water was applied to the filter paper. Ten last instar of *R. plicatus* larvae were transferred in each of the dishes and exposed to the nematodes. The test, with 3 replications for each isolate, was repeated three times. Larval mortality was assessed every 24 hr.

In the second experiment 10 last instar of the weevil grubs were deeply placed (about 15-18 cm from the soil surface) in each of the 60 pots around the plant roots and let settle for 7 days (Fig. 2). Afterwards, nematode suspensions counting approximately 30,000 IJs in 50 mL of tap water were spread with a 100 mL syringe on the soil surface on the pots. Fifteen pots of each strain and fifteen as control were used. Five pots for each nematode species and five for the control were checked every 5 days for larval mortality. All larvae used for the bioassays, after been washed with water, were dissected: the dead ones to determine whether death was caused by the presence of nematodes or another cause, live ones to find out whether they were infected with nematodes but not yet moribund.

Data on mortality of *R. plicatus* were analyzed by linear model procedure (ANOVA – analysis of variance) and significant differences among means were separated by HSD Tukey's test. All comparisons were made at 0.05 level of significance.

## Results and discussion

No EPNs were isolated from the soil samples collected in the savoy cabbage cultivation.

In the first experiment, *S. carpocapsae* and *H. bacteriophora* caused significantly higher mortality of the root weevil larvae, killing 100% of the larvae after 24 hr, than did *S. feltiae* (Fig. 1A). *S. feltiae*, in fact, controlled 57% after 48 hr and occurred 96 hr to obtain the complete mortality. No larvae died in the control Petri dishes.

In the second experiment, *H. bacteriophora* killed 97% of the grubs after 5 days and 100% mortality was reached after 10 days. *S. carpocapsae* controlled 73% of the larvae after 5 days and 80% after 10 days. Fifteen days were necessary to the nematode to kill 93% of the insect. *S.feltiae* controlled 26% of the grubs after 5 days and 27% after 10 days. Only 30% of the tested larvae were found moribund or killed by the nematode after 2 weeks (Fig. 1B). No larvae died in the control.

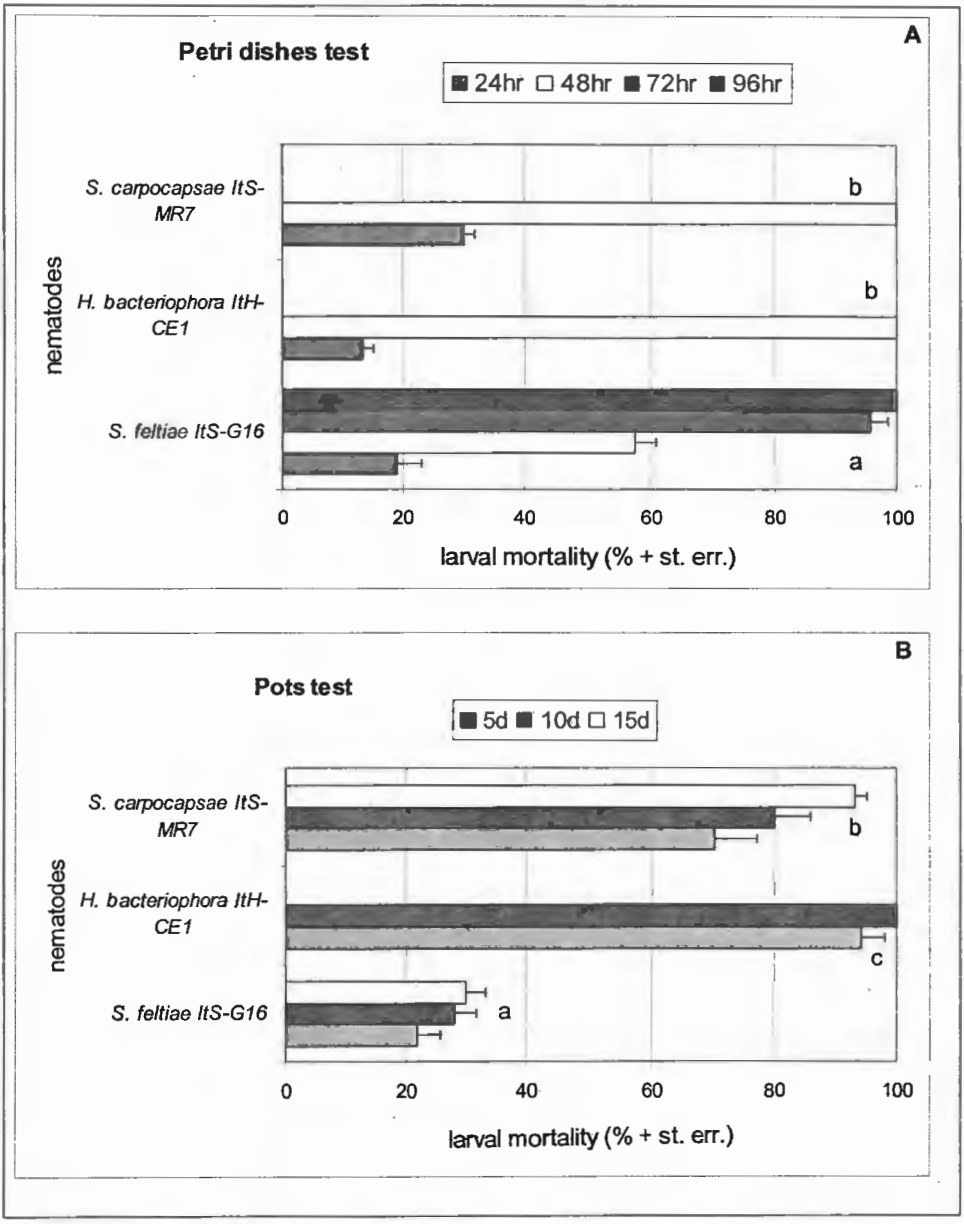


Figure 1. Percentage of *Rhytidoderes plicatus* larvae killed by different Italian nematode species in Petri dishes (A) and in potted savoy cabbage plants (B). Bars with the same letter are not significantly different ($P < 0.05$).

The results of the experiments have demonstrated that the EPNs tested on Petri dishes are able to kill the late instar larvae of the weevil in short time, in fact *H. bacteriophora* and *S. carpocapsae* controlled 100% of the grubs in 48 hr, while *S. feltiae* needs a longer time.

The same nematode species sprayed on the soil surface of potted savoy cabbages have also confirmed the great ability of *H. bacteriophora* to reach and kill the insects deepened in the soil and in a short time, as well as *S. carpocapsae* very active against the grubs in the soil, even with a slower action. While IJs of *S. feltiae* have showed no good results toward *R. plicatus* in Petri dishes and in soil.

The Italian nematodes previously tested in Petri dishes, have confirmed their capability of approaching the grubs even in the soil, where their action is limited by the soil structure and by the soil mixture.

The Italian *H. bacteriophora* and *S. carpocapsae* strains can represent a valid tool in the reduction of *R. plicatus* populations in the soil where pesticides are not active against the larvae. The sandy loam soil on which cabbage is often cultivated in Apulia Region (Southern Italy) is the right place for the nematode applications due to the fact that it is kept wet during the entire period since there are irrigated artificially or by natural rainfall.

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# Initial results in the application of entomopathogenic nematodes against the European cherry fruit fly *Rhagoletis cerasi* L. (Diptera, Tephritidae)

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**Abstract**: Within the framework of the "Bundesprogramm Ökologischer Landbau" (Federal Program for Organic Farming) initial experiments were carried out in 2002 under laboratory, semi-field and field conditions with the nematode species and strains *Steinernema bicornutum*, *S. carpocapsae*, *S. carpocapsae* strain China, *S. feltiae* und *Heterorhabditis bacteriophora*. The efficacy of different nematode dosages (50, 100, 150 nematodes per *R. cerasi*-larva and pupa) was examined in cell wells (24 cells filled with quartz sand) exposing *R. cerasi* instars individually, and in Petri dishes (filled with quartz sand or soil) with 5 *R. cerasi*-larvae and 50 nematodes per $cm^2$. For the semi-field experiments 10 *R. cerasi*-larvae and 50 nematodes per $cm^2$ were used in plastic fruit boxes, filled with soil. In the field tests 5 x 30 *R. cerasi*-larvae per nematode species (*S. carpocapsae*, *S. feltiae* and *Heterorhabditis bacteriophora*) were released in defined areas of 50 x 50 cm, within a total area of 20 $m^2$ of a cherry orchard. The nematodes were applied to the soil with water, giving an average dosage of 50 nematodes per $cm^2$. The cell well-tests resulted in an average infestation of the *R. cerasi*-larvae of 80 %, the highest rates being achieved with *S. carpocapsae*, *S. carpocapsae* strain China and *S. feltiae*. There were no significant differences between different dosages. The average infestation of *R. cerasi*-pupae was very low (2 %). When applying the nematodes in petri dishes, the average infestation rates were 71 % in quartz sand and 56 % in soil, respectively. *S. carpocapsae* and *S. feltiae* achieved the highest values. In the semi-field experiment, the average infestation of the *R. cerasi*-larvae was 54 %. The field tests will be evaluated in May/June 2003 by catching emerged adult fruit flies with photo eclectors. Further experiments to validate the results, to test different soil typs and lower dosages are planed in 2003.

**Key words**: *Rhagoletis cerasi*, European cherry fruit fly, *Steinernema carpocapsae*, *Steinernema bicornutum*, *Steinernema feltiae*, *Heterorhabditis bacteriophora*, entomopathogenic nematodes, biological control, organic farming

## Introduction

The European cherry fruit fly *Rhagoletis cerasi* L. is an important pest in cherry orchards. Dimethoate is the only compound registered for its control at present in conventionel farming in Germany (BVL, 2003; Vogt, 2002). There are two problems concerning the application. One, in using dimethoate, a waiting period of at least 21 days is required from the last application to harvest. Two, the compound is only registered till the end of December 2004. In organic farming, no sufficient control method against the pest is available. As a result, there is a need to find alternative methods of control, which are safe for both non-target organisms and the ecosystem. One possibility involving biological regulation, might be the application of entomopathogenic nematodes against the fly stages in the soil (Patterson Stark & Lacey, 1999). Entomopathogenic nematodes are already used to control different pest organisms in soil (e.g. Ehlers & Peters, 1998; Neubauer, 1997; Simser & Roberts, 1994;

Smits, 1992; Wilson *et al.*, 1999) . The univoltine *Rhagoletis cerasi* lays its eggs in maturing cherry fruits, where the larvae cause damage during feeding. At the end of the larval period the third instar larvae leave the fruit. They drop down, enter the soil and pupate. The obligatory diapause lasts for about 9 month till the next May or June, when the adults emerge (Wiesmann, 1933). Within the framework of the "Bundesprogramm Ökologischer Landbau" (Federal Program of Organic Farming) the first experiments using entomopathogenic nematodes as biocontrol agents against cherry fruit fly larvae and pupae were carried out in 2002.

## Material and methods

The experiments were carried out under laboratory, semi-field, and field conditions with the nematodes *Steinernema bicornutum* (S.b.), *S. carpocapsae* (S.c.), *S. carpocapsae* strain China (S.c.C), *S. feltiae* (S.f.) and *Heterorhabditis bacteriophora* (H.b.), provided by e-nema GmbH, Germany. For all trials, the cherry fruit fly larvae and pupae were obtained from field collected infested cherries. The detailed experiments are described below.

***Laboratory***

In laboratory the nematodes were tested against larvae and pupae of *R. cerasi* in cell wells®, with 24 single cells filled with moist quartz sand (moisture about 10 %). In each cell one larva or pupa was exposed individually to different numbers of nematodes, 50, 100 and 150 (corresponding to 25, 50 and 75 nematodes/cm$^2$). The test items were incubated at 20°C or 24°C at least for five days. Because of a lack of larvae only a few and different numbers of replicates for the nematode species and -strains could be made, ranging from 1 to 4 cell wells® à 24 cells per treatment. In a second laboratory assay, 50 nematodes per cm$^2$ (except *S. bicornutum*) and 5 *R. cerasi*-larvae were applied in petri dishes, filled with quartz sand or soil from the field close to the institute. In this assay 10 replicates were used. The Petri dishes were incubated at 20°C for at least 5 days too. To evaluate the infestation rate, *R. cerasi* instars were disected under the stereomicroscope.

***Semi field trial***

The semi field experiment was carried out in plastic fruit boxes (size: 29 x 39 x 10 cm, 5 replicates for each nematode species or strain) carefully filled with clods, exposing 10 *R. cerasi*-larvae to 50 nematodes per cm$^2$. For better finding at the end of the test, each *R. cerasi*-larva was placed in a little bag made of gauze and filled with soil. For this experiment the steinernematid and heterorhabdid nematodes except *S. bicornutum* were used. The semi field approach was exposed for four weeks in an open vegetation hall, moistening the soil every two or three days in the first week. Afterwards, the *R. cerasi* instars were digged out of the soil and, as described for the laboratory experiments, dissected under a stereomicroscope in order to evaluate the nematode infestation rate.

***Field trial***

The field trial was carried out in the sweet cherry orchard of the institute with the nematode species *S. carpocapsae*, *S. feltiae* and *Heterorhabditis bacteriophora* in July 2002. For each nematode species an area of 20 m$^2$ around a cherry tree with 5 ¼ m$^2$ sections in each was measured out and treated with 50 nematodes per cm$^2$ in average. 30 *R. cerasi* larvae were released into the soil of each ¼ m$^2$ section. For the control area, the same arrangements were made, without applying nematodes, but only water. The evaluation of the field approach will be made indirectly, trapping the emerging adult cherry fruit flies with photo-eclectors in May / June of the following year (2003).

## Results

***Laboratory***

The experiment in cell wells® resulted in an average infestation rate of *R. cerasi* larvae of 80 % in all treatments. Consistent significant differences between nematode species and -strains could only be found in the comparison of *S. bicornutum* and the other nematodes (Mann-Whitney test, Bonferroni correction), revealing a lower infestation rate for *S. bicornutum.* There were negligible significant differences with regard to the influence of the temperatures of 20°C and 24°C on the infestation rate (Mann-Whitney test). Following, the average infestation rates of the different nematode species and -strains are shown in one figure (Fig. 1). Comparing the different nematode dosages, no stable significant differences could be found (Kruskal & Wallis test). The highest rates of average infestation were achieved with *S. carpocapsae* strain China (88%) and *S. feltiae* (86%). The average infestation of pupae was very low (2%, Fig. 2) at both temperatures. A higher infestation with higher dosages could not be achieved for pupae either.

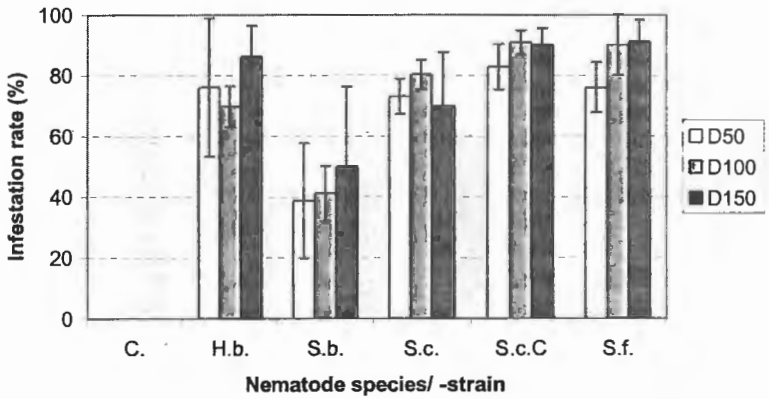


Figure 1. Cell wells®- average infestation rate (± standard deviation) of *Rhagoletis cerasi* larvae by various nematode species and strains. Results obtained at 20°C and 24°C pooled together (cf. text). C. = control, D = dosis.

The second laboratory assay exposing *R. cerasi* - larvae in Petri dishes with quartz sand or soil confirmed the good infestation rates by *S. carpocapsae* and *S. feltiae* in the small exposure units (cell wells). In quartz sand an average infestation rate of 80% with *S. carpocapsae* and 96% with *S. feltiae* was reached (Fig. 3). Comparing the two strains of *S. carpocapsae*, *S. carpocapsae* strain China was less effective with 69%. Significant differences in infestation rates could be found between *H. bacteriophora* and *S. carpocapsae* (0,01<P<0,05), *H. bacteriophora* and *S. feltiae* (P<0,01) as well as *S. carpocapsae* strain China and *S. feltiae* (P<0,01, Mann-Whitney test, Bonferroni correction). The use of soil resulted in a lower infestation rate than in sand for most nematodes.These differences were significant for *S. carpocapsae*, *S. carpocapsae* strain China and *S. feltiae* (Mann - Whitney test). Differences between nematodes in soil were significant comparing *H. bacteriophora* and *S. carpocapsae* strain China (0,01<P<0,05) as well as *S. carpocapsae* and *S. carpocapsae* strain China (P<0,01, Mann-Whitney, Bonferroni correction).

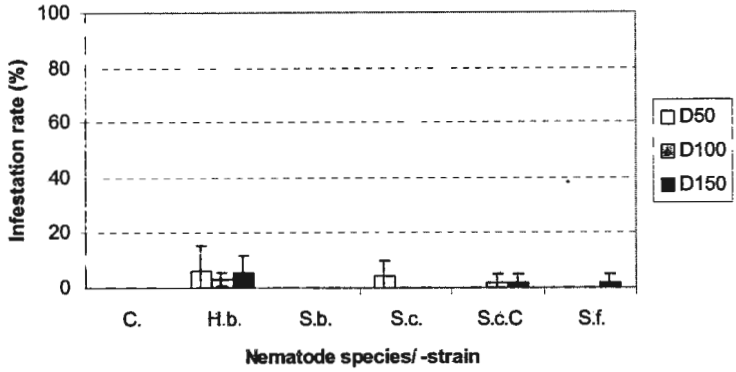


Figure 2. Cell wells®- average infestation rate (± standard deviation) of *Rhagoletis cerasi* pupae by various nematode species and strains. Results obtained at 20°C and 24°C pooled together (cf. text). C. = control, D = dosis.

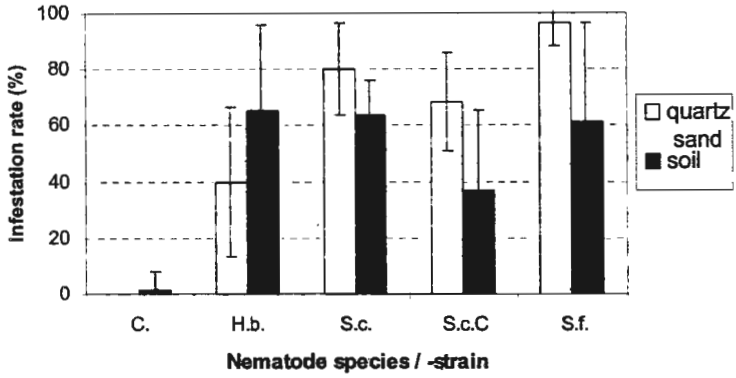


Figure 3. Petri dishes - average infestation rate (± standard deviation) of *Rhagoletis cerasi* larvae by various nematode species and strains at 20°C. C. = control.

***Semi field trial***

In the semi field experiment, the average infestation of the larvae was 54%, with *S. feltiae* being the most successful nematode species (Fig. 4). However, the differences were not significant (Kruskal & Wallis test).

## Discussion

The results indicate a high susceptibility of *R. cerasi* larvae to entomopathogenic nematodes. especially to steinernematid species *S. carpocapsae* and *S. feltiae* in laboratory and semi field. The *R. cerasi* pupae are not infectable by the nematodes. The nematodes are not able to enter the pupae according to the lack of body openings (Wiesmann, 1933). Renn (1998) demonstrated the penetration of nematodes into maggots, especially the house fly maggots (*Musca domestica*), via the anus. Futhermore the output of carbon dioxyde of larvae is an indication factor for host finding in nematodes (Kaya, 1990).

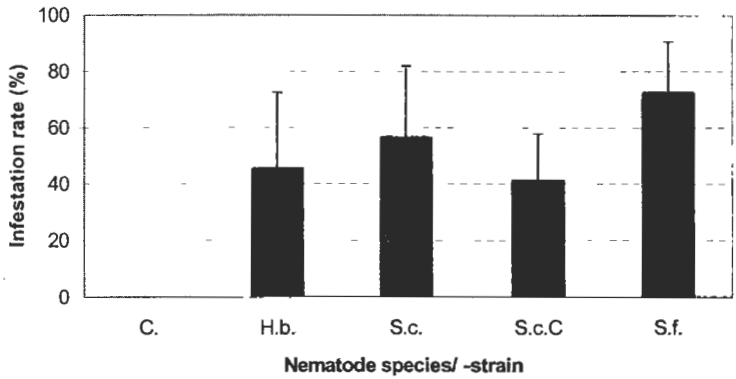


Figure 4. Average infestation rate (± standard deviation) of *Rhagoletis cerasi* larvae in semi field experiments by various nematode species and strains. C. = control.

Nematode species are following different foraging strategies (Ishibashi & Kondo, 1990; Lewis, 2002). According to our results, no special foraging strategy of the different nematode species can be seen as favourable for *R. cerasi* larvae.

Using temperatures of 20°C and 24°C no consistent significant difference in infestation of larvae could be found. Both temperatures are within the optimal temperature range of the nematodes (Grewal *et al.*, 1994). The temperature of 20°C was used as a standard and the temperature of 24°C was choosen according to the highest average soil temperature in a depth of 5 cm between 11 a.m. and 3 p.m. of the month June, July and August in 1999, 2000 and 2001, as recorded by the weather station of the research institute of BBA at Dossenheim. During this period *R. cerasi* larvae enter the soil for pupation.

The comparison of quartz sand and soil (from the field close to the institute, heavy clay soil) resulted in a lower infestation rate for most nematode species in soil. In heavy clay soil the nematode movement is impaired (Kaya, 1990). It is therefore necessary to analyze different standardized soil types for better characterization of *R. cerasi* infection by entomopathogenic nematodes.

Using soil, one problem became evident in laboratory and semi field tests. It was not easy to evaluate infested larvae and pupae, because of infestation symptoms but lack of nematodes, which might already have left the host. Furthermore, it was difficult to analyse those larvae or pupae which were infested by fungi.

Our results represent first indications for the control of the European cherry fruit fly *Rhagoletis cerasi* with entomopathogenic nematodes. Additional experiments are planed for the cherry season in 2003 to confirm the results. Experiments with different soil types will be considered. Furthermore, the field experiment 2002/2003 will be evaluated.

## Acknowledgements

We thank the staff of the Institute for Plant Protection in Fruit Crops (Federal Biological Research Centre for Agriculture and Forestry), Dossenheim, for assistance in experiments, the extension service for informations about infested cherry orchards, farmers and gardeners for providing infested cherries.

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# Developing entomopathogenic nematode delivery systems for biological control of oilseed rape pests

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**Abstract**: Tests were conducted to explore the potential of *Steinernema feltiae* for the control of oilseed rape pests *Meligethes aeneus* and *Phyllotreta* spp. Timing, dose, and method of nematode application were considered. A field test was carried out using optimum timing (beginning of pupation of *M. aeneus*, early July) and a 'sufficient' dose (1 million IJ/$m^2$), applied with a watering can. The treatment was extremely efficient against the pollen beetle: 93.8% control. Flea beetles were reduced by 50.1%. No non-target effects were detected, except that the pollen beetle parasitoid *Phradis morionellus* was dramatically reduced, by 94.4%. Problems in translating these results into practice include: (1) it is not possible for a farmer to treat at the optimum time (end of flowering), and (2) the high dose is prohibitively expensive. To overcome these, we constructed a low dose, slow-release nematode delivery system ('NemaBag'). In 2002 a field test on oilseed rape was conducted with 6 treatments, applied 1 week after sowing (end of May): control, *S. feltiae* spray at 450 kIJ/ $m^2$, *S. feltiae* in NemaBags at the rates of 450, 150 (2 dates), and 15 kIJ/$m^2$. The results were inconsistent. Three of the treatments significantly reduced the flea beetles, but none impacted pollen beetle numbers. The results indicate that one can get an equal or similar result from treating the soil with a high dose (450 000 IJ/$m^2$) in water solution, and from applying far less nematodes in a slow-release system at a rate as low as 15 000 IJ/$m^2$. Even with the lowest rate it was possible to establish nematodes in a plot, capable of killing bait larvae 3 months after the application. We conclude that EPN show good activity against key oilseed rape pests, and that the slow release, low rate delivery system merits intensified study as a possible way towards practical use of EPN in major outdoor crops.

**Key words**: application methods, integrated control, nematode establishment, non-target impacts, slow release systems, *Meligethes aeneus*, *Phyllotreta*, *Steinernema feltiae*

## Introduction

Biological control using entomopathogenic nematodes (EPN) has been with a few exceptions restricted to intensive cultivation systems in greenhouses and nurseries. So far, they have not found their place in the management of pests in any of the large-scale agricultural crops, and therefore, the markets for nematodes have remained small. To make progress in utilising EPN in agriculture, advances have to be made not only in finding effective strains of EPN capable of controlling major pests of field crops, but also in their formulation and application methods. Also, the possible (likely) long-term benefits of re-establishing or enhancing EPN populations in agricultural fields needs to be addressed.

Oilseed rape has many problematic pests – largely Coleoptera – that are not amenable to control by several classes of microbial control agents, such as viruses or bacteria. Only a few studies have been carried out using EPN in oilseed rape, but with rather encouraging results (C. Laumond, personal communication). Recently, interest in this possible use of EPN has been revived (e.g. Nielsen & Philipsen, 2004). We have been experimenting with storage and formulation of *Steinernema* –nematodes and were interested in applying these experiences to our long-term research target, the oilseed rape ecosystem and our main pests in Finland, the

pollen beetle (*Meligethes aeneus*) and the flea beetles (*Phyllotreta* spp.). Timing, dose, and method of nematode application were considered.

## Material and methods

***Biological materials***

*Steinernema feltiae* for these experiments was obtained from e-nema GmbH (Raisdorf, Germany), shipped on ice and maintained in aerated containers at +5 °C until use. *Tenebrio molitor* larvae of uniform size were obtained from our own continuous rearing.

***Field test 1***

A field test was carried out in 2001 using optimum timing (application at the beginning of pupation of *M. aeneus*, early July in Finland) and a 'sufficient' dose of nematodes (one million IJ/$m^2$), applied with a watering can (20 l of water) on a 1 $m^2$ plot. Eight replicates were used; the reference plots were treated with clean water. Photoeclectors (size 0.5 $m^2$) were erected on the plots two weeks after treatment, and emerging insects were collected for six weeks; sampling was continued in the following spring from the same precise spots to detect the impacts on overwintering parasitoids.

***Field test 2***

Before carrying out further field tests, we first constructed a low dose, slow-release nematode delivery system ('NemaBag'), which we tested in the greenhouse using *Tenebrio molitor* baits to monitor the nematode release and spread. The bags were made of standard nylon cheesecloth with 200 µm grid, and were about 0.6-0.7 cm thick and 2 cm long. The bags were filled with the desired dose of nematodes formulated in NemaLife™ (a superabsorbent gel formulation of Aasatek Ltd, Helsinki, Finland), and were buried at 2 cm depth in the soil. Nematodes easily moved out of the bags and quickly killed the test larvae even 9 cm away. In summer 2002 we carried out a comprehensive field test on oilseed rape field with six different treatments, applied one week after sowing of the rapeseed crop (end of May): water treated control, *S. feltiae* spray (watering can) at 450 kIJ per $m^2$, *S. feltiae* in NemaBags at the rate of 450, 150, and 15 kIJ/$m^2$; another application with NemaBags at 150 kIJ was carried out as the sixth treatment 10 days afterwards, due to the extremely hot (up to +30°C) and dry weather conditions at and after the first application. Each treatment was replicated five times in a randomised block design; each plot was 1 $m^2$. At the end of flowering, one photoeclector (0.4 $m^2$) was erected on each plot, and insects were collected for six weeks. In September soil samples were collected from each plot and baited with *T. molitor* in order to detect whether *S. feltiae* had established on the plots (3 larvae in 3 subsamples from each plot).

## Results and discussion

***Field test 1***

The nematode treatment was extremely efficient in controlling the pollen beetle, resulting in 93.8% control (Fig. 1). The flea beetle numbers were lowered by the treatment by 50.1%, although the timing of application probably was too late for their efficient control. As regards non-target effects, the overall numbers of 'macro-Diptera' were slightly affected (-37%), while those of 'Hymenoptera parasitica' (+66%), spiders (+19%), and 'micro-Diptera' (+3%) were affected positively, or were not affected at all (Fig. 1). However, the numbers of the pollen beetle specific parasitoid *Phradis morionellus* were dramatically affected: emergence in the following spring was reduced by 94.4%, practically equalling the reduction in unparasitised host numbers in the previous summer. Even in the spring of the following year,

no overall impact on the numbers of emerging insect parasitoids was detected (+23%, comparable to the +66% in the autumn sampling on the same plots).

There are (at least) two problems in translating these excellent results into practice: (1) it is not possible for a farmer to carry out a treatment at the optimum time (end of flowering), and (2) the high dose (one million per $m^2$) is prohibitively expensive. In order to make progress in the utilisation of EPN on large-scale outdoor crops, means of delivering an effective dose at a suitable time will have to be developed.

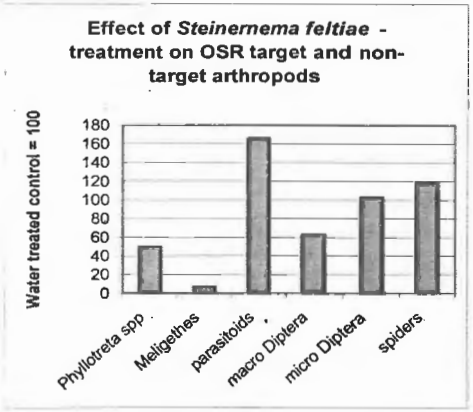


Figure 1. Relative effect of *Steinernema feltiae*-treatment on oilseed rape target and non-target arthropods in Finland, Field test 1. Water treated control = 100.

***Field test 2***

The results of the second field test were somewhat inconsistent. As compared with the water treated controls, three of the treatments significantly reduced the numbers of flea beetles: NemaBag at 150 kIJ/$m^2$ (second treatment time) by 46.4%, spray at 450 kIJ/$m^2$ by 43.2%, and NemaBag at 450 kIJ/sqm by 28.4%. The two other treatments did not reduce flea beetle numbers significantly (Fig. 2A). These results are similar to those from the previous year, now obtained with a considerably lower dose of nematodes. None of the treatments, however, had a significant impact on pollen beetle numbers, unlike the results from the previous year.

Interesting data were obtained from the soil samples on the establishment of *S. feltiae* on the study plots. *Tenebrio* mortality was highest in the samples from the plots treated with NemaBags at 450 kIJ/$m^2$ (57%), followed by spray at 450 kIJ/$m^2$ (41%), NemaBags at 150 kIJ/$m^2$ (28% for the later treatment, 25% for the first treatment time), NemaBags at 15 kIJ/$m^2$ (25%), and water treated control (12%) (Fig. 2B). No nematode replication occurred in the dead larvae from the soil samples collected from the control plots (i.e., no nematodes were present), whereas it took place in all other samples, where most dead larvae also produced nematodes. Nematode replication was clearly the highest in soils from the plots that had been treated with NemaBags at 450 kIJ/$m^2$ (41% of all bait larvae), followed by spray at 450 kIJ/$m^2$ (21%), NemaBags at 150 kIJ/$m^2$ (20% for the later treatment, 16% for the first), NemaBags at 15 kIJ/$m^2$ (20%), and the water treated control (0 %) (Fig. 2B).

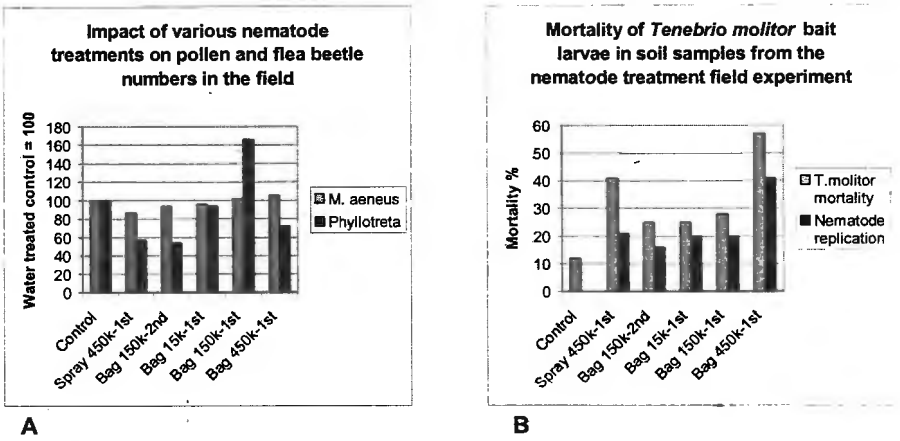


Figure 2. Data from field experiment 2.
2A: Impact on numbers of new generation pollen beetles and flea beetles of treatments with *Steinernema feltiae*: spray at 450k IJ/m$^2$, NemaBag releases at 15k IJ/m$^2$, 150k IJ/m$^2$, and 450k IJ/m$^2$. All treatments on May 30, 2002, except 'Bag 150k – 2$^{nd}$' on 9 June, 2002.
2B: Establishment and activity of *S. feltiae* in the treated plots three months after application. Mortality of *Tenebrio molitor* bait larvae in soil samples, and nematode replication in larvae as the percent of total number of bait larvae for each sample.

Another interesting aspect of these results is that one can get an equal or similar impact from treating the soil with a quite high dose (450 000 IJ/m$^2$) in water solution (watering can), and from applying far less nematodes in formulated slow-release systems, e.g. at a rate as low as 15 000 IJ/m$^2$. Even with the lowest application rate it was possible to establish a viable nematode stock in a plot, capable of killing effectively bait larvae at least three months after the application. The apparent modest impact of any of our treatments in this particular experiment may be due to hostile external conditions at the time of application, as well as the unconducive soil type available this time for our experiments (high clay content).

We conclude that EPN show good activity against key oilseed rape pests, and that the slow release, low rate delivery system merits intensified study as a possible way to making progress towards practical use of EPN in controlling pests on major outdoor crops.

## Acknowledgements

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# Preferences of Italian *Heterorhabditis* for different *Photorhabdus* bacterial types

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**Abstract**: *Heterorhabditis* spp. are mutualistically associated with bacteria of the genus *Photorhabdus*. Bacteria carried by the nematode infective juveniles (IJs) are inoculated into the haemocoel of the insect host, where they contribute to the death of the insect and provide a suitable nutritional environment for the nematodes. In this study, preferences of 2 *Heterorhabditis bacteriophora* Italian strains for 2 different *Photorhabdus* bacterial types were investigated. These strains of *H. bacteriophora*, isolated from different biotopes in Southern Italy, each carries a phenotypically different bacterium: ItH-CE1 is a "normal" nematode strain with red and strongly luminescent *Photorhabdus* while ItH-C6 has a green bacterium with weak luminescence. Two different choice assays were performed on agar with the aim to assess the preferences of these nematodes for one or the other bacterial type. In the first type of test, nematodes were released in the middle of an agar plate and 6 membrane filters (1,5 cm-diameter) with bacteria (2 red and 2 green *Photorhabdus*) + 2 filters without bacteria as control, were arranged around them; the distance between the middle of the plate and each filter was ~ 3 cm. After 2 hrs the membrane filters were transferred in tap water and IJs counted. In the second type of experiment, four 1 cm-diameter agar disks, with either red and green *Photorhabdus* (2 of each) or 2 *Photorhabdus* (red or green) + 2 similar control disks not containing bacteria, were positioned alternately in the middle of an agar plate, next to each other forming a square. IJs were pipetted in the middle of them; after 5 hrs the disks were placed in tap water and IJs counted. Both the assays had five replicates for each treatment.

In the first tests, nematodes seemed to prefer their own bacteria, but attraction to either bacterial type was quite weak and not a large difference between bacterial and control disks was observed. In the second tests there were large differences between bacterial and control disks and both nematode types preferred the red bacterium.

**Key words**: bioassays, entomopathogenic nematodes, symbiotic bacteria, host preference.

## Introduction

*Heterorhabditis* spp. are mutualistically associated with bacteria of the genus *Photorhabdus*. Bacteria carried by the nematode infective juvenile (IJs) are inoculated into the haemocoel of the insect host, where they contribute to the death of the insect and provide a suitable nutritional environment for the nematodes.

In this study preferences of 2 Italian *Heterorhabditis bacteriophora* strains for 2 different *Photorhabdus* bacterial types were investigated.

Strains of *H. bacteriophora* were isolated from different biotopes in Southern Italy (Tarasco & Triggiani, 1997; Triggiani & Tarasco, 2000). They carry a phenotypically different bacterium: ItH-CE1 is a "normal" nematode strain with red and strongly luminescent *Photorhabdus* while ItH-C6 has a green bacterium with weak luminescence.

## Material and methods

Two different choice assays were performed on agar with the aim to assess the preferences of these nematodes for one or the other bacterial type.

In the first type of test, nematodes (~ 300 IJs in 0.20 ml of water) were released in the middle of an agar plate and 6 membrane filters (1,5 cm-diameter) with bacteria (3 red and 3 green *Photorhabdus*) or 3 filters with red or green bacteria and 3 without bacteria as control, were arranged around them; the distance between the middle of the plate and each filter was ~ 3 cm. After 2 hrs the membrane filters were transferred in tap water and IJs counted.

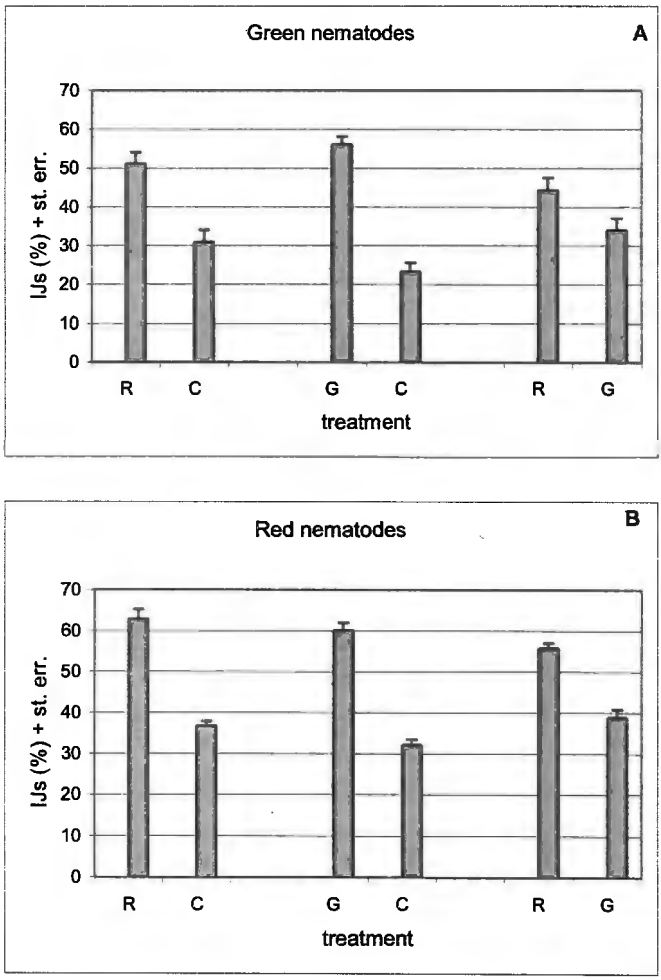


Figure 1. First choice test with green (A) and red (B) Italian *Heterorhabditis bacteriophora*. First pair: filter papers with red bacteria (R) and water (C) as control. Second pair: filter papers with green bacteria (G) and water (C). Third pair: filter papers with red bacteria (R) and green bacteria (G).

In the second type of experiment, bacterial lawns were previously cultured for both the red and green *Photorhabdus*. Starting from these lawns and following the methodologies of Balan (1985), four 1 cm-diameter agar disks, with either red and green *Photorhabdus* (2 of each) or 2 *Photorhabdus* (red or green) + 2 similar control disks not containing bacteria, were positioned alternately in the middle of an agar plate, next to each other forming a square. IJs (~ 300) were pipetted to the middle of them; after 5 hrs the disks were placed in tap water and IJs counted.

Both the assays had 5 replicates for each treatment and each experiment was repeated 3 times. The test temperature was 20°C.

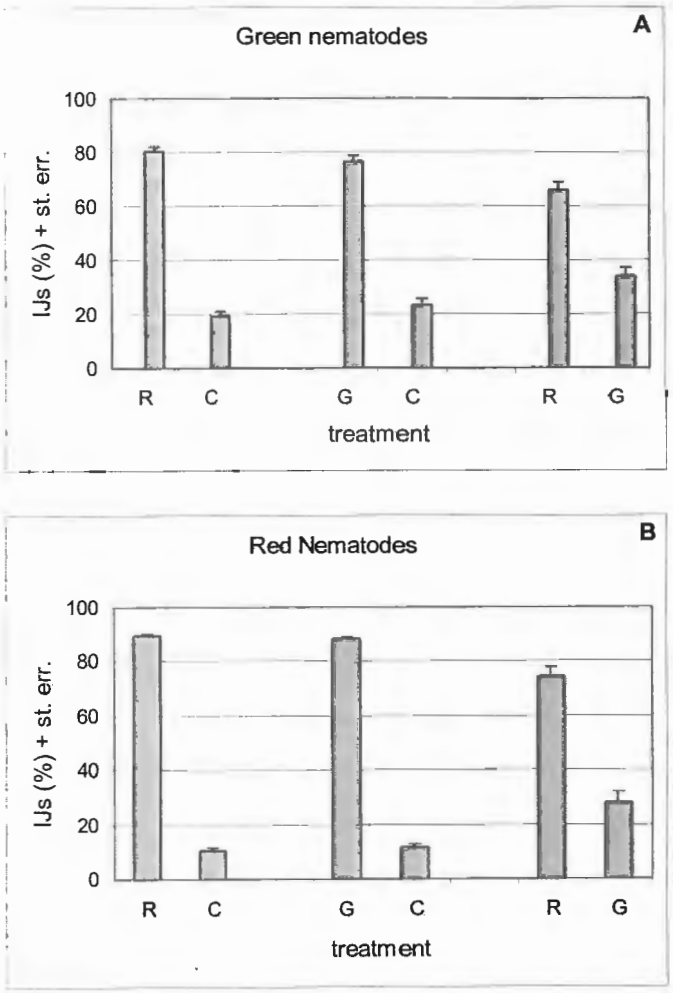


Figure 2. Second choice test with green (A) and red (B) Italian *Heterorhabditis bacteriophora*. First pair: filter papers with red bacteria (R) and water (C) as control. Second pair: filter papers with green bacteria (G) and water (C). Third pair: filter papers with red bacteria (R) and green bacteria (G).

## Results and discussion

In the first tests, both types of nematodes seemed to prefer the red bacteria, but attraction to either bacterial type was quite weak and not a large difference between bacterial and control disks was observed (Figs. 1A, 1B). In the second choice test, there were larger differences between bacterial and control disks and, again, both nematode types preferred the "red" bacterium and accumulated mainly on the "red" agar disks (Figs. 2A, 2B).

The difference between the two assays may have been due in part to the nature of the bacterial support (filter or agar disk), but probably a more important factor was the distance between nematodes and bacteria. Both tests demonstrated that "red" bacteria attract the IJs of both the *Heterorhabditis* strains more than the "green" ones. What is the cause of this attraction? Are there some kind of attractants? And where is the difference with the "green" bacteria? Interestingly, these questions deserve to be investigated.

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# Desiccation tolerance of six Italian strains of entomopathogenic nematodes

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**Abstract**: This study is a preliminary contribution on the desiccation tolerance of Italian entomopathogenic nematodes. We report the results of laboratory experiments on the survival of 6 Italian strains upon direct exposure of infective juveniles (IJs) to 2 relative humidities on membrane filters. These strains, isolated from different biotopes in Southern Italy, belong to 5 species: *Steinernema feltiae* (strain ItS-MA12), *S. affine* (ItS-AR1), *S. carpocapsae* (ItS-MR7), *Steinernema* sp. (ItS-TC5: a long nematode belonging to the *S. glaseri* Group) and *Heterorhabditis bacteriophora* (2 strains: ItH-CE1, with "red" *Photorhabdus* bacteria and ItH-C6, with "green" *Photorhabdus* bacteria). Infective juveniles were exposed to 84% and 76% relative humidity (rh) at ~ 20°C for up to five days, and the percentage of IJs surviving was recorded twice daily. There were 3 replicate desiccators per time interval for each nematode strain. The assays were repeated 3 times.

Survival decreased as rh decreased and with increasing exposure period. Tolerance to desiccation of these nematodes was generally low, except for *S. carpocapsae*, which showed the longest survival ability. At 84% rh the *S. carpocapsae* strain showed a desiccation tolerance of at least 5 days, with a survival percentage of 30%, while the other nematodes showed low percentage survival (11-26%) after 24h and no IJs alive after 28h. At 76% rh *S. carpocapsae* again survived for at least 5 days (23% survival) while the other strains survived for only 6-8 h (0-3% of IJs alive).

No significant differences in pathogenicity and ability to penetrate *Galleria mellonella* larvae were observed between non-desiccated IJs and rehydrated ones for any of the strains.

**Key words**: *Steinernema feltiae, Steinernema affinae, Heterorhabditis bacteriophora*, bioassays

## Introduction

Entomopathogenic nematodes (EPNs) (Steinernematidae and Heterorhabditidae) are obligate parasites of insects (Poinar, 1990) and have been given great attention because of their potential as a biological control agent. Their infectious activity varies with the species and strain and is affected by abiotic and biotic factors, especially temperature and humidity, which influence their infectivity as well as their survival, motility, development and reproduction.

Relative humidity (rh) is one of the most important factors limiting the success of EPN and low rh values may restrict their use.

Not many investigations have been performed on the desiccation survival ability of steinernematid and heterorhabditid nematodes and most of them have been focused exclusively on *S. carpocapsae* (Simons & Poinar, 1973; Ishibashi *et al.*, 1987; Womersley, 1990; Glazer, 1992; Piggot *et al.*, 2002). Some data are available regarding the *S. glaseri* group (Kung et al., 1991; Tarasco & Triggiani, 2002), *S. riobravis* (Baur *et al.*, 1995), *S. feltiae* (Menti *et al.*, 1997; Solomon *et al.*, 1999), *Heterorhabditis bacteriophora* (Liu & Glazer, 2000; Piggot *et al.*, 2002) and *H. zealandica* (Surrey & Wharton, 1995).

This study is a contribution on the desiccation tolerance of some Italian EPNs. We report the results of laboratory experiments on the survival of 6 Italian strains upon direct exposure of infective juveniles (IJs) to 2 relative humidities on membrane filters.

## Material and methods

The strains used for the bioassays were isolated from different biotopes in Southern Italy and belong to 5 species: *Steinernema feltiae* (strain ItS-MA12), *S. affine* (ItS-AR1), *S. carpocapsae* (ItS-MR7), *Steinernema* sp. (ItS-TC5: a long nematode belonging to the *S. glaseri* Group) and *Heterorhabditis bacteriophora* (2 strains: ItH-CE1, with "red" *Photorhabdus* bacteria and ItH-C6, with "green" *Photorhabdus* bacteria).

Infective juveniles (IJs) of these strains were reared in vivo on *Galleria mellonella* L. (Lepidoptera, Galleriidae) larvae and, after emerging, stored for 3-4 days at 14°C prior to being used in the experiments.

Relative humidity (rh) was controlled by the use of saturated salt solutions (Winston & Bates, 1960): potassium bromide (KBr) and sodium chloride (NaCl) were used to obtain respectively 84% and 76% rh at 20°C.

Approximately 200 IJs in 1 mL of water suspension were placed on a 2.5 cm cellulose filter (20 µm pore size) and then the water was removed with a vacuum pump. Each filter was then immediately transferred to a desiccation chamber. The chamber consisted of a square plastic box (10x10x5 cm), containing the upturned lid of a 5.5 cm Petri dish, placed onto the upturned base of the same Petri dish, and 60 mL of the saturated salt solution.

The desiccators were incubated at 20°C for 5 days and the survival percentage was recorded 2 times per day. Nine desiccators per time intervals were set up for each nematode strain and the desiccators were chosen at random. IJs were rehydrated at 20°C by adding 5 mL of tap water and their survival was assessed by microscopic observation of motility and response to probing after incubation at 20°C for 24 hours. The treatment temperature was 20°C.

Rehydrated IJs were used after the tests to check their infectivity and released on filter paper in a Petri dish with 3 *Galleria mellonella* larvae. Non-desiccated IJs were released in a similar Petri dish with 3 *Galleria* larvae to compare their infectivity with the rehydrated IJs. Only larval mortality was recorded in this case.

Data on percentage survival data were analyzed by linear model procedure (ANOVA – analysis of variance) and significant differences among means were separated by HSD Tukey's test. All comparisons were made at 0.05 level of significance.

## Results and conclusions

Survival decreased as rh decreased and with increasing exposure period.

Tolerance to desiccation of these nematodes was generally low, except for *S. carpocapsae*, which showed the longest survival ability. At 76% rh (Fig. 1A) *S. carpocapsae* survived for at least 5 days (23% survival) while the other strains survived for only 6-8 h (0-3% of IJs alive). At 84% rh (Fig. 1B) the *S. carpocapsae* strain again survived desiccation for at least 5 days, with 30% IJs alive, while the other nematodes showed low percentage survival (11-26%) after 24h and no IJs alive after 28h.

No significant differences in pathogenicity and ability to penetrate *Galleria mellonella* larvae were observed between non-desiccated IJs and rehydrated ones for any of the strains.

The data on the survival of *S. carpocapsae, S. feltiae* and *Heterorhabditis bacteriophora* agree with the published literature cited above, while the most significant of the findings are

those relating to *S. affine* and *Steinernema* sp. (belonging to the "*Steinernema glaseri* group") that represent a contribution to the knowledge of their behavior in relation to humidity variations.

When comparing our findings with the data reported by Kung *et al.* (1991) regarding the survival ability of *S. glaseri*, the Italian *Steinernema* sp. ItS-TC5, belonging to the "*Steinernema glaseri* group", *Steinernema* sp. appeared more susceptible to low rh.

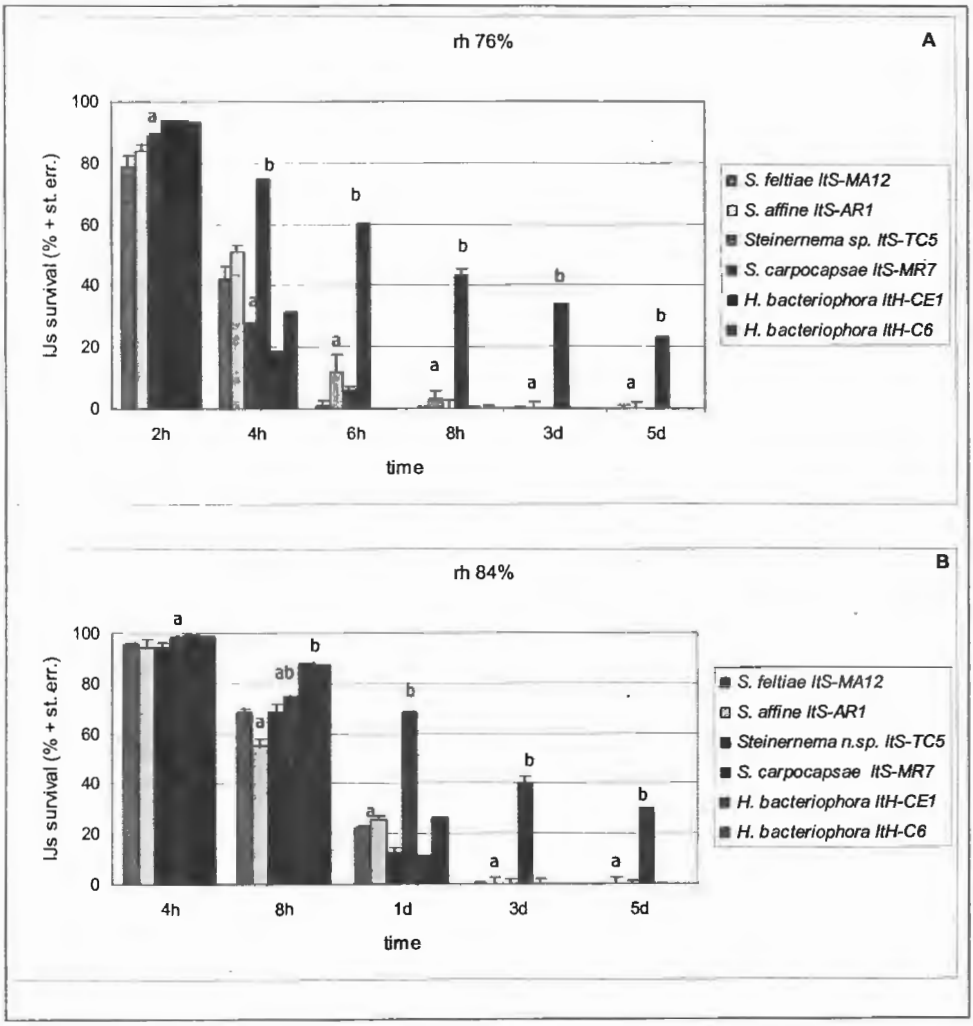


Figure 1. Survival rates of IJs of 6 Italian EPNs in 76% (A) and 84% (B) rh at 20°C. Within a time interval, bars with the same letter are not significantly different ($P < 0.05$).

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# Abundance of naturally occurring entomopathogenic nematodes and establishment of inoculated *Steinernema feltiae* in an organic cropping system

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**Abstract**: The abundance of entomopathogenic nematodes and soil dwelling stages of plant specific insects was studied in an organic cropping system for two years. In addition was the survival of an inoculated strain of *Steinernema feltiae* studied. The abundance of naturally occurring entomopathogenic nematodes was in general at a low level throughout the observation period (0-26% positive samples). Inoculated *S. feltiae* established well when pea was grown in the same or in the following year whereas the nematodes were less able or unable to establish in the other crops (cabbage, carrots, barley, alfalfa/clover). The successful establishment of nematodes, and the abundance in general, correlated well with the presence of plant specific insects.

**Key words**: *Delia radicum*, entomopathogenic nematodes, organic cropping system, population dynamics, *Psila rosea*, *Sitona lineatus*, *Steinernema affine*, *S. feltiae*.

## Introduction

Entomopathogenic nematodes are soil living organisms and are commercially available for biological control of pest. The key pests are scarabids, weevils, and sciarids in high value crops. The strategy here is often to release high numbers of nematodes in order to reduce the number of pest insects to an acceptable level (inundation biological control). This strategy would be too expensive in agricultural fields where large areas have to be covered and another strategy could be to favour the naturally occurring nematodes through cultural practise (conservation biological control). In some situations, however, the population size of the naturally occurring nematodes will be at a very low level and nematodes may have to be released first (inoculation biological control). The different terms for biological control mentioned above refers to the terminology suggested by Eilenberg *et al.* (2001). In the present study both the naturally occurring entomopathogenic nematodes and an inoculated isolate of *Steinernema feltiae* was followed over two years in an organic cropping system. The aim of studying entomopathogenic nematodes in this system was to gain knowledge on how cultural practise influences the populations of entomopathogenic nematodes. This knowledge may then be used to design or improve cropping systems that propagate entomopathogenic nematodes an in this way lower the risk of pest problems.

## Materials and methods

The organic cropping system that was studied was situated at Research Center Årslev (Danish Institute of Agricultural Sciences).The cropping system consisted of six 1-hectare fields that had been grown organically for six years without application of fertiliser or manure. The crops were grown in a fixed rotation of barley, green manure (alfalfa/clover), carrots/onions,

cabbage/leek and pea. The cropping system is further described by Thorup-Kristensen (1999). Entomopathogenic nematodes were quantified spring (May / June) and autumn (September / October) in 2001 and 2002 by baiting with *Tenebrio molitor* in four of the six fields. Baiting was performed on soil samples from 10 plots (7 $m^2$ each) in each of the fields. Five of the plots had been inoculated with *S. feltiae* (obtained from e-nema, Kiel, Germany) after the first bait in spring 2001. From each plot, 23-25 soil cores (approximately 400 ml of soil) were taken and stored at 5°C until baiting was performed (up to two months after sampling of soil). Each sample was baited twice by mixing and dividing the soil into two sub-samples. The sub-samples were baited in plastic jars that were placed in the dark at 18-24°C. One *T. molitor* larva was added per jar. The larvae were checked after one week. Dead larvae were replaced by a new larva and jars were checked again the following week where baiting was ended. Dead larvae were placed individually on water traps to collect nematodes emerging from the larvae. If one or more of the larvae from a sample was/were infected, the sample was denoted positive. In addition were soil living insects quantified once during the growing system by taking soil samples (1-4 l of soil per sample) around the root of the crop plants. Special attention was given to plant specific insects like cabbage root fly (*Delia radicum*) in cabbage, carrot root fly (*Psila rosae*) in carrots and the weevil *Sitona lineatus* in pea and alfalfa/clover. Cabbage root flies and carrot root flies were quantified when they were in the pupal stage in September whereas *Sitona lineatus* were quantified in July when the major part was in the larval stage. All insects were extracted by suspending the soil sample in water followed by flotation and sieving.

## Results

The bait results from the four fields are presented in Table 1 (Field A; data from not inoculated plots) and Figure 1 (Field B, C, D; data from all plots). The number of positive cores per plot in Field A ranged from 0-38 %. The highest level was observed in the autumn of 2001 after cabbage had been grown with a mean of 26 % positive cores. In the following year the numbers decreased again with a similar trend in all plots. The percentage of positive cores in not inoculated plots in Fields B, C, and D were generally at a low level. This was also the situation in Field C in inoculated plots whereas relatively high levels were seen in Field B and D in inoculated plots. The reason for these high levels could be the growing of pea which in 2001 in Field B resulted in 56 % positive cores and in 2002 in Field D raised the level from 22 % to 88 %. In field B, the number of positive cores remained at a high level in the autumn of 2002 (63 % positive cores).

Table 1. Percentage of soil cores in Field A (not inoculated plots) that were positive for entomopathogenic nematodes. The field was grown with cabbage in 2001 and barley in 2002. The nematodes were a mixture of *Steinernema affine* and *S. feltiae*.

| Year | Season | Positive cores (%) | | | | | Mean |
|---|---|---|---|---|---|---|---|
| | | Plot 1 | Plot 2 | Plot 3 | Plot 4 | Plot 5 | (%) |
| 2001 | Spring | 4 | 8 | 4 | 0 | 13 | **6** |
| | Autumn | 32 | 38 | 12 | 16 | 33 | **26** |
| 2002 | Spring | 8 | 24 | 20 | 8 | 12 | **15** |
| | Autumn | 4 | 20 | 0 | 4 | 0 | **6** |

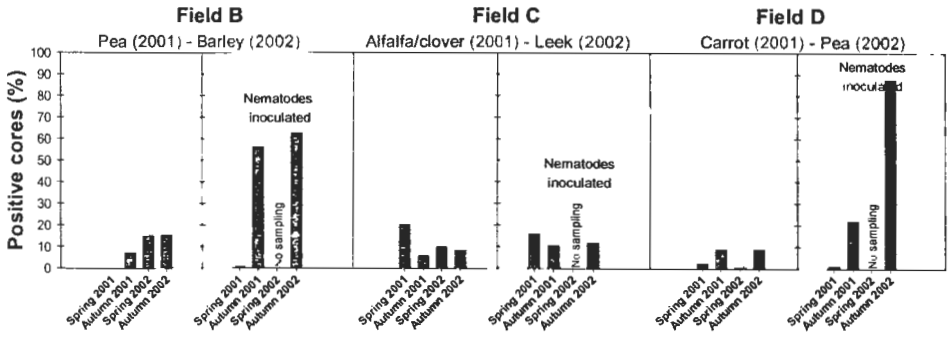


Figure 1. Entomopathogenic nematodes in Fields B, C and D in the organic cropping system at Årselv. Each bar is the mean of five plots. Results from Field A is shown in Table 1.

The successful establishment of *S. feltiae* in pea correlates well with the abundance of potential hosts in this crop with 15.6 and 11.9 *S. lineatus* larvae per plant in 2001 and 2002, respectively (Table 2). In the other crops, only cabbage was found to be infested with insects pests in higher numbers. The numbers of *D. radicum* were 7.2 and 11.8 per plant in 2001 and 2002 respectively (Table 2).

Table 2. Quantification of plant specific soil living insects in the organic cropping system at Årslev. Each soil sample contained one (cabbage, pea) or several roots (alfalfa/clover, carrots).

| Crop | Insects per sample[a] | | Insect species | Time |
|---|---|---|---|---|
| | 2001 | 2002 | | |
| Alfalfa/clover | 0.8 | . | *Sitona lineatus* larvae | July |
| Barley | . | . | No sampling | |
| Cabbage | 7.2 | 11.8 | *Delia radicum* pupae | September |
| Carrot | 0.1 | 0.0 | *Psila rosea* pupae | September |
| | 0.0 | 0.0 | *Agrotis segetum* | September |
| Pea | 15.6 | 11.9 | *Sitona lineatus* | July |

## Discussion

One of the most important factors for the abundance of entomopathogenic nematodes in the cropping system at Årslev seemed to be the number of potential hosts. *Sitona lineatus* is known to be susceptible to entomopathogenic nematodes (Wiech & Jaworska, 1990) and infected larvae were observed in field D at Årslev in 2002 (data not shown). The growing of pea was thus beneficial for the inoculated nematodes. The level of naturally occurring nematodes was also raised in pea although the general level was very low (from zero to 7% in 2001 and from 1 to 9% in 2002). An increase in the level of entomopathogenic nematodes was also seen during the growing of cabbage (Table 1) and the host here could be *D. radicum*. It has been shown that *S. feltiae* can reproduce in *D. radicum* (Nielsen, 2003; Nielsen & Philipsen, 2004).

The main conclusions of the present work is thus that entomopathogenic nematodes were present at a low level but inoculation of nematodes in relation to a crop infested with soil living pests could establish a higher level of nematode abundance.

## Acknowledgements

We wish to thank DARCOF (Danish Agricultural Research Center of Organic Farming) for financial support and the company e-nema, Kiel, Germany for providing nematodes. The technical assistance of Hanna Hansen and Anne Anttila is highly appreciated.

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# Sublethal effects of the parasitic nematode *Phasmarhabditis hermaphrodita* on the slug *Deroceras panormitanum* and the snail *Oxyloma pfeifferi*

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**Abstract**: The sublethal effects of the parasitic nematode *Phasmarhabditis hermaphrodita* on the slug *Deroceras panormitanum* and the snail *Oxyloma pfeifferi* were evaluated in bioassays and video experiments. Rates between 1/8 recommended rate and double rate were assessed. In the bioassays the slugs were significantly repelled from the treated surface of the plant pot. At the recommended rate, only 33% of slugs were found on the treated surface during the first week after treatment. Slugs fed significantly less than on untreated compost, depending on time after application and nematode rate between 25 and 100%.

In no-choice experiments the activity and mobility of *O. pfeifferi* on nematode-treated surfaces were significantly reduced (38 and 40% respectively) in comparison with untreated compost. Choice experiments with *D. panormitanum* did not show a significant overall repellence, but the slugs moved significantly less time and distance on treated than on untreated surfaces.

**Key words**: slugs, snails, slug-parasitic nematodes, biological control, repellence, feeding inhibition, irritation

## Introduction

Slugs and snails are common pests in agriculture and horticulture (Godan, 1979; Barker, 2002; South, 1992). In the Hardy Nursery Sector (HNS) of the British horticultural industry, the slug *Deroceras panormitanum* and the snail *Oxyloma pfeifferi* were found to be the most common mollusc pest species (Schüder *et al.*, 2002). Growers are interested in controlling these species with the parasitic nematode *Phasmarhabditis hermaphrodita.* In laboratory bioassays the nematodes caused significant mortality of slugs after three weeks and of snails after two weeks (Schüder *et al.*, 2003). This may be a too long a period to leave the crop unprotected. This paper presents experiments on the sub-lethal effects of nematode applications, which may protect the cropuntil mortality occurs.

## Material and methods

Slugs (*D. panormitanum*) and snails (*O. pfeifferi*) were collected from field sites and from HNS nurseries respectively.

***Bioassays in plant pots***

One-litre plant pots were filled with damp compost. The nematode suspension (Nemaslug®) was applied onto the damp compost (Table 1). A 4-cm$^2$ piece of Chinese cabbage leaf was also treated with nematodes and placed in the middle of each pot. One slug was added to each of 10 replicate pots. The pots were covered with muslin and polythene and kept at 10 °C and 12:12 hours light:dark. The position of the slug and feeding damage were assessed daily for the first week, then weekly until all slugs were dead.

Table 1. Nematode application rates used in the bioassays and abbreviations for future tables and figures.

| Treatment | tested | Abbreviation |
|---|---|---|
| Damp compost | Slug and snail | UNT |
| 150,000 nematodes per $m^2$ | Slug | 1/2x |
| 300,000 nematodes per $m^2$ | Slug and snail | 1x |
| 600,000 nematodes per $m^2$ | Slug | 2x |

***Video analysis of slug and snail behaviour***

The behavioural response of *O. pfeifferi* to treatment of compost with the recommended rate of nematodes was studied using low-light, time-lapse video in a no-choice experiment. One snail was placed in each of 16 replicate, 16 cm-diameter containers containing damp compost, onto which the nematodes were added in 30 ml of water. Snail behaviour was recorded for eight hours at 15 °C. The aim of the no-choice experiment was to assess irritant effects of nematode treated areas.

The same methods were used for a choice experiment with *D. panormitanum*, except that only half of each container was treated with 15 ml suspension of nematodes. The aim of the choice experiment was to assess repellent effects of nematode treated areas.

## Results and discussion

***Bioassays in plant pots***

All rates of nematodes repelled the slugs from remaining in contact with the treated compost or leaf, during the first week after treatment. The mean % slugs remaining in contact with the compost or leaf treated with recommended, double and half rates were significantly lower (33, 17 and 38% respectively, $P < 0.001$) than those treated with water (83%).

Over the five-week period of the experiment, all rates of nematodes significantly reduced mean percentage leaf feeding by the slugs when compared with the untreated compost ($P < 0.001$, Table 2). The double rate of nematodes reduced mean percentage feeding by significantly more (70%) than the recommended and half-rates (45 and 43% respectively). This result is consistent with work carried out with *D. reticulatum* (Glen *et al.*, 2000).

Table 2. Mean % reduction in feeding of *Deroceras panormitanum* compared with untreated compost.

| Week | % reduction in feeding | | |
|---|---|---|---|
| | 1/2x | 1x | 2x |
| 1 | 25 | 36 | 55 |
| 2 | 70 | 73 | 88 |
| 3 | 79 | 66 | 93 |
| 4 | 79 | 83 | 98 |
| 5 | 30 | 72 | 100 |

***Behavioural response of snails (no choice)***

On untreated compost the snails moved for as long as the slugs, but on nematode treated surfaces this time was significantly reduced by more than an hour or 38 % ($n = 16$, t-test: $P < 0.05$, Table 3). When moving, the velocity of snails was similar on treated and untreated

surfaces (n =16, t-test: P > 0.05, Table 3). In comparison with untreated surfaces the distance moved on nematode treated surfaces was significantly reduced to less than 1 m per night, which represented a reduction in track length by 40 %. A representative example for snail tracks on untreated and treated surfaces can be seen in Figure 1.

Table 3. Behavioural response of *Oxyloma pfeifferi* to nematode treatment in eight-hour no-choice test. Asterisks indicate a significant lower activity on treated than on untreated surfaces (* P < 0.05; ** P < 0.01).

| No choice | Moving activity | Distance moved (cm2) | Velocity |
|---|---|---|---|
| Untreated | 202 ± 25* | 141 ± 11** | 0.8 ± 0.1 |
| Nematodes | 126 ± 21* | 84 ± 15** | 0.9 ± 0.2 |
| Reduction (%)[a] | 38 | 40 | nil |

[a] reduction in activity in comparison to untreated replicates

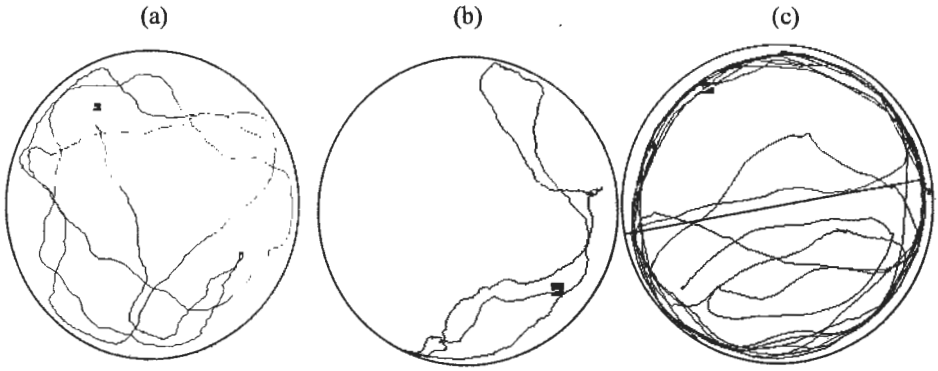


Figure 1. Representative tracks of an eight-hour period: (a) *Oxyloma pfeifferi* in a no-choice test on untreated compost, (b) *O. pfeifferi*, no-choice, treated with nematodes, (c) *Deroceras panormitanum*, choice (upper half nematode-treated) (arena 16 cm in diameter).

***Behavioural response of slugs (choice)***

When given a choice between untreated and treated surfaces the slugs showed a trend for avoiding the untreated areas, both in presence and moving activity. However, neither their presence in the untreated semi-circle nor their duration of moving activity was significantly higher than in the nematode-treated semi-circle (n = 16, paired t-test: P >0.05, Table 4). As in the no-choice test the slugs moved significantly slower when on nematode-treated surfaces (n = 16, paired t-test: P < 0.001, Table 4) with the consequence of significantly reduced track lengths in the treated semi-circles in comparison with the untreated semi-circles (n = 16, paired t-test: P < 0.01, Table 4). A representative example of a slug track in a choice-test can be seen in Figure 1c.

Both the results from the bioassay and the video experiments regarding repellence are consistent with work carried out with *D. reticulatum* (Glen & Wilson, 1997; Wilson *et al.*, 1999). In agriculture, the repellent effect may allow band application or circular application

around the stem of a plant (Glen & Wilson, 1997). In HNS, only the pots and not the entire greenhouse area could be treated. Repellence was not as strong as feeding inhibition. Therefore the feeding inhibition may be regarded as the major effect giving the crop immediate protection after application (Hass, *et al.*, 1999) until subsequent mortality occurs.

Table 4. Activity of *Deroceras panormitanum* in an eight-hour choice experiment. Asterisks indicate a significant difference between treated and untreated semi-circle. (* $P < 0.05$; ** $P < 0.01$).

| Choice | Presence | | Locomotor activity | | Distance moved | | Velocity |
|---|---|---|---|---|---|---|---|
| | (min) | (%) | (min) | (%) | (min) | (%) | (cm/min) |
| Compost | 273 ± 28 | 57 | 106 ± 13 | 54 | 213 ± 24** | 56 | 2.2 ± 0.1*** |
| Nematodes | 207 ± 28 | 43 | 89 ± 10 | 46 | 170 ± 23** | 44 | 1.8 ± 0.1*** |

## Acknowledgements

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# Slug parasitic nematodes in vegetable crops

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**Abstract**: Using nematodes, *Phasmarhabditis hermaphrodita*, for control of the grey field slugs *Deroceras reticulatum* in green asparagus, Brussels sprouts and iceberg lettuce. Results are described of slug control in green asparagus in 2000, Brussels sprouts in 1999 and iceberg lettuce in 2002. Significant protection of the green asparagus crop during the harvest period was reached with nematodes applied three times in the row at a dose of 50,000 $m^{-2}$. Successful protection of the Brussels sprout crop during the period that the buttons (sprouts) are on the plants was reached with nematodes applied seven times in the row at a dose of 150,000 $m^{-2}$. Significant protection of the iceberg lettuce against the grey field slug was reached until five weeks after transplanting with nematodes applied once at a dose of 150,000 per $m^{-2}$.

**Key words**: *Deroceras reticulatum*, *Phasmarhabditis hermaphrodita*, green asparagus, Brussels sprouts, iceberg lettuce, field trails, semi-field trial.

## Introduction

Slug pests are difficult to control in a wide range of horticultural crops (Port & Ester, 2002). In green asparagus, Brussels sprouts and iceberg lettuce slugs decrease yield or cause loss of quality due to presence of slugs or their faeces in the harvested product (Glen *et al.*, 2000). The grey field slug *D. reticulatum* is the most harmful species in the Netherlands.

Green asparagus (*Asparagus officinalis* L.) spears are deformed by tiny feeding marks at the growing tips, resulting in an unmarketable product (Ester *et al.*, 2003a). Slugs are the most serious pests of Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* (D.C.) Schulz) in Western Europe (Glen *et al.*,1996). Slug damage results in loss of product quality as slugs attack the young buttons by eating the outer leaves (Ester *et al.*, 2003b). In iceberg lettuce (*Latuca sativa*) presence of slugs, feeding damage and faeces must be avoided in the saleable head.

During harvesting time of green asparagus and after planting iceberg lettuce chemical application is restricted due to residue problems. Chemical protection of Brussels sprouts is often insufficient during growth. This research shows the potential of the nematode *P. hermaphrodita* protecting high valuable crops against slugs. The parasitic nematode is currently sold as Nemaslug® in different countries and recommended in a dose of 300,000 nematodes $m^{-2}$ (Glen *et al.*, 2000).

## Materials and methods

### *Green asparagus*

From April until the beginning of June field experiments were carried out at Oudkarspel (clay soil with 22 % silt) in the western part of the Netherlands. The asparagus variety Gijnlim was planted in 1997. A randomised block design was conducted with five replicates. Each plot consisted of a 5 m asparagus and 1.5 m width. All the treatments were 0.3 m wide row applied with a total of 1.5 $m^2$ treated area. The first treatment (11 April) was carried out at the

time the soil surface crust was breaking as the first asparagus spears emerged followed by treatments on 18, 25 April and 2 May. The nematodes and metaldehyde bait pellets were applied as a band application. Nematodes were applied in dosages of 10,000 (3 times applied), 50,000 (3), 100,000 (3) and 300,000 (1) per square meter as a suspension by a watering can at the end of the day. One treatment consisted of metaldehyde (a.i. 5 %) as a chemical reference. The asparagus crop was harvested daily cutting the spears at 21 cm length at soil level. The spears were counted and divided in affected (slug-damaged) and unmarketable spears (Ester and Rozen, 2003).

***Brussels sprouts***

From July until October 1999, a field experiment were carried out on clay soil of 20 – 30 % silt in the south western part of the Netherlands. Brussels sprout variety Romulus was planted in the first week of May. A randomised block design was conducted with four replicates. Plots consisted of 7.5 m x 6 m = 150 plants. The previous crop was Perennial ryegrass. Nematodes were applied in a 0.6 m wide band application (between the crop rows) as a suspension by Spraymatic 10 S sprayer (tank content 15 L, 2 Teejet XR 11006 nozzles, 2.5 bar at the nozzle without a filter) at the end of the day in a two week interval. For each treatment 10 mm water was used. Metaldehyde (a.i. 6.4 %) was broad applied by hand. Nematodes and metaldehyde were applied 7 times (start at 15 July) and 5 times (start at 12 August). Crop damage by slugs was assessed during the autumn every four weeks by cutting the stems and counting the number of sprouts damaged by slugs the number of undamaged sprouts on 10 plants in each plot.

***Iceberg lettuce***

A semi-field trial was conducted of 10 treatments and 4 replicates to assess feeding inhibition of *D. reticulatum* with *P. hermaphrodita*. An iron fence provided with copper barrier at the top surrounded plots of one square meter. Fourty slugs were added per plot, accept for one control without slugs. Nematodes were applied with a watering can and one treatment the nematodes were sprayed with a Spraymatic 10 S (nozzlenumber 8006 with a pressure of 2.5 bar at the nozzle). Nematodes were diluted in 2000 L water $ha^{-1}$. After nematode treatment 16 seedlings with 4 leaves were transplanted of the variety Anouk. Plant distance was 0.2 m. As a chemical reference iron-III-phosphate (a.i. 1 %) and metaldehyde (a.i. 6.4 %) were applied after planting. Iceberg lettuce plants were replaced after 2 weeks. The number of leaves and the number of healthy (no feeding damage) leaves were counted 4 and 11 days after the second transplantation and calculated into percentages.

***Data analysis***

Data were analysed using analysis of variance (ANOVA) in Genstat 5. From the ANOVA means, least significant differences (LSD) and F-probabilities are presented. LSD's are calculated with Student's t-distribution. Means followed by the same letter do not differ significantly ($p = 0.05$).

## Results and discussion

***Green asparagus***

Eight weeks after the first treatment, all the nematode treatments as well as the metaldehyde treatment resulted in significant control of slug damage, except for 10,000 nematodes $m^{-2}$ three times applied (Table 1). *P. hermaphrodita*, applied three times at 50,000 per $m^2$ as a row application may reduce the total application rate of only 10% of the recommended dose. In green asparagus as a high value crop especially in the first four weeks of harvesting, *P. hermaphrodita* can be introduced as a new biological agent against slugs (Ester *et al.*, 2003a).

Table 1. Number of harvested spears $m^{-2}$, percentage of slug-damaged spears and percentage of unmarketable spears up to 6 June, 2000.

| Treatment | Dose | No. | No. of spears | % affected | % unmarketable |
|---|---|---|---|---|---|
| Nematodes | 10,000 $m^{-2}$ | 3 | 40 | 41.0 b | 26.7 a |
| | 50,000 $m^{-2}$ | 3 | 46 | 15.6 a | 7.5 b |
| | 100,000 $m^{-2}$ | 3 | 42 | 14.6 a | 8.6 b |
| | 300,000 $m^{-2}$ | 1 | 42 | 13.7 a | 6.9 b |
| Metaldehyde | 350 g a.i. $ha^{-1}$ | 4 | 47 | 8.4 a | 4.8 b |
| Untreated | 0 | - | 36 | 44.9 b | 26.5 a |
| F-probability | | | 0.786 | < 0.001 | < 0.001 |

Table 2. Mean percentage leaf damage to Brussels sprouts in 1999.

| Treatment | Dose $m^{-2}$ | No. | 26August | 23 September | 21 October | 19 November |
|---|---|---|---|---|---|---|
| Untreated | 0 | 0 | 8.9 a | 37.8 a | 63.9 a | 52.1 a |
| Nematodes | 150,000 | 7 | 1.0 c | 5.7 b | 11.8 c | 9.6 c |
| | 300,000 | 7 | 2.3 bc | 5.8 b | 11.2 c | 10.9 c |
| | 450,000 | 7 | 1.9 c | 1.9 b | 7.8 c | 5.5 c |
| Metaldehyde | 448 g a.i.$ha^{-1}$ | 7 | 1.4 c | 5.5 b | 9.0 c | 12.9 c |
| Nematodes | 300,000 | 5 | 7.6 a | 7.4 b | 10.0 c | 7.3 c |
| | 450,000 | 5 | 2.6 bc | 3.0 b | 4.2 c | 1.8 c |
| Metaldehyde | 448 g a.i.$ha^{-1}$ | 5 | 7.2 ab | 24.7 a | 34.2 b | 35.0 b |
| F-probability | | | 0.009 | < 0.001 | < 0.001 | < 0.001 |

Table 3. Mean percentage healthy leaves of iceberg lettuce, 2002.

| Treatment | Dose $m^{-2}$ | No. of slugs | Treatment dates | Planting dates | % healthy leaves | |
|---|---|---|---|---|---|---|
| | | | | | 23 July | 30 July |
| Untreated | 0 | 0 | - | 5 & 19 July | 97 a | 97 a |
| | 0 | 40 | - | 5 & 19 July | 35 e | 36 e |
| Nematodes (sprayer) | 300,000 | 40 | 5 July | 5 & 19 July | 83 abc | 75 abc d |
| Nematodes (can) | 300,000 | 40 | 11 July | 19 July | 84 abc | 85 ab |
| | 300,000 | 40 | 5 July | 5 & 19 July | 92 ab | 93 a |
| | 150,000 | 40 | 5 July | 5 & 19 July | 76 bcd | 77 abc d |
| | 75,000 | 40 | 5 July | 5 & 19 July | 64 d | 61 d |
| | 37,500 | 40 | 5 July | 5 & 19 July | 65 d | 65 cd |
| Iron-III-phosphate | 0.0500 g | 40 | 5 July | 5 & 19 July | 87 abc | 82 abc |
| Metaldehyde | 0.0448 g | 40 | 5 July | 5 & 19 July | 75 cd | 67 bcd |
| F-probability | | | | | < 0.001 | < 0.001 |

***Brussels sprouts***

All nematode treatments reduced damage of sprouts by slugs in opposite to the untreated plots, except 5 times 300,000 nematodes on 26 August (Table 2). Treatments with nematodes decreased the sprout damage by slugs independent of the initial treatment start. These treatments resulted in levels of damaged sprouts similar to metaldehyde pellets at a dose of

448 g a.i. $ha^{-1}$. Slug pellets applied 15 July show a significant better protection comparable to the August treatments.

***Iceberg lettuce***

Wilson *et al.* (1995) reported that 300,000 nematodes $m^{-2}$ significantly reduced the percentage of lettuce plants damaged by slugs. Table 3 shows the capability of lower numbers of nematodes to keep significant healthier (not damaged) leaves of iceberg lettuce by slugs. A dose response effect is evident. Reducing slug presence and damage in the saleable head of iceberg lettuce, as the outer leaves will be removed, is still in research.

In green asparagus, Brussels sprouts and iceberg lettuce nematodes showed strong potential protection against slug damage by *D. reticulatum*.

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# Entomopathogenic fungi

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# Natural occurrence and spatial distribution of *Beauveria bassiana* and *Metarhizium flavoviride* in a Danish agro-ecosystem

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**Abstract**: The frequencies and spatial distribution of entomopathogenic fungi were investigated in the soil of a single field and adjacent hedgerow. Soil samples were collected using a grid based on GIS (Geographical Information Systems) and GPS (Global Positioning System) making it possible to relocate sample points in consecutive years. *Beauveria bassiana* was the most frequent fungus in the field soil (29% in 2001; 42% in 2002), while *Paecilomyces fumosoroseus* was most frequent in soil from the hedgerow (81% in 2002). *Metarhizium flavoviride* was more common in the field soil than *M. anisopliae* in 2001 (22% vs. 7%) and 2002 (17% vs. 3%). Both *B. bassiana* and *M. flavoviride* had a clumped distribution in the two years of investigation.

**Key words**: Soil, *Galleria* bait method, GIS, GPS, spatial distribution, *Beauveria bassiana*, *Metarhizium flavoviride*, local species composition

## Introduction

Entomopathogenic fungi naturally present in the soil constitute a reservoir of inoculum of antagonists for insect pests. Knowledge of the local occurrence of these fungi in soil is important to assess the potential of the natural occurring species for regulating insect populations on the locality.

The occurrence of entomopathogenic fungi in soil has been investigated in several countries, usually on a national or regional scale (e.g. Kleespies *et al.*, 1989; Vänninen, 1995; Chandler *et al.*, 1997). Steenberg (1995) made a survey of the naturally occurring fungi in soil sampled from different parts of Denmark. However, no investigation has focused on the occurrence and distribution within a single field using a high number of samples.

In the present study soil samples collected from one organic grown field and the associated hedgerow were analysed for entomopathogenic fungi with special reference to *Beauveria bassiana*. In addition, the horizontal distribution of fungi in the field was investigated. Preliminary data are presented below.

## Materials and methods

### *Field site and soil sampling*

The field site was situated on an experimental farm at Taastrup, 20 km west of Copenhagen, Denmark. In 1999, the production of the 27.5 ha farming area was converted from conventional to organic and a crop rotation system was implemented. In the spring 2001 and the autumn 2002, soil samples were collected from a grid of sample points, 25 m apart, covering the whole field using GIS (Geographical Information Systems). Each point could be relocated by GPS (Global Positioning System) with a precision of a few cm in successive years. Samples at each point were collected as 25 soil cores (diameter 1 cm) to a depth of 10 cm. The cores in each point were taken within a 50 cm x 50 cm grid, thus every soil sample

represented an area of 0.25 $m^2$. In between samplings at different points, the core sampler was washed in water and 70% ethanol. Samples were also collected from a hedgerow bordering the field to the southeast. A total of 70 samples, 5 m apart, were collected as described above along a transect in the middle of the hedgerow.

***Isolation of fungi***

Soil from each sample was placed in separate plastic containers and insect pathogenic fungi were isolated by the insect bait method (Zimmermann, 1986), using larvae of *Galleria mellonella* as bait insect. Each soil sample was baited with 10 medium sized larvae of *G. mellonella* at room temperature and during the first week the containers were turned upside down frequently to insure high level of contact between larvae and soil. During four weeks of baiting the mortality was assessed once every week, and dead larvae were rinsed in demineralised water and thereafter incubated individually under moist conditions.

Emerging fungi were identified by macroscopical features as colour and conidia formation, and identifications were verified by use of light microscopy. The fungi were isolated on solid media (SDA) and stored in the culture collection at the department.

## Results

***Species composition***

There was no significant difference in the frequency of samples from the field soil positive for insect pathogenic fungi between the two years ($\chi^2$=0.566; P=0.452). In both 2001 and 2002, *B. bassiana* was the most common species in the field soil while *Paecilomyces fumosoroseus* was more common in soil from the hedgerow (Fig. 1). *Metarhizium anisopliae* was relatively rare in the field soil while *M. flavoviride* was more common. The latter was also the only *Metarhizium* species found in the hedgerow soil (Fig. 1). *P. farinosus* was equally common in both field and hedgerow soil. In general, the frequencies of the different fungi were quite consistent between 2001 and 2002 in the field, although there was a significantly higher frequency of *B. bassiana* in autumn 2002 than in spring 2001 ($\chi^2$=9.620; P=0.002).

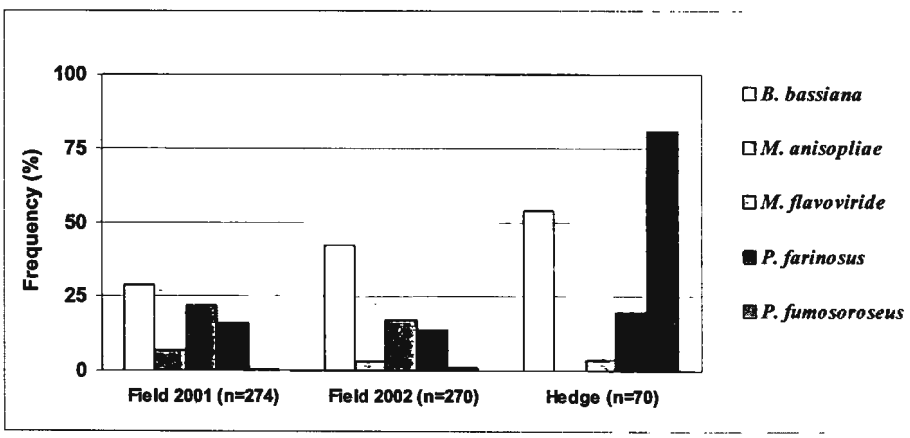


Figure 1. Frequencies of entomopathogenic fungi in soil samples collected from the organic field in spring 2001 and autumn 2002 and the associated hedgerow in autumn 2002.

In addition to the species mentioned in Figure 1, *Lecanicillium* (= *Verticillium*) *lecanii* and *Conidiobolus coronatus* were found very rarely in the field soil.

***Spatial distribution***

As the location of each soil sample in the field was known, it is possible to depicture the horizontal distribution of the fungi. Of the two most common fungi, *B. bassiana* was found more frequently in the westernmost cropping system (barley) in the field site in 2002 ($\chi^2$=15.631; P=0.0013) while no significant difference in the horizontal distribution of *M. flavoviride* could be found ($\chi^2$=7.580; P=0.0556).

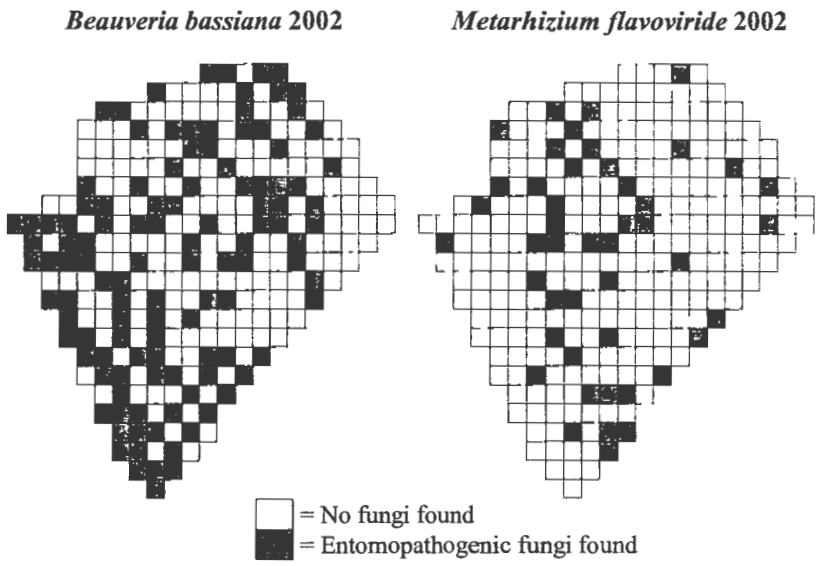


Figure 2. Horizontal distribution of positive soil samples in the field site for *Beauveria bassiana* (left) and *Metarhizium flavoviride* (right). Each square represents 25x25 m.

To compare the distribution patterns of *B. bassiana* and *M. flavoviride* indices of aggregations were calculated (Krebs, 1999). In Table 1 both Lloyd's index of patchiness and standardised Morisita index suggest clumped distributions for the two species at the investigated scale of 25 m between sampling points. Both indices were quite consistent between the two years of investigation.

## Discussion

The present results suggest that if soil sampling is performed thoroughly within a single field site all the expected soil borne entomopathogenic fungi can be found. Despite the very short distance from the field to the hedgerow, *P. fumosoroseus* was almost only found in soil from the hedgerow, while *B. bassiana* was common in both places. Chandler *et al.* (1997) found *P. fumosoroseus* in hedgerow and woodland soils but not in field soil. Likewise, Steenberg (1995) never found *P. fumosoroseus* in Danish field soil. This suggests that arable fields do not provide optimal conditions for *P. fumosoroseus*

Table 1. Lloyd's index of patchiness >1 indicates clumped distributions. At 95% confidence level, standardised Morisita index >0.5 indicates clumped distributions.

| Site | Lloyd's index of patchiness | | Standardized Morisita index | |
|---|---|---|---|---|
| | *B. bassiana* | *M. flavoviride* | *B. bassiana* | *M. flavoviride* |
| Field 2001 | 3.52 | 3.02 | 0.504 | 0.503 |
| Field 2002 | 3.22 | 2.57 | 0.504 | 0.502 |

Surprisingly *M. flavoviride* was the second most frequent fungus in the field soil and the species was more common than *M. anisopliae*. Steenberg (1995) only found *M. flavoviride* in a single soil sample using *Tenebrio molitor* as bait insect and characterized the fungus as a rare species in Denmark. In general, *M. anisopliae* is more common in Danish soils (Steenberg, 1995) and it is commonly found in agricultural soils in the UK (Chandler, 1997), Finland (Vänninen, 1995), and Germany (Kleespies *et al.*, 1989). However, this investigation demonstrated that *M. flavoviride* can be locally common and outlines that the local species composition cannot be predicted from investigations compiling several field sites into habitat types. Whether the high population level of *M. flavoviride* in the field soil of the investigated site could influence the local population of *M. anisopliae* causing it to decline due to competition has to be evaluated further by additional experiments.

Based on the present sampling method and scale of investigation it can be concluded that the two most common insect pathogenic fungi (*B. bassiana* and *M. flavoviride*) had a clumped distribution and could be found in all parts of the investigated field. In addition, it is important to collect a large number of separate soil samples to make sure that all occurring species are found and thus generate a representative picture of the natural occurrence at a field site.

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# Observations on the occurrence of Entomophthorales in Austria

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**Abstract**: Observations on the occurrence of Entomophthorales in Austria (Vienna, Lower Austria, Upper Austria, Styria and Burgenland) in the year 2001 brought evidence of eight different species identified from aphids (*Erynia neoaphidis, Erynia erinacea, Entomophthora planchoniana, Neozygites fresenii, Neozygites cinarae, Conidiobolus obscurus, Zoophthora aphidis* and an unidentified *Zoophthora* sp.). Five different entomophthoralean species were found on Diptera (*Erynia conica, Erynia dipterigena, Eryniopsis transitans, Entomophthora muscae* and an unidentified *Batkoa* sp.).

**Key words**: Entomophthorales, aphids, Diptera, Austria

## Introduction

Entomophthorales are considered to be the most important pathogens of aphids. They are known to have a great potential as biological control agents (Latgé & Papierok, 1988) and have been object of several studies (ref. in Pell *et al.*, 2001). All over the world 30 species of the order Entomophthorales are known from aphids with 23 species of European distribution (Keller, 1987, 1991, 1997; Humber, 1990; Balazy, 1993). From Austria, there is only scanty information on the occurrence of Entomophthorales, a preliminary study brought evidence of several different species on aphids (Barta *et al.*, 2003). Therefore, we intended with this further study to get more detailed information on the occurrence of Entomophthorales on dipteran and especially on aphid hosts from different regions in Austria.

## Material and methods

Sampling was performed in the year 2001 during five time periods: in June (6. and 27.-28.), July (10.-13.), September (20.-25.) and in November (8.-11.). Insects were collected at 19 different localities in Austria: Vienna (10 sites), Lower Austria (6 sites, at two different areas: eastern Lower Austria and western Lower Austria, separated by a distance of approx. 100 km), Upper Austria (1 site), Styria (1 site) and Burgenland (1 site). Scenery varied including urban areas, agricultural areas, lowland forests and Alpine forests. The insects were collected from crops, plants in the herbaceous layer, shrubs, bushes and trees, wherever they could be found excluding root aphids. Dead aphids or Diptera suspected to be killed by fungi were collected on a random basis. Cadavers with external symptoms of an entomophthoralean disease were collected, as well as living aphids from colonies close to infected individuals.

## Results and discussion

Thirty five different aphid species resp. subspecies of the families Aphididae, Anoecidae and Drepanosiphidae were found with an entomophthoralean infection. Eight different entomophthoralean species were identified: *Erynia neoaphidis* (from 21 aphid species), *Erynia erinacea* (from 1 aphid species), *Entomophthora planchoniana* (from 7 aphid species), *Neozygites fresenii* (from 11 aphid species), *Neozygites cinarae* (from 1 aphid species), *Conidiobolus obscurus* (from 2 aphid species) and *Zoophthora aphidis* (from 1 aphid species) and one unidentified *Zoophthora* species (on *Cinara pilicornis* from Upper Austria). Especially interesting is the record of *Erynia neoaphidis* (in eastern Lower Austria) on *Diuraphis noxia*, an aphid species recently reported new to Austria (Cate, 2000) (Table 1).

Table 1. Entomophthorales identified from different aphid species in Austria.

| Entomophthoralean species | Aphid species |
|---|---|
| *Erynia neoaphidis* | *Aphis fabae cirsiiacanthoidis, Aphis nasturtii, Aphis craccivora, Aphis sambuci, Capitophorus horni horni, Brachycaudus cardui, Acyrthosiphon pisum, Diuraphis noxia, Microlophium carnosum, Macrosiphum funestum, Hyperomyzus lactucae, Rhopalosiphum padi, Acyrthosiphon neerladicum, Acyrthosiphon pelargonii, Rhopalosiphum maidis, Phorodon cannabis, Brachycorynella asparagi, Uroleucon aeneum, Metopolophium dirhodum, Myzus persicae, Capitophorus elaeagni* |
| *Erynia erinacea* | *Aphis umbrella* |
| *Entomophthora planchoniana* | *Aphis fabae, Aphis pomi, Aphis sambuci, Eucallipterus tiliae, Cavariella pastinacae, Cavariella theobaldi, Capitophorus elaeagni* |
| *Neozygites fresenii* | *Aphis acetosae acetosae, Aphis fabae, Aphis fabae fabae, Aphis fabae cirsiiacanthoidis, Aphis fabae mordwilkoi, Aphis nasturtii, Brachycaudus cardui, Hyalopterus pruni, Rhopalosiphum maidis, Rhopalosiphum padi, Capitophorus elaeagni* |
| *Neozygites cinarae* | *Cinara pilicornis* |
| *Conidiobolus obscurus* | *Acyrthosiphon pisum, Rhopalosiphum padi* |
| *Zoophthora aphidis* | *Anoecia corni* |
| *Zoophthora* sp. | *Cinara pilicornis* |

*E. planchoniana* was the only species found on aphids (*Aphis sambuci*) during the first sampling period in early June; furthermore, this species was found during the second sampling period in June too, and in July as well as in November. *E. neoaphidis* (the most abundant species) and *N. fresenii* were found in the course of all other sampling periods on different host species. All the other species were found only in very few cases (Tables 1 and 2). A great variety of species was found during the second sampling period in June (4 different species), in July (4 different species, plus one *Zoophthora* sp.) and in November (5 different species) (Table 2).

The occurrence of these fungal species varied with regard to sampling period and sampling area, even differences within different eastern and western Lower Austria were

found. Only *E. neoaphidis* was found on aphids from all five federal states, *E. planchoniana* and *N. fresenii* were found in four federal states (not in Burgenland). *N. cinarae* occurred on *Cinara pilicornis* from Upper Austria only, all the other species were found in one federal state (*E. erinacea*, *Zoophthora* sp.), in two (*C. obscurus*), or in three federal states (*Z. aphidis*).

Table 2. Occurrence of different entomophthoralean species on aphids (numbers of records) during the five sampling periods (s.p.: 1.-5.) and total numbers of records (Tot. Σ).

| Month | s.p. | *E. neoaphidis* | *E. erinacea* | *E. planchoniana* | *N. fresenii* | *N. cinarae* | *C. obscurus* | *Z. aphidis* |
|---|---|---|---|---|---|---|---|---|
| June | 1. | - | - | 1 | - | - | - | - |
| | 2. | 7 | - | 3 | 2 | - | 1 | - |
| July | 3. | 3 | - | 3 | 5 | 1 | - | - |
| Sept. | 4. | 10 | 1 | - | 4 | - | - | - |
| Nov. | 5. | 13 | - | 5 | 2 | - | 1 | 3 |
| Tot. Σ | | 33 | 1 | 12 | 13 | 1 | 2 | 3 |

Observations on the occurrence of Entomophthorales on Diptera from Austria were performed in the year 2001 for the first time. Five different entomophthoralean species were found on Diptera during the first and second sampling period in June: *Erynia conica, Erynia dipterigena, Eryniopsis transitans, Entomophthora muscae* and *Batkoa* sp. The occurrence of *E. transitans* (on *Limonia tripunctata*) is the first record that does not originate from the type locality (Keller & Eilenberg, 1993)(Table 3).

Table 3. Entomophthorales on different Diptera, from localities in Lower Austria (LA).

| Date | Locality | Host | Entomophthoralean species |
|---|---|---|---|
| 06.06.01 | Orth a.d. Donau (LA) | Chironomidae | *Erynia conica* |
| 06.06.01 | Orth a.d. Donau (LA) | unidentified fly | *Erynia dipterigena* |
| 06.06.01 | Orth a.d. Donau (LA) | *Limonia tripunctata* | *Eryniopsis transitans* |
| 06.06.01 | Orth a.d. Donau (LA) | unidentified fly | *Batkoa* sp. |
| 28.06.01 | Fuchsenbigl (LA) | unidentified fly | *Entomophthora muscae* |

Species diversity was much higher in the study performed in 2001, compared to the results found in the year 2000 (Barta *et al.*, 2003). Three species (*E. neoaphidis, E. planchoniana,* and *N. fresenii*) were dominant and widely distributed in the sampling areas of Austria. *E. neoaphidis* and *E. planchoniana*, which are very common pathogens of aphids with worldwide distribution (Keller, 1991; Balazy, 1993), can be responsible for epizootics in aphid populations (Milner *et al.*, 1980). In Austria, no *E. planchoniana* epizootics were recorded. *N. fresenii* is known from nearly all continents including the south Pacific region (Keller, 1997), this species is considered to be best adapted to tropical conditions (Steinkraus *et al.*, 1991; Keller, 1997), although it is effective in the subpolar region too (Nielsen *et al.*, 2001). All the other species were observed in one to three sampling sites, respectively were identified from one to two aphid species. *E. erinacea* is known for more than 20 years from the type locality in Israel (Ben-Ze'ev & Kenneth, 1979), but now this species is known from

central Europe too. Even if this study was focused mainly on aphids, some diseased Diptera were found during sampling; this study is the first report on the occurrence of Entomophthorales on Diptera from Austria.

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# Insect species used as baits for isolation of entomopathogenic fungi from the soil

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**Abstract**: The aim of this study was to compare usefulness of different insects as a baits for isolation of entomopathogenic fungi from the soil. Imago of two species from Coleoptera (*Acanthoscelides obtectus, Tribolium destructor*), and larvae of four species from Lepidoptera (*Galleria mellonella, Ephestia kühniella, Laspeyresia pomonella* and *Plodia interpunctella*), one species from Hymenoptera *Pteronidea ribesii*), and three species from Coleoptera (*Agriotes ustulatus,Tenebrio molitor, Tribolium destructor*) were selected for detection of entomopathogenic fungi from the soil. The imago of *T. destructor*, larvae of *P. ribesii* and *A. ustulatus* were not infected by entomopathogenic fungi in the soil. High level of resistance to fungal infection showed the imago of *A. obtectus*. Only 2% of specimens were infected by *Metarhizium anisopliae*. Fife species of insect pathogenic fungi (*Conidiobolus major, Beauveria bassiana, M. anisopliae, Paecilomyces farinosus* and *P. fumosoroseus*) were found on *T. destructor* larvae. *M. anisopliae* was the most frequent. Only three fungal species were found on lepidopteran larvae. The fungus *B. bassiana* appeared most frequently on *G. mellonella* larvae; *P. fumosoroseus* on *E. kühniella* and *G. mellonella* but *M. anisopliae* on *P. interpunctella* and *L. pomonella*. The most useful as baits for isolation of fungi were larvae: *G. mellonella, E. kühniella, P. interpunctella, L. pomonella* and *T. destructor*.

**Key words**: entomopathogenic fungi, insect bait method, isolation

## Introduction

Soil is the main reservoir of entomopathogenic fungi, which play a major role in the regulation of pest population (Ferron, 1981; Ignoffo *et al.*, 1978). The common techniques for isolation of saprophytic soil fungi are of little efficiency in relation to entomopathogenic strains because most of the latter either requires of richer composition of nutritive substances or is not able to compete with typical saprophytes.

The "insect bait method", developed by Zimmermann (1986) has been widely used as a standard for isolating entomopathogenic fungi from soils in many countries. This method facilitates quick and easy isolation and enable to detect more virulent isolates, as compared with selective medium method. The spectrum of isolated fungal species depends on temperature, type of soil and species of insect bait used ( Mietkiewski *et al.*, 1992; Tkaczuk & Mietkiewski, 1996; Tkaczuk *et al.*, 2000). The aim of this study was to compare usefulness of different insects as a baits for isolation of entomopathogenic fungi from the soil.

## Material and methods

Imago of two species of Coleoptera (*Acanthoscelides obtectus, Tribolium destructor*), and larvae of four species of Lepidoptera (*Galleria mellonella, Ephestia kühniella, Laspeyresia pomonella* and *Plodia interpunctella*), one species of Hymenoptera (*Pteronidea ribesii*), and three species of Coleoptera (*Agriotes ustulatus, Tenebrio molitor, Tribolium destructor*) were selected for detection of entomopathogenic fungi from the soil.

Soil was sampled using a 20 mm diameter steel core to a depth of 200 mm and was taken from arable field near Siedlce (Poland). The soil was air dried for 2 days at 20°C and sieved in order to remove stones and other debris. Soil (about 250 mL) were placed into plastic boxes and ten bait insects were added to each of the box. Ten boxes for each treatment were prepared and all were incubated at 21°C for 25 days. The insect bait mortality was monitored first at day 6 and then every 3 days afterwards until the end of incubation time. Dead larvae were surface sterilized in 1% sodium hypochloride for 30 second, then rinsed in sterile water and placed in humid conditions to encourage fungal out growth and sporulation. Factors causing mortality were identified where possible. Preliminary identification of fungi was confirmed by slide preparation.

## Results and discussion.

The results are shown in Table 1. Imagines of *T. destructor*, larvae of *P. ribesii* and *A. ustulatus* were not found infected by entomopathogenic fungi in the soil. High level of resistance to fungal infection was shown by adults of *A. obtectus*: only 2% of specimens were infected by *Metarhizium anisopliae*. Fife species of insect pathogenic fungi (*Conidiobolus major*, *Beauveria bassiana*, *M. anisopliae*, *Paecilomyces farinosus* and *P. fumosoroseus*) were found on *T. destructor* larvae. *M. anisopliae* was the most frequent fungus and colonised 48,2% larvae of *T. destructor* (Table 1).

Table 1. Mortality (%) of different bait insects after 25 days of incubation in the soil (sandy soil).

| **Bait insects** | | | **Isolated entomopathogenic fungi** | | | | | **Fungi with unproved entomopatho-genic abilities** | **Nematodes** |
|---|---|---|---|---|---|---|---|---|---|
| Order | | Species | *B.b* | *C.m* | *M.a* | *P.fa* | *P.fu* | | |
| COLEOPTERA | imago | *Acanthoscelides obtectus* | – | – | 2,0 | – | – | 2,0 | 2,0 |
| | | *Tribolium destructor* | – | – | – | – | – | – | – |
| | larvae | *Agriotes ustulatus* | – | – | – | – | – | – | – |
| | | *Tribolium destructor* | 2,7 | 0,5 | 48,4 | 0,5 | 2,1 | 21,6 | 7,0 |
| LEPIDOPTERA | larvae | *Ephestia kühniella* | 6,0 | – | 29,5 | – | 16,4 | 19,0 | 10,4 |
| | | *Galleria mellonella* | 12,4 | – | 13,4 | – | 16,2 | 22,1 | 11,3 |
| | | *Laspeyresia pomonella* | 2,7 | – | 33,0 | – | 2,2 | 29,7 | 11,5 |
| | | *Plodia interpunctella* | 3,2 | – | 64,8 | – | 3,2 | 15,1 | 4,4 |
| HYMENO-PTERA | larvae | *Pteronidea ribesii* | – | – | – | – | – | – | – |

Explanation: *B.b.* : *Beauveria bassiana*, *C.m* : *Conidiobolus major*, *M.a* : *Metarhizium anisopliae*, *P.fa* : *Paecilomyces farinosus*, *P.fu* : *Paecilomyces fumosoroseus*

Table 2. Insect species used as baits for isolation of entomopathogenic fungi from the soil (data from the literature).

| Bait insects | | | Isolated entomopathogenic fungi | | | | | | Other fungal species | References (Country) |
|---|---|---|---|---|---|---|---|---|---|---|
| Order | | Species | *B.ba* | *B.br* | *M.an* | *M.fl* | *P.fa* | *P.fu* | | |
| COLEOPTERA | imago | *Acanthoscelides obtectus* | - | - | + | - | - | - | - | Mietkiewski *et al.* 1991a * |
| | | *Tribolium destructor* | - | - | - | - | - | - | - | Mietkiewski *et al.* 1991a * |
| | larvae | *Agriotes ustulatus* | - | - | - | - | - | - | - | Mietkiewski *et al.* 1991a* |
| | | *Tenebrio molitor* | + | - | + | + | + | + | *Tolypocladium* sp., *Verticillium* sp. | Vanninen *et al.* 1989 (Finland) |
| | | *Tribolium destructor* | + | - | + | + | + | + | *P.i., Verticillium* sp. | Mietkiewski *et al.* 1994* |
| LEPIDOPTERA (larvae) | | *Carposina niponensis* | + | - | + | - | + | + | - | Yaginuma 1990 (Japan) |
| | | | - | - | - | - | - | - | *Paecilomyces cicadae* | Yaginuma 2002 (Japan) |
| | | *Depressaria nervosa* | + | - | + | - | - | + | - | Mietkiewski *et al.* 1991/1992* |
| | | | + | - | - | + | - | - | - | Mietkiewski (unpublished) |
| | | *Ephestia kühniella* | + | - | + | - | - | + | - | Mietkiewski *et al.* 1991a* |
| | | | + | - | + | - | - | + | - | Mietkiewski, Ignatowicz 1995 (Bulgaria) |
| | | | + | - | + | - | - | + | *Conidiobolus major* | Mietkiewski *et al.* 1993* |
| | | | + | + | + | + | + | + | *P. i.* | Mietkiewski *et al.* 1994* |
| | | *Galleria mellonella* | + | - | + | - | + | + | *V. lecanii, C.c., E.v.* | Chandler *et al.* 1996 (UK) |
| | | | + | - | + | - | + | + | - | Bidochka *et al.* 1998 (Canada) |
| | | | + | - | + | - | + | - | *Verticillium* sp. | Brownbridge *et al.* 1993 (USA) |
| | | | + | - | + | - | - | - | - | Hummel *et al.* 2002 (USA) |
| | | | + | + | + | - | - | + | *Conidiobolus* sp. | Keller *et al.* 2003 (Switzerland) |
| | | | + | - | + | - | + | + | - | Kleespies *et al.* 1989 (Germany) |
| | | | + | - | + | - | - | - | - | Klingen *et al.* 2002 (Norway) |
| | | | + | - | + | - | - | - | *Paecilomyces* sp., *Verticillium* sp. | Maranhao, Santiago-Alvares 2003 (Spain) |
| | | | + | - | + | - | - | + | - | Mietkiewski *et al.* 1991a* |
| | | | + | - | + | + | - | + | *Conidiobolus major* | Mietkiewski *et al.* 1997 (UK) |

Table 2 (continued). Insect species used as baits for isolation of entomopathogenic fungi from the soil (data from the literature).

| Bait insects | | Isolated entomopathogenic fungi | | | | | | Other fungal species | References (Country) |
|---|---|---|---|---|---|---|---|---|---|
| Order | Species | *B.ba* | *B.br* | *M.an* | *M.fl* | *P.fa* | *P.fu* | | |
| LEPIDOPTERA (larvae) | *Galleria mellonella* | + | - | + | + | + | + | *Verticillium* sp. | Bajan *et al.* 1995 * |
| | | + | - | + | + | + | + | - | Tkaczuk, Miętkiewski 1996* |
| | | + | - | + | - | + | + | - | Tkaczuk, Mietkiewski 1998* |
| | | + | - | + | - | - | + | - | Tkaczuk, Renella 2003 (Italy) |
| | | + | + | + | + | + | + | - | Steenberg 1995 (Denmark) |
| | | + | - | + | - | - | - | *Peacilomyces lilacinus,* | Tarasco *et al.* 1997 (Italy) |
| | | + | - | + | - | + | + | - | Vanninen 1996 (Finland) |
| | | + | - | + | - | + | + | Entomo-phthorales | Zimmermann 1986 (Germany) |
| | *Laspeyresia pomonella* | + | - | + | - | + | + | - | Mietkiewski *et al.* 1991/1992* |
| | | + | - | + | - | - | + | - | Mietkiewski *et al.* 1992* |
| | *Plodia interpunctella* | + | - | + | - | - | + | - | Mietkiewski *et al.* 1991b* |
| | | + | - | + | - | + | + | - | Mietkiewski *et al.* 1991/1992* |
| HYMENO-PTERA (larvae) | *Hoplocampa minuta* | + | - | + | - | - | + | - | Mietkiewski *et al.* 1995* |
| | *Pachynematus pumilio* | + | - | - | - | - | + | - | Tkaczuk, Mietkiewski 1995* |
| | *Pteronidea ribesi* | - | - | - | - | - | - | - | Mietkiewski *et al.* 1991a * |
| DIPTERA (larvae) | *Calliphora vemitoria* | - | - | - | - | - | + | - | Tkaczuk (unpublished) |
| | *Dasyneura ribis* | + | - | + | - | - | + | - | Tkaczuk, Mietkiewski 1995* |
| | *Delia floralis* | - | - | + | - | - | - | *Tolypocladium cylindrosporum* | Klingen *et al.* 2002 (Norway) |

Explanation: *B.ba*: *Beauveria bassiana*, *B.br*: *Beauveria brongnartii*, *M.an*: *Metarhizium anisopliae*, *M.fl*: *Metarhizium flavoviride*, *P.fa*: *Paecilomyces farinosus*, *P.fu*: *Paecilomyces fumosoroseus*, *P.fi*: *Paecilomyces inflatus*, *C.c*: *Conidiobolus coronatus*, *E.v.*: *Erynia virescens*; * :Poland

Only three fungal species were found on lepidopteran larvae. The fungus *B. bassiana* appeared most frequently on *G. mellonella* larvae and *P. fumosoroseus* on *E. kühniella* and *G. mellonella* but *M. anisopliae* on *P. interpunctella* and *L. pomonella* (Table 1).

When considering data of other authors on isolation of entomogenous fungi from soils using bait insects, one can notice that the methods used were generally slightly different, mainly as regards the insect species and its stage, the number of insect larvae placed to the soil and quantity of soil samples. Comparative results provided from the literature were

compiled in Table 2, where the occurrence of fungal species is given according to the insect species and stage.

According to Klingen *et al.* (2002), it may be necessary to use a range of bait insect species or bait methods with complementary susceptibilities to pathogen in order to sample a more representative spectrum of insect pathogenic fungi from soil. The same authors stated for instance, that *Tolypocladium cylindrosporum* was isolated more frequently from the soil in Norway when using *Delia floralis* larvae as the bait insect than when using *G. mellonella.* The spectrum of fungal insect pathogenic species isolated from the soil, by means of bait insects, may be also influenced by incubation temperature (Tkaczuk *et al.*, 2000) and depth of soil sample (Mietkiewski *et al.*, 1995).

The data presented in Table 2 show that larval stages of insects are the most useful baits for the detection of entomopathogenic fungi from the soil. Imagines of insects appeared to be of interest only in the case of Coleoptera, but their usefulness is rather low.

Interestingly, larvae of insects species, which do not have natural contact with soil in their development cycle, are more susceptible to infection caused by fungi occurring in the soil. The most useful as baits for isolation of fungi were: *G. mellonella, E. kühniella, P. interpunctella, L. pomonella* and *T. destructor*. Biodiversity studies of entomopathogenic fungi in soils were usually conducted by use of *Galleria mellonella* or *Tenebrio molitor* as bait insects. The most frequently isolated species belong to the genera *Beauveria, Metarhizium* and *Paecilomyces* (Hyphomycetales). Fungi from the order Entomophthorales were isolated more rarely.

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# Factors affecting viability of entomophthoralean fungi

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**Abstract**: The morphological, cytological and biochemical properties of local strains of entomophthoralean species, i.e. *Conidiobolus obscurus, C. thromboides,* and *Basidiobolus ranarum* were determined. The aim of this research was to estimate factors that influenced viability of the entomopathogenic fungi. Cytological studies show that dehydration causes structural changes in resting spores and spore walls. We observed that accumulation of proteins, carbohydrates and lipids depend on nutrient conditions. *C. obscurus, C. thromboides,* and *B. ranarum* growth rate and sporulation intensity were low, if they were grown on less nutrient media.

**Key words**: *Conidiobolus obscurus, C. thromboides, Basidiobolus ranarum,* viability, resting spores

## Introduction

Entomophthoralean fungi play a significant role in the regulation of pest insect populations. Persistence of fungi in the environment is important for driving infection in pest population. The persistence of pathogens in the environment is guaranteed mainly by resting spores in anabiosis.

The morphological, cytological and biochemical properties of entomophthoralean species were studied. The aim of research was to estimate factors that influenced viability of the entomopathogenic fungi *Conidiobolus obscurus, C. thromboides,* and *Basidiobolus ranarum.* Attention was paid to resting spores, which insure persistence of fungi in the environment. The paper presents the results of a study on influence of cultivation processes and drying of spores on viability of fungi.

## Materials and methods

***Fungal cultures***
Local strains of *Conidiobolus coronatus, C. obscurus, C. thromboides,* and *Basidiobolus ranarum* were used in these investigations. Cultures were maintained on malt extract agar.

***Insect cultures***
Aphids (*Aphis fabae, Macrosiphum rosae* and *Anuraphis subterraneae*) and flies (*Musca domestica* and *Delia brassicae*) collected in the environment and reared in laboratory were used in investigations. Aphids used in investigations were infected by *C. obscurus* (titre of working solution $3.6 \times 10^5$ conidia/mL, and $5.5 \times 10^5$ resting spores/mL), *C. thromboides,* ($3.7 \times 10^5$ conidia/mL, and $5.7 \times 10^5$ resting spores/mL) and *B. ranarum* ($5.5 \times 10^5$ conidia/mL).

***Histological, citochemical and biochemical investigations***
For histological examination, the insects were fixed in Bouin; Schaffer, Zenker, Carnoy, Chapy solutions and 10% buffered formalin. Serial sections of preparations were stained with safranine, azure-eosin after Romanovsky, picro-indigo carmine after Becker and Smith. The carbohydrates were revealed by the periodic acid-Schiff test (PA/S) and metachromatic

substances with toluidine blue. The histochemical identification of lipids was performed with Sudan III and IV, Sudan Blac B (Sumner & Sumner, 1969; Lilli, 1969).

For biochemical research, the cultures were grown on Czapek's agar, glucose - mineral salt agar (20.0 g glucose, 0.5 g $MgSO_4.7H_2O$, 1.0 g $KH_2PO_4$, 20.0 g agar); glucose - peptone - mineral salt agar (20.0 g glucose, 2.0 g peptone, 0.5 g $KH_2PO_4$, 2.0 g $MgSO_4.7H_2O$, 20.0 g agar) and malt extract - yeast extract - glucose agar (40.0 g malt extract, 4.0 g yeast extract, 20.0 g glucose, 1.0 g $K_2HPO_4$, 20.0 g agar). The biochemical composition of cultures was determined in the Institute of Biochemistry and Physiology of Microorganisms following methods described by Dyatlovitskaja *et al.* (1969) and Sulganovic *et al.* (1969).

***Microscopy***

Cytological and histological preparations were investigated using a Laboval light microscope.

For scanning electron microscopy, the spores were fixed in a 3% solution of glutaraldehyde, then fixed in 1 % osmium tetroxide, dehydrated in a graded series of 30-100% ethanol, druid, and coated with gold. Specimens were examined with a JEM-100 C electron microscope and ASID-4D scanning equipment.

For transmission electron microscopy, mechanically disrupted spores were fixed in a 3% solution of glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in a graded series of 30-100% ethanol, cleared in propylene oxide, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined under JEM-100C electron microscope.

***Determination of colony growth rate and sporulation***

Fungal cultures were grown at 22 – 24 °C on Czapek's agar, Sabouraud's agar glucose - mineral salt agar, malt extract - yeast extract - glucose agar and glucose- peptone - mineral salt agar. As indicator of growth intensity the diameter of culture colonies on the 5$^{th}$ day after inoculation was used. Growth rate was estimated by measuring the culture diameter (both directions) in Petri dishes. Average mean from 15 Petri dishes were calculated. The intensity of sporulation was estimated by counting the number of spores in a counting chamber.

***Drying of spores***

Entomophthoral spores were dried at 29°C, 30°C and 42°C for 4-6 hrs with additional ventilation. Some were lyophilised.

## Results and discussion

Results obtained during this study allow concluding that nutrient media and growing conditions significantly affected viability of entomophthoral fungi. Observation of cytological preparation obtained from infected insects, shows that development of mycelia, conidia and resting spores, synthesis of proteins, lipids and carbohydrates depends on physiological condition of host insect. Fat cells in infected insect body have an important role in development of entomophthoral fungi. Fat cells provide favourable nutrient conditions. Examination of host insect sections shows that development of mycelia and spores and accumulation of proteins, carbohydrates and lipids have high intensity if insect body is abundant with fat cells. We found that the *C. coronatus* have metachromatic substances not only in mycelia and spores. These substances accumulated in hair-like appendages of the spores.

***Development of fungi on different nutrient media***

Growth rate, sporulation and pathogenicity were used as characteristics for culture viability. We found that colonies of the cultures *C. obscurus, C. thromboides, B. ranarum* grown on rich media, cover all Petri dishes during 3 - 5 days. Growing on Czapek agar size of *C.*

*obscurus* colonies was only 3.2 ± 0.2 x 3.5 ± 0.5 cm, *C. thromboides* - 3.3 ± 1.1 x 4.6 ± 1.0 cm, *B. ranarum* – 4.7 ± 1.1 x 5.6 ± 1.1 cm.

Development of conidia and resting spores regardless from nutrient media starts at 3 - 4 days after inoculation. Diameter of *C. obscurus* resting spores grown on Czapek media was 41.7 ± 1.5 μm, grown on Malt extract agar – 44.3 ± 0.8 μm. Germination of conidia differs significantly, if the cultures were grown on Czapek agar (51.8%) and Malt extract agar (87.1%). Mortality of *A. fabae* infected with cultures grown on Czapek agar and Malt extracts were 55.8% and 73.9%, respectively.

We found that growth rate and sporulation intensity of the cultures *C. obscurus, C. thromboides, B. ranarum* are low, if they were grown on less nutrient media, for example Czapek agar. On less nutrient media accumulation of amino acids, lipids and carbohydrates and lipolytic and proteolytic activity of mentioned cultures decrease. As follows decrease the ability of cultures to infect host insect. Sudan black colours neutral lipids in mycelia and spores intensively, if the cultures were grown on rich media. Fungus *C. obscurus* grown on glucose-peptone-agar produce arachin acid (0.3-13.2% from total content of fat acid), addition of mineral salts inhibit production of this fat acid.

***Anabiosis of resting spores***

Development of cultures ends with formation of resting spores, which insure persistence of fungi in the environment. Important role on viability of entomophthoral fungi has an anabiosis, when resting spores are characterised with very low metabolic processes or quiescence. The resting spores of *C. obscurus* and *C. thromboides* were used for electron microscopy. Viability of resting spores depends on processes guarantied anabiosis; processes remaining anabiosis; factors interrupting anabiosis and renewing metabolic processes. Considerable changes occur in spores during transference into anabiosis.

In first step of anabiosis resting spores have significant structural and biochemical changes, and maturation of spores happens (Cudare, 1990, 2001). Beginning of anabiosis depends on nutrient and growing conditions. At the initial stage of anabiosis there are a nucleus, lysosomes and mitochondria in the central part of resting spores (Cudare, 1993). During anabiosis we observed changes in spore and spore wall structure: for example, accumulation of lipids, moving of organelles to the periphery of central part. Lipids occupy the central part of matured resting spores, and nucleus was not visible in thin sections of spores. Cytological preparations showed that resting spores developed in insect's body usually have a positive PS/A reaction. Very intensive PS/A reactions were observed in spore wall.

Dehydration is one of the possibilities to cause anabiosis of resting spores. Viability of resting spores during dehydration depends on species, genotype and phenotype of isolates, nutrient conditions and methods of dehydration. Dehydration caused visible morphological, structural and biochemical changes. Morphological, structural and biochemical properties of dehydrated resting spores of the *C. obscurus* and *C. thromboides* depend on stage of maturation. Diameter of dehydrated resting spores decreases by 2to5 μm. Dehydration appears to be a reason for a change of spore form as well. The surface structure of resting spores appears wrinkled (Cudare, 1990, 1993, 1998, 2001). During drying, condensation of chromatin occurs in the nucleus, which loses its spherical form and becomes angular. When spores are rapidly dehydrated the plasmatic membrane ruptures. Cellular substance accumulates on the spore wall. Cytochemical investigations and electron microscopy shows that lipids of various consistency occupy the central part of matured resting spores. Viability of resting spores decreases by lyophilisation. Spores were dramatically deformed, and their walls were ruptured.

Very important during anabiosis process are environmental factors, like as humidity. Presence of water restores the anabiosis.

Humidity and optimal temperature conditions are important for interrupting anabiosis and renewing metabolic processes in resting spores. Cytological preparations allow concluding that contents of lipids and mucopolysacharides in membranes are important for bounding of water. Neutral lipids are sources of energy, enhansing germination of spores, viability of cultures and thereby persistence in the environment (Cudare, 1998). Comparison of different fungal cultures shows that *B. ranarum* loses viability in dehydration processes faster than others.

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# Influence of the temperature of reisolation on the virulence of the entomopathogenic fungus *Verticillium lecanii*

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**Abstract**: Four strains of *Verticillium lecanii* of different geographical provenance were passaged through *Frankliniella occidentalis* three times at the temperature of reisolation of 28°C. Then these reisolates were tested at optimal and suboptimal temperatures on the same host. In the case of reisolates, mortality increased in the first days after inoculation compared to the original strains at the same temperatures. No effect of reisolation temperature was noticed but differences appeared between strains.

**Key words**: *Verticillium lecanii*, entomopathogenic fungus, reisolates, virulence, temperature

## Introduction

It is assumed, that the virulence of strains of entomopathogenic fungi depends highly on the ecological conditions. Four strains of *Verticillium lecanii* (Zimmermann) Viégas of different geographical provenance were passaged through larvae of the western flower thrips *Frankliniella occidentalis* three times at 28°C, which was considered as the highest possible temperature of reisolation. Then, the stability of virulence of these reisolates has been tested at optimal and suboptimal temperatures on the same host.

## Materials and methods

Four strains kept in mycological media for a more or less long period were studied (Table 1). They were compared to their reisolates following 3 host passages at 28°C and 80 % humidity on the larvae of *F. occidentalis* without an agar passage. The virulence of strains has been recorded as a degree of the pathogenicity (mortality, in %) owing to a bioassay where larvae I of *Frankliniella occidentalis* were submitted to a direct application of 5,5 mL of a blastospore suspension (concentration: $1.0x10^6$ spores / mL) at the temperatures of 20°C, 10°C and 30°C and 95% humidity.

## Results

The reisolates of all strains have achieved a higher virulence than the original strains in the majority of the experiments.

The temperature of 28°C proved as the highest possible for reisolations without intermediate culture on agar. Such an high temperature during the reisolation promoted saprophytic organisms and impeded an active development of mycelium of *V. lecanii* on the cadavers of *F. occidentalis*. For that reason only three reisolation levels could be realized.

In bioassays with the "original" strains (without reisolation) at 20°C, the strain V24 was the most virulent (Fig. 1). The strain Vs was highly virulent as well. The strains Vb3/50 and Vn exhibited only a middle virulence. They didn't differ significantly from each other.

Table 1. Strains of *Verticillium lecanii* used in the study

| Strains | Year | Original host | Geographical location | Further development |
|---|---|---|---|---|
| Vb3/50 | 1978 | *Trialeurodes vaporariorum* | Bulgaria | 50 x passaged through *T. vaporariorum*. After 10 passages 1 x agar medium; since 1990 maintained on malt extract agar |
| Vs | Approx 1985 | *Trialeurodes vaporariorum* | Siberia | 10 years only on Czapek-Dox complete medium |
| Vn | 1997 | *Tetranychus urticae* | Siberia | At first passed through original insect host repeatedly. Then cultivated on Sabouraud's dextrose agar and malt extract agar |
| V24 | 1989 | *Myzus persicae* | Germany | Since isolation no host passage of the strain. Maintained on malt extract agar. |

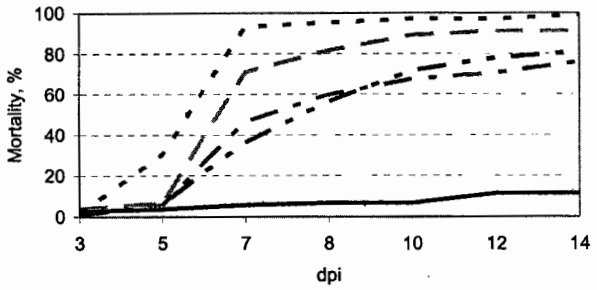


Figure 1. Mortality of larvae I of *F. occidentalis* after application of a blastospore suspension (1.0 x $10^6$ Sp./mL) of original strains of *V. lecanii* at 20°C.

In comparison to the original strains a reisolation at 28°C led to a more increased virulence for all the tested strains. At the test-temperature of 20°C, all thrips larvae died rapidly (Fig. 2). A mortality of approximately 70% was obtained on the 5$^{th}$ day already. The virulences of the reisolates of all strains differ hardly.

Bioassays with the original strains at 10°C showed a differentiated reaction of the strains at this low temperature (Fig. 3). In these conditions the strain V24 proved its high pathogenity to the thrips larvae. The strain Vs had an unexpected high virulence. The strains Vn und Vb3/50 reached only a middle virulence level.

After three reisolations of the original strains at 28°C, all strains caused a faster increase of the mortality under the test temperature of 10°C (Fig. 4). The reisolates seemed to be more balanced than the original strains at the low temperatures. An especially strong increasing of the mortality of strain V24 has been noticed. The reisolate V24($R3_{28°C}$) showed a mortality higher than 50% already seven days after the application. The virulence of the other reisolates didn't differ considerably among each at that time. All reisolates achieved a comparably high mortality after approximately 15 days.

The mortality of insects differed significantly in the trials with the reisolates of the strains V24 and Vs, while no significant difference was observed between the strains Vn and Vb3/50.

At 30°C the original strains showed a very slow, slight increase of mortality and didn't differ among themselves and the control.

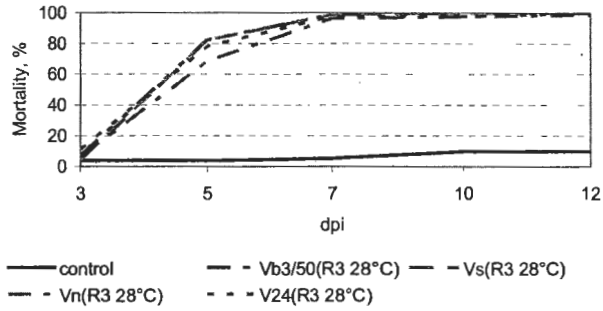


Figure 2. Mortality of larvae I of *F. occidentalis* after application of a blastospore suspension (1.0 x $10^6$ Sp./mL) of reisolates ($R3_{28°C}$) of *V. lecanii* strains at 20°C.

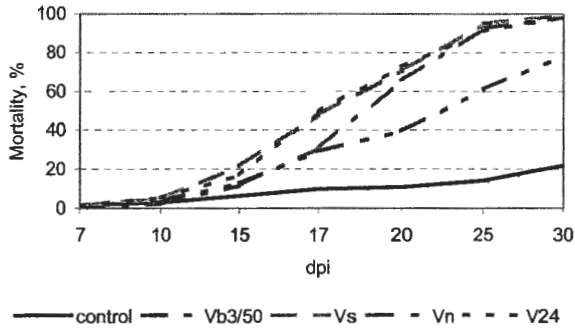


Figure 3. Mortality of larvae I of *F. occidentalis* after application of a blastospore suspension (1.0 x 106 Sp./mL) of original strains of *V. lecanii* at 10°C.

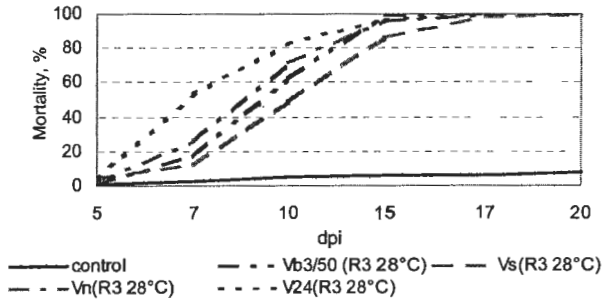


Figure 4. Mortality of larvae I of *F. occidentalis* after application of a blastospore suspension (1.0 x $10^6$ Sp./mL) of reisolates ($R3_{28°C}$) of *V. lecanii* strains at 10°C.

The reisolates ($R3_{28°C}$) did not react with an acceleration of the mortality at the high test temperature of 30°C (Fig. 5). A differentiated reaction of the reisolates to the high

temperature was observed only 7 days after application. The reisolate of the strain V24 demonstrated the highest mortality again. The strains Vb3/50 und Vs caused a middle mortality in the insects. The strain Vn did hardly differ to the control.

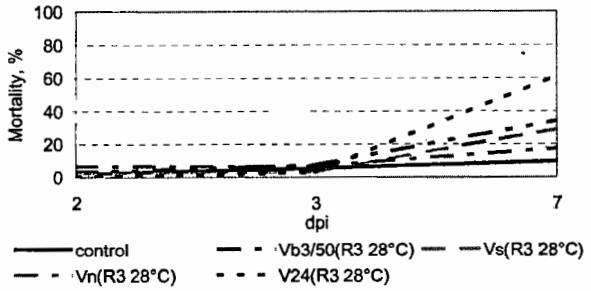


Figure 5. Mortality of larvae I of *F. occidentalis* after application of a blastospore suspension (1.0 x $10^6$ Sp./mL) of reisolates ($R3_{28°C}$) of *V. lecanii* strains at 30°C.

## Discussion

In the literature it is assumed, that the host passages lead to an increase of the virulence of the entomopathogenic fungi. Ogarkov & Ogarkova (1999) have succeeded in achieving a doubling of the virulence in strains of *Beauveria bassiana* after the fourth and eighth passage. Contrary data are given very rarely. Hall (1980) had observed that strains of *V. lecanii* didn't show any increase of virulence after a passage. Pavlyushine (1998) explained the positive effects of the reisolation with the selection of the high pathogenic clones and the elimination of the low pathogenic clones.

The passage of *V. lecanii* through the larvae of *F. occidentalis* caused an increase of the virulence of the four tested strains. The high reisolation temperature of 28°C has led to the selection of generally high pathogenic clones. But it couldn't be proved, that the reisolation temperature had a purposeful influence. The reisolates of all strains reacted with a visible increase of the virulence under optimal as well as under sub optimal temperatures against *F. occidentalis*. As regards the effect of the reisolation on the virulence, a strain specific character could be considered. The strain V24 has a constantly high, stable virulence in a wide temperature range, which can be increased through host passages only at the beginning of an infection. Such strains with a natural balance are especially valuable for the use under field conditions.

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# Records of aphidophagous Entomophthorales in Slovakia

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**Abstract**: In Slovakia colonies of various aphid species were investigated in order to determine a spectrum of entomophthoralean fungi parasiting aphids. 70 different localities were visited throughout the country during 1999-2002. The survey was focused on aphid fauna of agricultural crops and non-production sites. Altogether 14 different entomophthoralean species out of three families were identified from aphids; *Erynia neoaphidis, Erynia nouryi, Erynia erinacea, Entomophthora planchoniana, Zoophthora aphidis, Zoophthora radicans, Zoophthora phalloides, Zoophthora occidentalis* (Entomophthoraceae); *Neozygites fresenii, Neozygites microlophii, Neozygites cinarae, Neozygites turbinata* (Neozygitaceae); *Conidiobolus obscurus, Conidiobolus thromboides* (Ancylistaceae). The fungal species were recorded from 69 aphid species belonging to three families (Aphididae, Drepanosiphidae and Anoecidae). Out of the 14 fungal species 10 are the first record from Slovakia. *E. neoaphidis* was a dominant fungal species and caused epizootics in aphid colonies. *E. planchoniana* was the second most frequent pathogen but no epizootics were recorded. Conversely, *N. fresenii* had a strong tendency to establish epizootics in dense aphid colonies, especially in those of *Aphis fabae*. Species *E. nouryi, E. erinacea, Z. phalloides, Z. occidentalis, C. thromboides* were identified from single aphid species despite they are known to have a broader host spectrum. *N. microlophii* and *N. cinarae* are probably monophagous species.

**Key words**: Aphids, Entomophthorales, Slovakia

## Introduction

Entomophthoralean fungi have been found to be important mortality factors for aphids in fields (e.g. Latgé & Papierok, 1988). Three tens of the fungi are known as aphid pathogens at present (Keller, 1987, 1991, 1997; Humber, 1990, Balazy, 1993). Intensive studies have been done to contribute to understanding of the disease distribution, epidemiology, and management in agricultural crops (Pell *et al.*, 2001). The pathogens possess a great potential as biological control agents due to their ability to develop strong epizootics that result in natural regulation of aphid populations (Latgé & Papierok, 1988). Unfortunately, very limited information in respect to the group of fungi is available in Slovakia. Up to know only 4 entomophthoralean fungi have been reported from aphids in fields; *Erynia neoaphidis* Remaudière et Hennebert, *Entomophthora planchoniana* Cornu, *Neozygites fresenii* (Nowakowski) Remaudière et Keller, and *Conidiobolus obscurus* (Hall et Dunn) Remaudière et Keller. They were observed in the pea aphid, the black bean aphid or cereal aphid populations (Weismann, 1961; Stary, 1974; Stalmachová & Cagán, 2000; Cagán & Barta, 2001).

The main goal of the study was to determine the species spectrum of entomophthoralean fungi in aphid populations under conditions of Slovakia.

## Material and methods

From 1999 through 2002 numerous localities were visited in Slovakia and colonies of various aphid species were observed in order to determine their entomophthoralean enemies. Each year the observations started at the beginning of March and usually finished at the end of November. 70 different localities were irregularly visited and different types of sites were surveyed. The visited sites varied for sampling occasions and the survey was mainly focused on aphids infesting agricultural and horticulture crops, but observations were extended to include also non-production sites. All cadavers of aphids (fungus-killed aphids) were collected on a random basis. If plants were found infested with aphids, the aphid colonies were simply searched and cadavers were collected. Only cadavers with external symptoms of entomophthoralean disease were collected. The fungus-killed aphids were used for pathogen identification as soon as possible. Some part of the samples were deposited in a herbarium prepared as air-dried cadavers and stored at approximately +4°C in dark conditions. During the survey samples of living aphids were also collected from aphid colonies where a disease was detected. These samples were used for aphid identification.

## Results and discussion

69 aphid species were found killed by one or more fungi of the order Entomophthorales in their natural habitats during the survey in Slovakia. Majority of the aphid species belonged to the family Aphididae (59 species) and the remaining ten were from the family Drepanosiphidae (9 species) or the family Anoecidae (one species). Overall, 14 different fungal species out of three families were identified from the aphids in Slovakia; *Erynia neoaphidis, Erynia nouryi* Remaudière et Hennebert, *Erynia erinacea* (Ben-Ze'ev & Kenneth) Remaudière et Hennebert, *Entomophthora planchoniana, Zoophthora aphidis* (Hoffmann in Fres.) Batko, *Zoophthora radicans* (Brefeld) Batko, *Zoophthora phalloides* Batko, *Zoophthora occidentalis* (Thaxter) Batko (Entomophthoraceae); *Neozygites fresenii, Neozygites microlophii* Keller, *Neozygites cinarae* Keller, *Neozygites turbinata* (Kenneth) Remaudière et Keller (Neozygitaceae); *Conidiobolus obscurus, Conidiobolus thromboides* Drechsler (Ancylistaceae).

*E. neoaphidis* was the predominant species. The fungus was identified from 47 aphid species. It could be encountered from the beginning of April to the half of November and in some sites an epizootic level of disease was observed resulting in a collapse of host population. The fungus seasonal distribution fully corresponds with observations of other authors made in central Europe (e.g. Keller, 1991; Balazy, 1993). There is no doubt that *E. neoaphidis* is truly the most important and most frequent pathogen observed in aphid populations in Slovakia. It has a worldwide distribution and has being recorded from nearly all continents (Keller, 1991; Balazy, 1993). The fungus is characterized by a potential for epizootic development and a great effectiveness to control of various aphid populations (e.g. Dean & Wilding, 1971).

*E. planchoniana* was the second most frequent pathogen. It was identified from 27 aphid species. The pathogen attacked several pest aphids but it was a typical pathogen of *Aphis sambuci* L. The seasonal dynamics of the disease was the same as that of *E. neoaphidis* but no epizootics were recorded. *E. planchoniana* is known worldwide (Keller, 1987; Balazy, 1993) and is frequently reported as a causal agent for epizootics (e.g. Milner *et al.*, 1980; Feng & Nowierski, 1991). It is of interest that despite its rather great prevalence, no epizootics were recorded in host populations in Slovakia. The majority of aphids were infected at dryer sites. Keller (1987) also noticed that the fungus preferred relatively dry habitats and did not occur in dense humid crops.

*N. fresenii* was identified from 24 aphid species in 21 localities. A seasonal dynamics of the disease was similar to those of *E. neoaphidis* and *E. planchoniana*, but the infection usually appeared one month later. *N. fresenii* had a strong tendency to establish epizootics in dense aphid colonies, especially in those of *Aphis fabae* Scop., *Aphis nasturtii* Kaltenbach and *Microlophium carnosum* (Buckton). The species is known from nearly all continents including the South Pacific region (Keller, 1997). It is considered to be better adapted to tropical conditions (Steinkraus *et al.*, 1991; Keller, 1997) although it is effective in the subpolar region as well (Nielsen *et al.*, 2001). If a coexistence of *N. fresenii* with more fungi was present in the colonies, *N. fresenii* usually gained dominance over the other fungus species in a short time. A great effectiveness of the species was presented by many authors (e.g. Steinkraus *et al.*, 1991; Nielsen *et al.*, 2001). The species was identified from less number of host species when compared with *E. planchoniana*, although it was recorded in more localities. This may imply a narrower host spectrum of *N. fresenii*, however, this may probably relate with different types of niches occupied by both species. While *N. fresenii* was frequent in humid habitats at ground dense vegetation, *E. planchoniana* infected aphids living in relatively dryer microclimates at upper levels of vegetation. Resting spores were generally observed during dry and hot periods in the summer.

*C. obscurus* is next important aphid pathogenic fungus. In Slovakia it infected 17 aphid species and the infection was normally recorded during June and October. The fungus was present within aphid colonies at low levels as an occasional species alongside other fungi and the disease development never reached epizootic character. This is in agreement with observations of Keller & Suter (1980). At the end of vegetation period resting spores were usually produced. From a viewpoint of natural aphid control in Slovakia *C. obscurus* is considered not as important control agent as the previous ones.

*C. obscurus* closes the foursome of the most important aphid pathogenic Entomophthorales characterised by a worldwide distribution, an obligate specificity to aphids, a pathogenity to rather great variety of aphid species, a high prevalence in aphid colonies, and finally by the ability to cause epizootics in host populations.

Remaining species are usually considered as minor pathogen of aphids for various reasons, e.g. for a broader host spectrum (*Z. radicans, C. thromboides*) or, on the other hand, an obligate specificity to simple aphid species (*N. microlophii, N. cinarae*), an absence of epizootics or for a rare presence in aphid populations (*E. nouryi, E. erinacea, Z. aphidis, Z. phalloides, Z. occidentalis, N. turbinata*). All these species represent the first records from Slovakia. They were mostly identified from one aphid species with a very low prevalence, however two *Neozygites* species, *N. microlophii* and *N. turbinata* caused severe epizootics in populations of *Microlophium carnosum* and *Tuberolachnus salignus* (Gmelin), respectively. *Z. radicans* was observed on three aphid species but epizootic was recorded only in the cabbage aphid population. Epizootics of *C. thromboides* were not observed in Slovakia, but experimental applications of resting spores usually resulted with high infections in tested aphid populations (Cudare, 1990).

We found a great species diversity of entomophthoralean fungi attacking aphids in Slovakia. Out of the 30 species known from aphids, 23 have been recorded from Europe (Keller, 1987, 1991; Balazy 1993). The 14 species observed during the survey in Slovakia surpass the number of pathogens reported from the aphid fauna by other authors in different countries or regions all over the world. Regional list of aphid pathogenic fungi were published for instance in France (Thoizon, 1970), Australia (Milner *et al.*, 1980), Finland (Papierok, 1989), Poland (Balazy et al., 1990), or Iceland (Nielsen *et al.*, 2001). We found a set of four fungus species to be the most important pathogens in the country. They were *E. neoaphidis, E. planchoniana, N. fresenii,* and *C. obscurus*. These species were the most frequent in aphid colonies and they were identified

from many aphid species. Plenty of findings were from agricultural crops including important pestiferous aphids. This was because fields were specifically targeted in our survey.

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# Why does *Beauveria bassiana* predominate over *Metarhizium anisopliae* in soils from southern Spain?

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**Abstract**: Previous surveys made in Andalucía (Spain) showed the prevalence of *Beauveria bassiana* in soils compared to *Metarhizium anisopliae*. Bioassays were therefore carried out in laboratory, using local strains, larvae of *Galleria mellonella*, and sand as substratum at 20 and 25 °C. The mortality due to *B. bassiana* appeared not depend on the temperature unlike *M. anisopliae*. Furthermore, the mean survival time of the larvae infected by the former fungus was lower compared to the latter. These results are discussed in relation to the data collected in the field.

**Key words**: Anamorphic fungi; entomopathogen fungi, *Galleria* bait trap

## Introduction

The entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*, which are currently being used or considered as commercial biocontrol agents, are soil inhabitants (Keller & Zimmermann, 1989) that infect a great variety of insect species from different orders (Mugnai *et al.* 1989; Zimmermann, 1993).

In previous surveys for entomopathogenic fungi diversity in soils all over Andalucía (southern Spain) using the *Galleria mellonella* bait method (Zimmermann, 1986), 138 soil samples (on a total of 181) were found positive for *B. bassiana* and/or *M. anisopliae* (Maranhão, 2003; Maranhão & Santiago-Alvarez, 2003). More precisely, *B. bassiana* was the most commonly detected fungus; it was isolated from 131 soil samples (72,38 %), By contrast, *M. anisopliae* was only isolated from 55 soil samples (45,86 %), whereas both fungi were coincidental in 48 soil samples (26,52%) Moreover, in samples yielding the two fungi fungi, the percentage of *G. mellonella* larvae infected by *B. bassiana* was 2- fold higher than that of *M. anisopliae* (Maranhão, 2003).

The aim of this contribution is to study a possible competition between *B. bassiana* and *M. anisopliae* to infect the wax moth larvae in the soil.

## Material and methods

The two isolates used in this assay, (*B. bassiana*: isolate EABb 01/07-SU, and *M. anisopliae*: EAMa 01/99-SU), were originally obtained from a meadow and a cotton field soils respectively (Maranhão, 2003).

The isolates were grown on Malt extract agar at 25 °C in darkness. After 11 days conidia were harvested, suspended on sterile water plus 0,05 % Tween 80, and the concentration was estimated using the Malassez camera. Two bioassays were conducted using sterile sand as substratum. In the first experiment, one plastic cups containing 100 g of sand contaminated

with $1x10^3$ or $1x10^5$ conidia/g of sand were baited with 10 last instar larvae of *Galleria* and then incubated at 20 and 25 °C. In the second experiment, the sand received the same amount of conidia per g but from a mixture of both isolates at three different rates: 3:7; 1:1 and 7:3 Bb:Ma respectively baited with 10 *Galleria* larvae and then incubated at 25 °C. For each conidia combination 5 cups were used. As a control 5 cups with sand added with sterile water plus 0,05 % tween 80 were used.

Mortality was recorded daily and survival was analysed using Kaplain Meier survival analysis in SPSS for Windows. Cadavers were immediately removed, surface sterilized in an 1 % sodium hypochlorite solution for 3 min, washed in sterile water for 3 min and incubated at 25 °C under conditions of high humidity to favour fungal outgrowth.

## Results

The mortality of *Galleria* larvae due to mycosis by the *B. bassiana* isolate apparead to not depend on the temperature. On the contrary, mortality due to *M. anisopliae* was found to depend on temperature at the two dose levels tested as well as on inoculum level at both temperatures (Table 1).

Table 1. Mortality of *Galleria* larvae in sand inoculated with *Beauveria bassiana* or *Metarhizium anisopliae* isolates at different temperatures (N = number of larvae).

| Treatment | | | 20°C | | | | | 25°C | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose (conid./ plate) | Isolate | N | Mortality | | | | N | Mortality | | | |
| | | | Mycosis | | Other causes | | | Mycosis | | Other causes | |
| | | | n | % | n | % | | n | % | n | % |
| 0 | Control | 50 | 0 | 0 | 5 | 10 | 50 | 0 | 0 | 5 | 10 |
| $10^5$ | EaBb 01/07-Su | 50 | 48 | 96 | 1 | 2.0 | 50 | 49 | 98 | 0 | 0 |
| | EAMa 01/99-Su | 50 | 0 | 0 | 8 | 16.0 | 50 | 32 | 64 | 3 | 6.0 |
| $10^7$ | EaBb 01/07-Su | 50 | 50 | 100 | 0 | 0 | 50 | 50 | 100 | 0 | 0 |
| | EAMa 01/99-Su | 50 | 7 | 15.2 | 14 | 31.4 | 50 | 41 | 82 | 6 | 12 |

Furthermore, the Mean Survival Times (MST) of the larvae infected by *B. bassiana* at the two dose levels and at both temperature were significantly lower than that of the larvae infected by *M. anisopliae* (Table 2).

The bioassay with co-inoculated sand was conducted at 25 °C, the mortality of the *Galleria* larvae was caused by *B. bassiana*, and only a few larvae were killed by *M. anisopliae* (Table 3).

Table 2. Mean Survival Times (MST) of the larvae infected by entomopathogenic fungi.

| Treatment | | 20°C | 25°C |
|---|---|---|---|
| Dose (conid./plate) | Isolate | MST±SE days | MST±SE days |
| 0 | Control | 23.94±0.50a | 14.40±0,28a |
| $10^5$ | EaBb 01/07-Su | 11.26±0.36b | 8.36±0.22b |
| | EAMa 01/99-Su | 23.70±0.51a | 12.68±0.29c |
| 0 | Control | 23.94±0.50A | 14.40±0,28A |
| $10^7$ | EaBb 01/07-Su | 7.60±0.07B | 6.16±0.06B |
| | EAMa 01/99-Su | 20.91±0.78C | 10.98±0.31C |

Table 3. Mortality of *Galleria* larvae in sand co-inoculated with both fungal isolates at 25 °C (N = number of larvae).

| Total dose (conid./ plate) | Treatment | | N | Mortality | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *B. bassiana* | | *M. anisopliae* | | Other causes | |
| | EaBb 01/07-EAMa 01/99 (conid./plate) | | | n | % | n | % | n | % |
| 0 | 0 | 0 | 50 | 0 | 0 | 0 | 0 | 5 | 10 |
| $10^5$ | $3x10^4$ | $7x10^4$ | 50 | 46 | 92 | 2 | 4 | 1 | 2 |
| | $5x10^4$ | $5x10^4$ | 50 | 47 | 94 | 0 | 0 | 2 | 4 |
| | $7x10^4$ | $3x10^4$ | 50 | 47 | 94 | 0 | 0 | 3 | 6 |
| $10^7$ | $3x10^6$ | $7x10^6$ | 50 | 50 | 100 | 0 | 0 | 0 | 0 |
| | $5x10^6$ | $5x10^6$ | 50 | 49 | 98 | 1 | 2 | 0 | 0 |
| | $7x10^6$ | $3x10^6$ | 50 | 50 | 100 | 0 | 0 | 0 | 0 |

## Discussion

The germination rate of entomopathogenic fungi spores, which is interfered by the temperature (Hywel-Jones & Gillespie, 1990; Yeo *et al.*, 2003), is a key factor in the expression of the virulence of anamorphic fungi. The *Galleria* bait trap detected probably better spores of the virulent strains, which adhere onto the insect cuticle, germinate fast and then penetrate to establish into the body cavity (Altre *et al.*, 1999).

The results gained in our experiments are probably due to differences in germination behaviour between spores of *B. bassiana* and *M. anisopliae*, onto the insect cuticle, under the same temperature regime, which is likely to have repercussions on host-pathogen outcomes. According to our results, the absence of interactions between temperature and dose in the case of *B. bassiana*, unlike *M. anisopliae*, could explain why the former fungus could increase its

inoculum load in the soil by infecting soil-dwelling insects more frequently regardless of temperature (Soares *et al.*, 1983). Furthermore, the fact that *B. bassiana* kills the host faster than *M. anisopliae* could be another reason for increase inoculums load in the soil.

In soils where both fungi are present, most of the larvae can be killed by *B. bassiana.* Nevertheless, we do not discard the possible co-infection of larvae but the *M. anisopliae* infection can be arrested by the higher growth speed of *B. bassiana* or by the production of an array of metabolites (Vey *et al.*, 2001). However, during our field study, we have isolated both fungi from the same samples, at the same temperatures (Maranhão, 2003), in contrast to Bidochka *et al.* (1998), using a range of temperatures. Such results could be explained because in these soil samples, the *M. anisopliae* natural infestation was higher than the *B. bassiana* one and the number of germinated propagules was also high to overcome the interference caused by those of *B. bassiana.* In conclusion, the predominance of *B. bassiana* in soils could be the result of the increasing of the inoculum load owing to the faster germination of spores and more rapid action, compared to *M. anisopliae.*

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# The efficacy of *Verticillium lecanii* against whitefly in sweet pepper and tomato in Spain

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**Abstract**: MYCOTAL® is microbial insecticide, a formulation of a specific strain of the entomopathogenic fungus *Verticillium lecanii*. Four trials were carried out in order to study the effect of MYCOTAL® on the mortality of *Bemisia tabaci* and *Trialeurodes vaporariorum* in commercial sweet pepper and tomato crops in the South of Spain. In each one of the trials, three applications were carried out with weekly intervals. The mortality of the immature whitefly stages was quantified and compared with the mortality in untreated plots and in plots treated with a reference product (i.e. *imidacloprid* in sweet pepper and *piriproxifen* in tomato).

In all trials, there was a big, and very significant, increase of the whitefly mortality in the *V. lecanii* treated plants compared to the mortality in the untreated plots. In comparison with the reference products, the effect of *V. lecanii* resulted to be similar in two cases, once in sweet pepper and once in tomato. In the other two cases, the mortality caused by the reference products did not differ from the mortality in the untreated plots. These observations illustrate the high level of acquired resistance among the whiteflies against the available chemical insecticides in the Southern Spanish horticultural area. The results of *V. lecanii* indicate that Mycotal can be a valuable alternative in the control of whitefly in Southern Spain, as well as an excellent tool in a resistance management programme.

**Key words**: *Bemisia tabaci*, biological control, efficacy, sweet pepper, Spain, tomato, *Trialeurodes vaporariorum, Verticillium lecanii.*

## Introduction

The development of *Verticillium lecanii* (Zimmermann) Viégas as a biological pesticide began at the Glasshouse Crops Research Institute (GCRI), Littlehampton, United Kingdom, in 1972. On the basis of the research of Dr. Richard Hall the first commercial product was developed in United Kingdom, for control of aphids on chrysanthemums. Mycotal was registered in the United Kingdom in 1986 for the control of whitefly in greenhouse crops. Following the registration in the United Kingdom registration in other European countries was applied for.

*V. lecanii* has a natural habitat as a soil pathogen (Domsch *et al.*, 1981), as a hyper parasite on rusts (Spencer & Atkey, 1981), as a parasite on cyst-nematodes (Hanssler & Hermans, 1981), as a saprophyte on ripening grain (Hill & Lacey, 1983) and various insects, especially on aphids and scales (Hall, 1981). Thus *V. lecanii* is not an obligate parasite, but it also occurs general saprophytically. Whiteflies are also parasitized by *V. lecanii* by nature in greenhouses (Ekbom, 1979; Hall, 1975), but under practical circumstances epidemics seldom occur. Inoculum is often available in the soil (Rombach & Gillespie, 1988).

The entomopathogenic fungus *V. lecanii* occurs worldwide (Bradly, 1979), in temporal and tropical zones (*e.g.* Klingen *et al.*, 2002; Lo & Chapman, 1998).

About the mode of action, the spores of *V. lecanii* germinate on the insect cuticle between 12-48 hours. Strong hyphal growth on the cuticle is observed before penetration of the host. The cuticle is penetrated, and tissue has been affected within 48 hours after infection. Once in the host, *V. lecanii* forms blastospores which spread through the haemolymph of the arthropod host and lead to further infection. After 7-10 days, a great number of hyphal bodies have been formed inside the body cavity (Walter *et al.*, 1988; Shreiter *et al.*, 1994). At a high relative humidity the fungus can grow outside through the cuticle and can start to sporulate there. This can already occur at 50% of the larvae a week after spraying with *V. lecanii*.

In 2001 a revision has been made by R. Zare and W. Gams of *Verticillium* section Prostata, concluding that the fungus hitherto known as *Verticillium lecanii sensu lato* is renamed *Lecanicillium muscarium* (Petch) Zare et Gams with a distribution mainly in the temperate climates (UK, Italy, etc.), whereas *Lecanicillium lecanii* (Zimmermann) Zare et Gams has a distribution mainly in the tropical countries (West Indies, Dominican Republic, Peru, Jamaica, USA, Sri Lanka, Indonesia, Turkey, etc.). Two strains of *Verticillium lecanii sensu lato* have developed as biological control agents of glasshouse pests are now classified as *Lecanicillium muscarium.*

Objective of the study was to determine the efficacy of Mycotal® (*Verticillium lecanii*) for control of *Bemisia tabaci* (Gennadius) and *Trialeurodes vaporariorum* (Westwood) on sweet pepper and tomato in Spain with 3 applications, and to compare the performance of the test item with a standard product used at the normal dosage rate.

## Material and methods

***Test site information***

Four trials were carried out in order to study the effect of *Verticillium lecanii* on the mortality of *Bemisia tabaci* and *Trialeurodes vaporariorum* in commercial sweet pepper and tomato crops in the South of Spain, in Nijar, Santa María del Aguila, La Mojonera and Orihuela. The trials were done in November of 2001, Mars, February-Mars and April-May of 2002 respectively and according to EPPO guidelines.

***Information on test item and reference***

Before application the weighed amount of Mycotal is presoaked in water for 2-4 hours. The proportion of water recommended is 500 g Mycotal in 3-4 L, with a temperature between 15-20 °C. The adjuvant Addit, which improves the effect of Mycotal, is added just before application (2.5 cc/ L). The reference products were imidacloprid (Confidor 20 LS 20 %) in sweet pepper and piriproxifen (Atominal 10 EC 10 %) in tomato. Dose for Mycotal of 100 g / hL and for reference chemical of 75 cc / hL. Both Mycotal and the reference product were applied 3 (in case 4) times with an interval of one week.

***Assessments***

Assessments were done one day per treatment and 4 assessments per trial, in one case 5.

25 leaves per plot are taken to the laboratory in order to determine the amount of individuals of whitefly at every stage, under the binocular. With help of a sharp cylinder (35mm Ø) a disk is cut from every leaf, and all larvae stages are counted, divided into two categories L1-L2 and L3-L4, determining if they are dead or alive. If there are less than 10 larvae of each category in the disk, more disks are cut and counted. The amount of eggs and the number of adults and pupae are also determined at each evaluation.

With these data the percentage of mortality is calculated for each plot and evaluation, this percentage is analysed statistically.

The whitefly found in the trials was *Bemisia tabaci* with exception the trial on tomato of Orihuela where the species of whitefly was *Trialeurodes vaporariorum.*

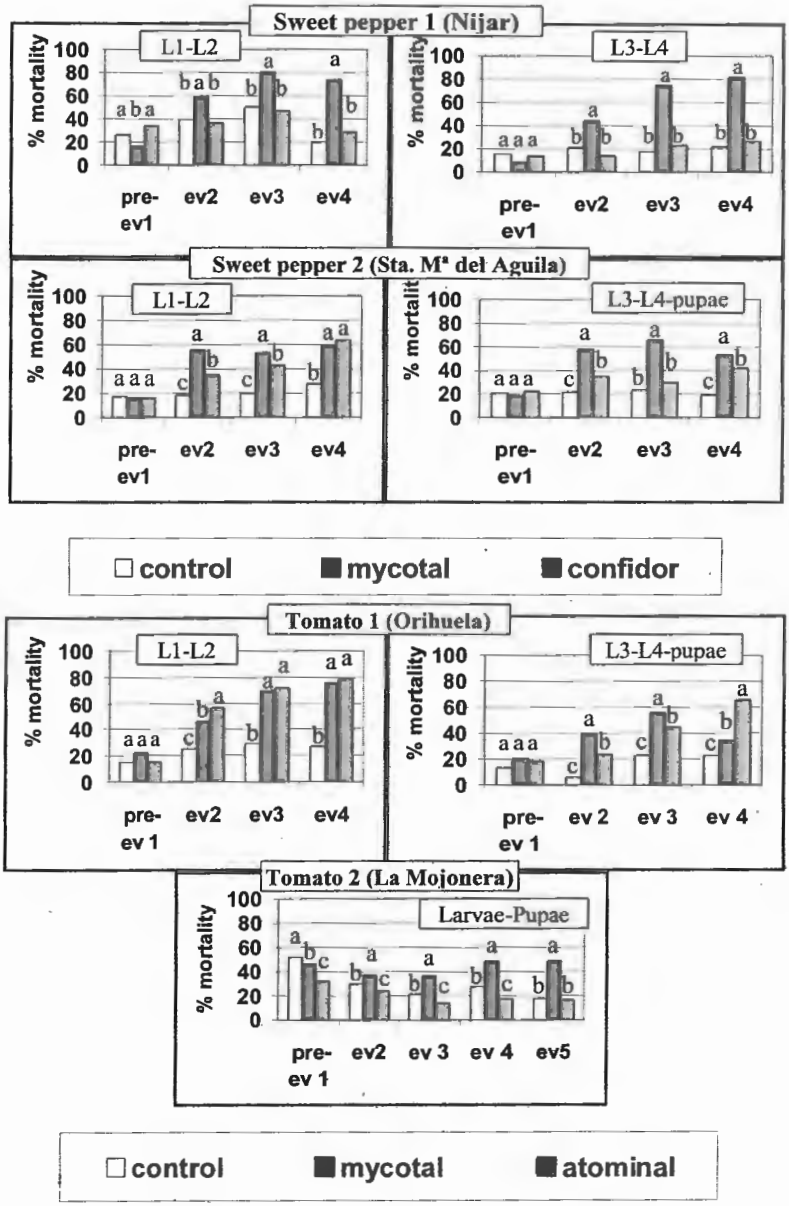


Figure 1. Mortality of immature whitefly stages, quantified and compared by treatments in the trials on sweet pepper and tomato before applications and in the different evaluations Different letters indicate significant difference (LSD test, P<0.05). (pre-ev1 = mortality before applications; ev2 = evaluation after first week of applications; ev3 = evaluation after second week of applications; ev4 = evaluation after third week of applications; ev5 = evaluation after fourth week of applications).

## Results and discussion

In all trials, there was a big, and very significant, increase of the whitefly mortality in the *V. lecanii* treated plants compared to the mortality in the untreated plots (Fig. 1). In comparison with the reference products, the effect of *V. lecanii* in L1-L2 stages resulted to be similar in two cases, once in sweet pepper and once in tomato. In the other cases, the mortality caused by the reference products did not differ from the mortality in the untreated plots, while mortality caused by *V. lecanii* was much higher and differed significantly from the other two treatments. These observations illustrate the high level of acquired resistance among the whiteflies against the available chemical insecticides in the Southern Spanish horticultural area.

As a global conclusion, all observations of the trials show the efficacy of *V. lecanii* (Mycotal) against *B. tabaci* and *T. vaporariorum* compared to a non-treated variant.

The results of *V. lecanii* indicate that Mycotal can be a valuable alternative in the control of whitefly in Southern Spain, as well as an excellent tool in a management program to conquer whitefly resistance to insecticides.

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# Western Flower thrips: Biological control in greenhouse with *Verticillium lecanii*

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**Abstract**: Effective control of *Frankliniella occidentalis* populations in greenhouse has not been successful until now. In this contribution we investigated the effect of the entomopathogenic fungus *Verticillium lecanii* against this thrips under greenhouse conditions. We have found a significant effect of the fungus on the reduction of the insect population (88,9% efficiency) during the trial, depending on the environmental conditions. The efficiency reached 88,9%.

**Key words**: *Frankliniella occidentalis, Verticillium lecanii,* biological control, greenhouse

## Introduction

*Frankliniella occidentalis* is an important sucking pest organism in grennhouses. The control of this insect has not been solved satisfactorily until now.

Trials to establish a biological control method using entomopathogenic fungi such as the Deuteromycete *Verticillium lecanii* (Zimmermann) Viégas has been sucessful in the laboratory. The aim of this contribution was to prove the efficacy of the pathogen against the insect under non-ideal conditions and to investigate the special effet of a ground application of the fungus.

## Material and methods

*Frankliniella occidentalis* was raised on bush beans (*Phaseolus vulgaris* "Marona") at 20°C, 60-70% relative humidity (rh) and 16h of light. In the trial we used 4-5 female adults per plant to start growth of the insect population on the plant.
The fungal strain used was V24K2. The blastospores were produced using the method of Hirte *et al.* (1989).

Trials have been carried out in 2002 (April to July) on ground beds with drop irrigation system, in two small greenhouses. The crop plant used has been *Phaseolus vulgaris* "Rakker" with 30 plants per variant. The fungus was applied weekly on leaves and ground (2.5 L suspension with $1x10^9$ blastopspores/mL). Spore titre on leaves and ground has been monitored during the trial (Beyer *et al.*, 1997).

For the weekly scoring we have taken three leaves per plant from 5 plants per variant and have monitored the number of living and dead insects, their developmental stage and state of health, and the existence of mycelium on the insect surface.

## Results

Temperature and humidity ranged from 15 to 30°C, and from 50 to 95% respectively on average in both greenhouses during the experiments.

The development of the thrips population started slowly in both variants. In the beginning no population decrease due to the fungus could be seen. From the end of May a distinct increase of the population was noticeable in both variants. From the 24th of June, the population density in the control variant laid highly significantly above the density in the variant which has been applied with the fungus (Fig. 1). At the end of the trial, the limited number of plants led to a deficiency in nutrition ant to a decrease of the thrips population in the control variant as well (Fig. 1).

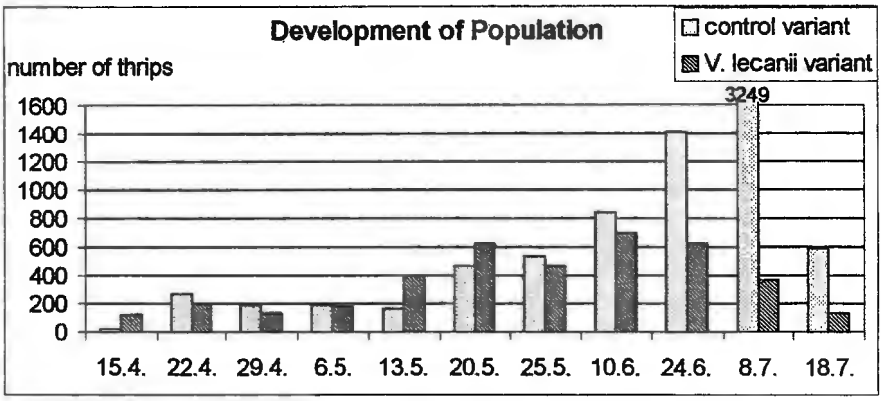


Figure 1. Development of a population of *Frankliniella occidentalis* on *Phaseolus vulgaris* "Rakker" after a leave and ground application of a blastospore suspension of *Verticillium lecanii* (strain V24K2).

Table 1. Mortality of *Frankliniella occidentalis* after a leave and ground application of a blastospore suspension of *Verticillium lecanii* (strain V24K2).

| **Date of monitoring** | **Number of dead insects / mortality in %** | | | |
|---|---|---|---|---|
| | Control variant | | *V. lecanii* variant | |
| 15th Apr. (3 dpa) | 0 / 0 | a | 0 / 0 | a |
| 22nd Apr. (10 dpa) | 7 / 2.6 | a | 17 / 7.7 | a |
| 29th Apr. (17 dpa) | 9 / 4.4 | a | 34 / 20.4 | a |
| 06th May (24 dpa) | 11 / 5.5 | a | 55 / 23.6 | b |
| 13th May (31 dpa) | 14 / 7.7 | a | 40 / 9.0 | a |
| 20th May (38 dpa) | 12 / 2.5 | a | 75 / 10.8 | b |
| 25th May (43 dpa) | 9 / 1.7 | a | 74 / 13.8 | b |
| 10th June (59 dpa) | 28 / 3.2 | a | 279 / 28.5 | b |
| 24th June (73 dpa) | 36 / 2.5 | a | 138 / 18.3 | b |
| 08th July (87 dpa) | 58 / 1.8 | a | 140 / 27.9 | b |
| 18th July (97 dpa) | 224 / 27.4 | b | 58 / 31.2 | a |

The monitoring of the number of dead insects was difficult, because especially in the beginning of the trial, a lot of them stayed inside the substrate, where they have been killed by the fungus. Starting on May the 20th and lasting until June the 24th, the number of dead thrips

on the leaves reached a significantly higher level in the *V. lecanii* variant than in the control. At the end of scoring there appeared a large natural mortality in the control variant (Table 1).

At the end of the trial an efficiency level of 88,9% was reached related to the number of living insects in the control variant.

The average necrotic part of the leaf area has been approximately 30% higher in the control variant than in the fungus applied variant during the whole trial, except on the 25th of May.

The spore titre has fluctuated strongly especially on the leaves, despite a regular application of the fungus. At first, the titre was very high, with a value of $10^5$ spores/cm$^2$. Later on it ranged from $10^3$ to $10^4$ on the ground and from $10^4$ and slightly under $10^5$ on the leaves, respectively.

## Discussion

The host/pathogen system and thus the success of controlling the population of the former is especially influenced by climatic conditions. These conditions, above all the temperature, fluctuated very strongly during the experiment. The temperature reached extreme values of 2 and 46°C respectively. That fact could have influenced the effect of *V. lecanii* against the thrips on the leaves in an unfavourable way. Hsiao *et al.* (1992) also proved the important role of the temperature on the level and speed of the control effect of *V. lecanii* against aphids. In these trials, the mortality reached only 39.7% at 15°C after 7 days, but achieved 100% after 3 days at 21 and 27°C.

The relative humidity is very important for the development of the fungus as well. In our trial the humidity fell temporarily under 60%, which has benn noticed by Ravensberg *et al.* (1990) as a limit in successful greenhouse trials with the aim of controlling *F. occidentalis* populations in cucumber and tomato cultures. Unfavourable climatic conditions can be compensated to a certain amount and the infection can be transmitted successfully to the upper parts of plants by using ground application. This was demonstrated by finding adult insects covered with mycelium on the leaves, which had promoted the dissemination process of the fungus (see also Meyer *et al.*, 2002).

At first we did'nt detect a population regulating effect of the fungus. This was caused maybe to a certain part by the initial period, which the fungus needs to get established in the insect population and in the greenhouse, and by the conditions prevailing during this period. Only after insects covered with mycelium were found on the leaves, transmission of the infection was supported inside the insect population and a rise of the fungal effect was observed.

Another reason for the gradually rising control effect was the density of the thrips population. The increase of the population has been very slow at the beginning of the trial. At first, only a few insects could have been infected for this reason. With increasing density of the population, a considerable effect caused by the fungus was observed. The strong mobility of thrips and a lot ot contact between them led to the transmission of the infection in the population. The higher density of the insects has promoted probably the dissemination of the fungus in the host population.

To establish a successful biological control method of F. *occidentalis* with help of *V. lecanii*, it is necessary to monitor and control all environmental factors and maybe to combine different biological organisms depending on their characteristics and demands as temperature, humidity and day lenght (Jacobson, 1995). A visible effect of *V. lecanii* on the thrips in our experiment could only be observed after the fast develoment of the host population had started. Eventually we have reached a distinct population decimating effect at the end of the trial despite partially suboptimal conditions for the fungus.

## Acknowledgements

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# Field testing of new biocontrol strategies to decrease the population density of *Melolontha hippocastani*, an important scarab species in Germany

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**Abstract**: The current outbreak of the forest cockchafer, *Melolontha hippocastani*, starting in the eighties in Germany, particularly in the Southern Federal States of Hessen, Rheinland-Pfalz and Baden-Württemberg, has reached an enormous extent (a total of approx. 7.500 ha are damaged). Therefore, different application methods of *Beauveria brongniartii* were tested in the laboratory and in the field during the main flight period in 2002.

First, barley kernel products were used to protect young seedlings. In one trial, the fungus kernels were applied into the planting hole (20g/plant) whereas in another trial, the kernels were distributed in rows (dosage of 50 kg and 100 kg $ha^{-1}$), mechanically pre-treated by a rotary tiller. Pine trees have been planted into these rows in spring 2003. Data analysis of these trials still is in progress. Second, an experimental product was tested in different formulations in the laboratory. The efficacy after application of either 10 µL onto the pronotum of single specimens ($5x10^8$ spores $mL^{-1}$) (n=10) or 50 mL onto groups of 10 (n=5), was 57, 78 and 87% or 75, 80 and 68% respectively for two oil/lecithin emulsions and one ProNet-Alfa suspension, respectively. Mid-May, *B. brongniartii* was applied as 0.1% ProNet-Alfa suspension (conc. of $5x10^{13}$ spores $ha^{-1}$), on the crowns of red oak trees on 2000 $m^2$. Cockchafers collected from the treated area and from an untreated one were kept in cages until death. Mycosis by *B. brongniartii* was recorded in 41% of the treated, but only in 1% of the untreated cockchafers. Third, a combination of funnel traps baited with a recently developed cockchafer attractant and equipped with *B. brongniartii* spores was used to investigate the possibility to smuggle *B. brongniartii* in the cockchafer population by this means. It was shown that captured males having passed the traps transmit the pathogen to females; four weeks after death, 26% of the specimens (17% females) were mycosed. Further studies for optimisation of this method will be conducted this year.

**Key words**: *Melolontha hippocastani*, *Beauveria brongniartii*, biocontrol, formulations, field testing

## Introduction

In Germany, particularly in the southern Federal States Hessen, Rheinland-Pfalz and Baden-Württemberg, a recent outbreak of the forest cockchafer, *Melolontha hippocastani*, has led to tremendous levels of damage. The estimated extent of damage, due to loss of trees of varying age and in different habitats (approx. 7.500 ha), amounts to several millions euros. The main damage is caused by the white grubs, that feed on the fine roots of nearly all tree species during their three years lasting life in the soil. The application of conventional control strategies is impossible, neither against the white grubs, nor the beetles, due to the size of the forest area, which is too big to become treated with a non-selective insecticide, and generally due to the lack of a product registered for the control of *M. hippocastani*.

Therefore, during the main flight period in 2002, different application methods of *Beauveria brongniartii* barley kernel products and conidiospores have been tested in field and accompanying laboratory trials, i.e. soil application, spray application and transfer of conidiospores from males via females into the soil and onto the offspring by means of pheromone traps.

## Material and methods

***Soil application***

*B. brongniartii* barley kernel products, Melocont® (Agrifutur srl., Italy) and Beauveria Schweizer ® (Eric Schweizer Samen AG, Switzerland), were used to protect young seedlings from damage. In the first trial, either one of the products was applied into the planting whole (20 g/plant, 120 plants/replicate, 4 replicates/treatment). 480 seedlings remained untreated. In a second trial, Melocont® alone was applied on the soil at two different dosages, corresponding to 50 kg and 100 kg $ha^{-1}$ (40x0.5 m, 3 replicates) pre-treated by a rotary tiller. A corresponding area remained either untreated or mechanically pre-treated alone. In spring 2003, young pine trees have been planted into the rows. In both trials, damage of roots and seedlings together with spore concentration in the soil are assessed in regular intervals.

***Spray application: laboratory trials***

An experimental product of *B. brongniartii* conidiospores (Fytovita, Czech. Rep.; BBA-isolate B.br. 56; $1x10^{10}$ conidia $g^{-1}$) was tested in three formulations (Kwizda = soy bean oil-Tween 80-lecithin, fluid bed dried; oil+lecithin = aqueous emulsion of soy bean oil and lecithin; ProNet-Alfa = registered wetting agent). Five males and five females (5 replicates) received 10 µL each ($5x10^8$ conidia $mL^{-1}$) dorsally onto the pronotum, whereas another five males und five females (5 replicates) in cylinders, where sprayed with approx. 50 mL of the same suspensions by using a hand sprayer. A corresponding number of beetles treated with water served as control. Dead beetles were incubated in a moist chamber at room temperature. Percentage effect was calculated from mortality data.

***Spray application: field trials***

On May, 15$^{th}$ the experimental product of *B. brongniartii* conidiospores was applied as aqueous ProNet-Alfa-formulation by a motorized backpack sprayer from the ladder of a fire engine on the top of an American red oak stand, treating an area of approx. 2000 $m^2$ (dose corresponding to $5x10^{13}$ spores $ha^{-1}$). Subsequently, 50 males and 50 females were collected both from the treated and an untreated area and placed into cages on potted oak trees. Dead beetles were incubated in a moist chamber at room temperature. Rate of mycosis was calculated based on the number of beetles recovered.

***Pheromone traps: beetle catches***

Funnel traps "with" and "without a lid" to protect the spores against rainfall, equipped with improved lure substances (Ruther *et al.*, 2000, 2001), were compared. Traps were placed into the trees about 1.5 h before the flight period during dusk. Catches were counted the next morning. Data were analysed statistically by SAS.

***Pheromone traps: rate of mycosis***

After passage through a funnel trap equipped with the experimental product of *B. brongniartii* conidiospores (0.1 g/20 beetles), 10 beetles were either washed off with 10 mL Tween 80 each, in order to determine the acquired spore dose, or transferred directly in boxes onto leaves. Beetles were incubated in a moist chamber at room temperature, after death.

***Pheromone traps: transfer of spores***

After passage through a funnel trap equipped with the experimental product of *B. brongniartii*-conidiospores (0.1 g/25 beetles), 10 males and 10 untreated females were

placed in plastic pots (0.63 $m^2$ surface; n=5) half filled with soil. Correspondingly, 50 untreated couples served as control. After death, beetles were incubated in a moist chamber at room temperature. Based on the number of beetles recovered, the rate of mycosis was calculated.

## Results

***Soil application***

Analysis of soil samples, taken in autumn 2002 and spring 2003 is in progress. Results on the establishment of *B. brongniartii* in the soil and the success of protection of young seedlings are expected in spring 2004. So far, no *Melolontha* grubs could be recovered.

***Spray application: lab trials***

After 10 days, percentage effect of the Kwizda, oil+lecithin and ProNet-Alfa formulations was 57, 78 and 87% respectively, when applied with a pipette, and 75, 80 and 68% respectively, after spray application (Fig. 1). The highest rate of mycosis (62%) was achieved by spray application of the ProNet-Alfa formulation (30 and 12% for Kwizda and oil+lecithin,respectively, as well as 44, 46 and 40% respectively for Kwizda, oil+lecithin and ProNet-Alfa by pipette application) (Fig. 1).

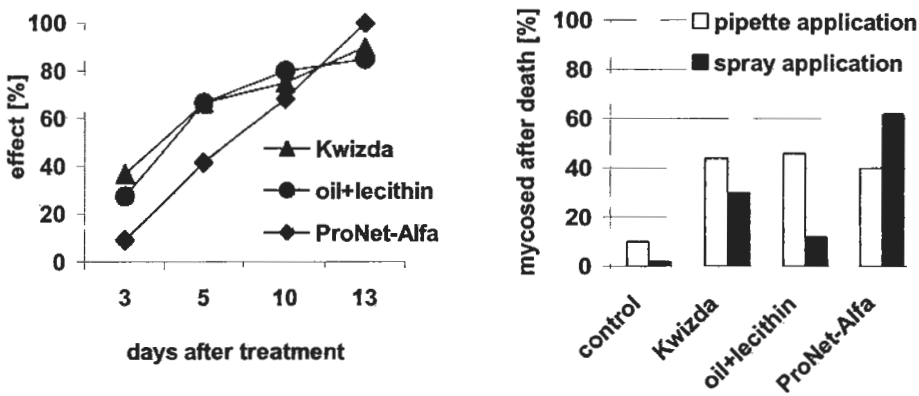


Figure 1. Effect of a *Beauveria brongniartii* conidiospore-product in three different formulations on adults of *Melolontha hippocastani*. Left: Percentage effect after spray application. Right: Rate of mycosis after pipette and spray application. See text for details.

***Spray application: field trials***

41% of the treated and 1% of the untreated beetles were mycosed by *B. brongniartii*. Nevertheless, within the soil of potted oaks of the treated and the untreated beetles a total of 9 $L_1$ and 76 eggs compared to 73 eggs were found, respectively.

***Pheromone traps: beetle catches***

Statistically significant more beetles were caught „without a lid" ($\alpha \leq 0.05$) (mean of 117, n=10, compared to 84 with a lid, n=12) when the catches of two nights were analysed together (Fig. 2). During several other trials with pheromone traps, performed by the group of Ruther, up to 500 beetles/trap were caught during one evening.

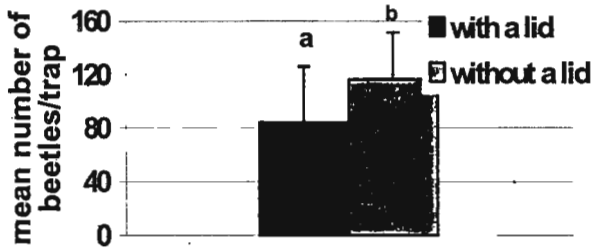


Figure 2. Beetle catches (mean of two assessment dates) by pheromone traps: comparison of funnel traps "with" (n=10) and "without a lid" (n=12) to protect *Beauveria* conidiospores against rainfall. Bars with a different letter are significantly different ($\alpha \leq 0.05$).

***Pheromone traps: rate of mycosis***

After washing off contaminated beetles, only few or no spores could be recovered. Yet, a mean of 30 and 70% of beetles (n=20), either washed off or directly placed onto leaves respectively, was still mycosed by *B. brongniartii,* after three weeks of incubation.

***Pheromone traps: transfer of spores***

From couples where the males had been passed a funnel trap with conidiospores, 17% of the females were mycosed (total of 26%).

## Discussion

Since the onset of the current maybeetle gradation, these are the first experiences with a spray application of *B. brongniartii* conidiospores against *M. hippocastani* in the field. Moreover, new formulations have been tested for the first time. The good results of the laboratory trials have to be confirmed in the field. Transfer of the pathogen from males to females could be demonstrated successfully. This promising method will be improved (e.g. trap design) in the following years. Presumably, a sustainable reduction of the population might be achieved at medium-term by the application of *B. brongniartii* in three different methods; the soil application in reforestation areas, the specific spray application of affected stands, and the infiltration of *B. brongniartii* into the population via pheromone traps.

## Acknowledgements

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# Advances in the development of novel control methods against chicken mites (*Dermanyssus gallinae*)

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**Abstract:** CHIMICO, a four-year EU-financed project, aims at developing new control methods against the chicken mite *Dermanyssus gallinae*, a major ectoparasitic pest in European egg production facilities. The ultimate aim is to combine entomopathogenic fungi with semiochemicals in a lure-and-infect trapping system. This paper reports the progress of notably the work on entomopathogenic fungi. A survey of naturally occurring entomopathogens in *D. gallinae* in Denmark and in Spain have not yet shown any records of fungal pathogens. In contrast, several species of Hyphomyctetes from other arthropods have been shown to be virulent against bloodfed females, although high spore concentrations are necessary to obtain control. Five isolates of *Beauveria bassiana* and *Metarhizium anisopliae* have been selected for further studies, including transmission, persistence and effect on fecundity.

**Key words**: *Dermanyssus gallinae*, entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, pheromones, lure-and-infect

## Introduction

The bloodfeeding chicken mite, *Dermanyssus gallinae*, is a major problem for egg producers all over Europe. The mites spend most of the time in hiding places in the poultry house and come out only to feed briefly on the birds, mainly during the night. Several life stages need to feed in order to moult and the adult female mite needs a blood meal for every batch of eggs (Wood, 1917). This implies that the mites repeatedly must locate a host and return to a hiding place, with other mites, for mating. This behaviour is partly the reason for the difficulty in controlling chicken mites, as mites in cracks and crevices are difficult to reach with acaricides or other control agents. However, at the same time it might provide a solution to the problem, provided that the mites during their search phase can be manipulated into some kind of trap. The EU CHIMICO project aim at developing a new control method for *D. gallinae* based on a combination of entomopathogenic fungi and semiochemicals in a lure-and-infect device.

The background for the work is as follows: there is very scarce information in the literature concerning the presence of entomopathogens in *D. gallinae*. Concerning semiochemicals, a number of known (but not identified) and other putative pheromones are involved, when the mites locate a host and return to a hiding place, along with other mites (Fig. 1).

## Materials and methods

Live bloodfed females were collected from poultry houses infested by *D. gallinae*. Each sample consisted of 500 mites. The mites were placed in groups of 50 specimens in filter

paper arenas surrounded by sticky paper to avoid mites escaping. Arenas were incubated at 25°C, 85% r.h. and a photoperiod of 12D:12L for 10 days. This incubation system allowed the daily removal of dead specimens which were surface-disinfected in sodium hypochlorite followed by rinsing in sterile water. This procedure was necessary in order to avoid fast colonisation of cadavers by saprophytic fungi. Mite cadavers were then transferred to humid chambers, and checked daily for presence of entomopathogenic fungi for a period of 5 days.

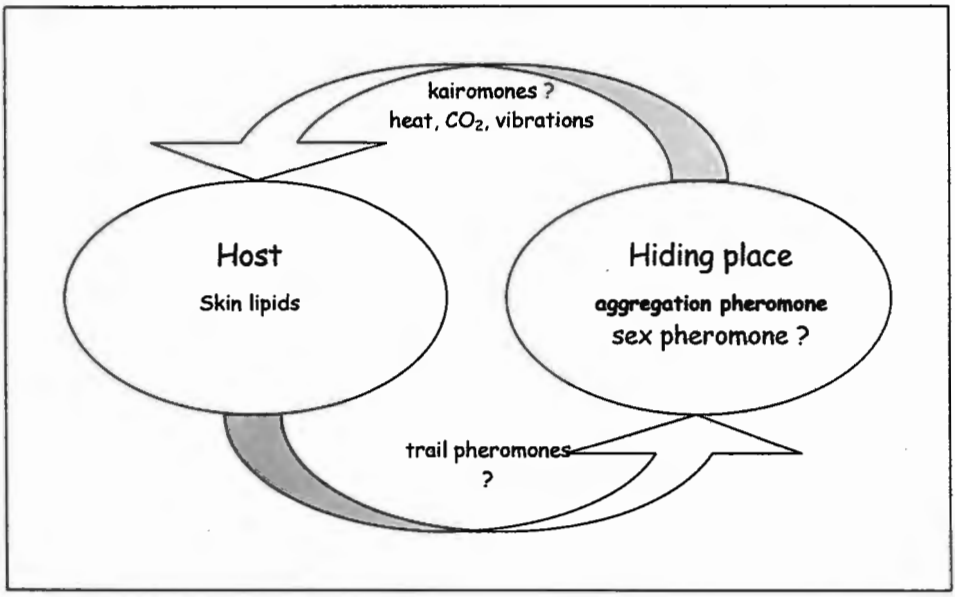


Figure 1. Possible and known cues involved in the progress of locating a host and returning to a hiding place for *Dermanyssus gallinae*.

Twelve fungal isolates from the collection at the Danish Pest Infestation Laboratory (collected from a broad range of arthropods) were screened for activity against bloodfed females using massive spores doses as described in Steenberg & Kilpinen (2003). The test included isolates of *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus* and *Verticillium lecanii*. Mites were incubated in ventilated glass tubes at 25°C, 85% r.h. and a photoperiod of 12D:12L for 10 days with daily records of mortality.

## Results and discussion

The survey for fungal pathogens have not yet revealed any entomopathogenic fungi. Previous surveys for fungal pathogens in arthropods in livestock farms have documented the presence of entomopathogenic Hyphomycetes in arthropods in broiler houses and in dairy cattle farms, but prevalences were low (Steenberg *et al.*, 2001; Skovgård & Steenberg, 2002). Based on the limited occurrence of fungus-infected arthropods in general in stables and on the intermediate susceptibility of *D. gallinae* to fungal infection in bioassays, it is not surprising that entomopathogenic fungi are not commonly found in field populations of *D. gallinae*.

The screening of isolates showed that most isolates produced mortality in females, but very high doses were necessary to cause high mortality (data not shown). Three isolates of *B. bassiana* and two isolates of *M. anisopliae* were selected for further studies including transmission, persistence and fecundity effects.

In conclusion, several virulent fungal isolates are available for future combination with a semiochemical in a mite trap; future studies will show whether these isolates are appropriate for use in the poultry house environment.

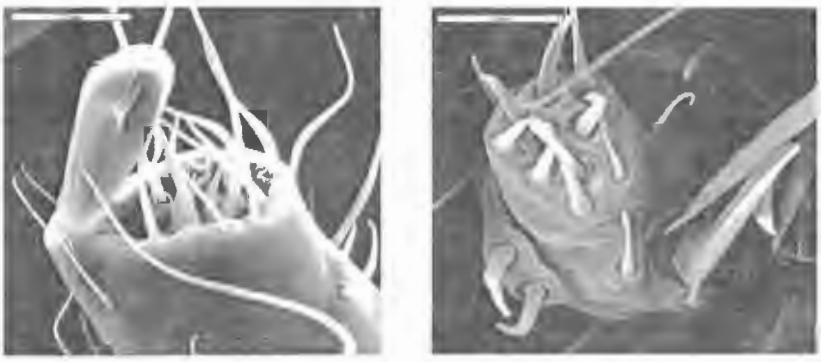

Figure 2. Scanning electron microscopy pictures of the tarsi on the first of the first pair of legs (left) and of the palps (right). Both from an adult female chicken mite, *Dermanyssus gallinae*.

In addition, extracts based on simple washings of mites and of their hiding places have been produced and shown to contain biologically active semiochemicals. Also air entrainment has been applied as a way of collecting semiochemicals (Agelopoulos & Pickett, 1998), and scanning electron microscopy has produced a mapping of those sensilla, present on the palps and the tarsi of the first pair of legs, that are the most likely locations for sensory sensilla involved in chemo-reception (Figure 2). After extracts have been produced and fractionated, active components will be isolated using electrophysiological recordings in combination with GC, and also using behavioral bioasssays for verification of activity.

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# Risk assessment of *Beauveria brongniartii* for three carabid beetles

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**Abstract**: The entomopathogenic fungus *Beauveria brongniartii* was tested as Melocont-Pilzgerste® for its pathogenity for the three carabid beetle species *Poecilus versicolor*, *P. cupreus* and *Clivina fossor*, representing soil dwelling non-target-organisms. Only low infection rates (< 10%) with *B. brongniartii* were observed in the carabid species but the provision of barley kernels enhanced mortality of *Poecilus* larvae drastically. This may have masked detrimental effects of the fungus and points to the necessity of further experiments evaluating the exclusive influence of the fungus. In feeding experiments with *B. brongniartii* infected *M. melolontha* cadavers no negative effects on the development of *P. versicolor* could be observed. This shows that this way of infection, which is probably the most important infection source in the field, is of negligible risk for this carabid species under field conditions.

**Key words**: *Beauveria brongniartii*, Carabidae, non-target organisms, risk assessment

## Introduction

The entomopathogenic fungus *Beauveria brongniartii* is used to control larvae of *Melolontha* spp. and is supposed to be highly host specific. Field experiments, however, have shown that besides the primary host *Melolontha* spp. also other groups of arthropods can be infected by the fungus (Baltensweiler & Cerutti, 1986; Back *et al.*, 1988). Probably the most susceptible non-target-arthropods are soil dwelling Coleoptera and their larval stages. Laboratory experiments revealed that third instar larvae of the carabid species *Nebria brevicollis* and *Amara aulica* could be infected with high doses of *B. brongniartii*-spores (Traugott *et al.*, 2001). Thus it was the aim to investigate the potential hazards of this entomopathogen in more detail for the three predatory ground beetle species *Clivina fossor*, *Poecilus cupreus* and *P. versicolor*, which are widespread and abundant dwellers of arable habitats throughout the Northern Hemisphere. All stages of *C. fossor* live in the soil, whereas only the larvae and pupae of *P. cupreus* and *P. versicolor* are found in this habitat (Traugott, 1998).

## Material and methods

### *Insect rearing*

Larvae of *P. cupreus* and *P. versicolor* were bred for the experiments in the laboratory to study the influence of the fungus on the development from the first larval instar to adult stage. $L_1$ larvae were 2-4 days old when entering the experiment. Only specimens which appeared to be vital and healthy were used for the experiments. In the case of *C. fossor* only adults were used. Specimens of *C. fossor* were captured in the field and underwent a quarantine period of two weeks before the experiment started. Field collected *M. melolontha* $L_3$ larvae were used to show the pathogenity of the Melocont-Pilzgerste®. Scarab larvae were kept for two weeks in quarantine before they entered the experiments.

Specimens of all four species were kept individually in single plastic containers (42 mL), filled with a soil-peat substrate, which was tested to be free of *B. brongniartii*. Trials were run in a climatic chamber at constant 18°C and darkness. Carabidae were checked and fed with pupae of *Tenebrio molitor* and *Calliphora* sp. every third day, *M. melolontha* larvae were checked and fed with carrot slices twice a week.

***Experimental design***

Three experiments were run: (1) 0.5 g of Melocont-Pilzgerste® (M-PG), a *B. brongniartii*-formulation of *B. brongniartii* cultivated on sterile barley kernels, were applied to each container. The concentration used is equivalent the 100-fold concentration recommended for field use. (2) An experiment with 0.5 g of sterilized barley (SB) kernels per container was run to test the influence of the barley grain on the carabid larvae. The kernels were pre-soaked in distilled water before they were mixed into the substrate. (3) A feeding experiment was carried out to test if *P. versicolor* larvae could be infected by feeding on *B. brongniartii* infected *M. melolontha* larvae. *B. brongniartii* infected *M. melolontha* larvae were cut into pieces and feed once to third instar *P. versicolor* larvae.

Control groups were run either without applying Melocont-Pilzgerste®, barley kernels or larvae were fed with pieces of uninfected cockchafer larvae. Table 1 provides an overview of the three experiments carried out.

Table 1. Overview on the experiments carried out with Melocont-Pilzgerste® (M-PG), sterile barley (SB) and the feeding experiment with B. brongniartii-infected M. melolontha larvae (M. m-feeding).

| | **Control group** | **Treated group** | **Group size (N)** |
|---|---|---|---|
| M-PG | soil-peat substrate | soil-peat substrate with 0.5 g M-PG | *P. cupreus* larvae (96)<br>*P. versicolor* larvae (96)<br>*C. fossor* adults (15)<br>*M .melolontha larvae* (26) |
| SB | soil-peat substrate | soil-peat substrate with 0.5 g SB | *P. versicolor* larvae (41) |
| M. m-feeding | uninfected *M. melolontha larvae* | *B. brongniartii* infected *M. melolontha* larvae | *P. versicolor* larvae (65) |

Parameters recorded in each experiment were mortality rate, duration of development from $L_1$- adult stage in the *Poecilus* species and the body mass of the *Poecilus* tenerals. All dead specimens were visual examined for characteristics of a *B. brongniartii* infection, which was indicated by a dehydrated, reddish coloured cadaver with a white, dense mycelium.

## Results, discussion and conclusion

***Experiment with Melocont-Pilzgerste®***

Figure 1 shows the mortality rates of all three carabid species and of *M. melolontha* third instar larvae. In the treated group both *Poecilus* species and the chockchafer larvae showed a mortality of 100%, wheras in the control groups a significantly reduced mortaly rate occurred (mortality of 37.5%, 39.5%, and 10% of the *P. cupreus*, *P. versicolor* and *M. melolontha*,

respectively). Characteristics of a *B. brongniartii*-infection were observed for <10% of the treated *Poecilus* larvae. In the case of *C. fossor* mortality was not significantly enhanced when Melocont-Pilzgerste® was applied (Fig. 1).

As indicated in Figure 2 both *Poecilus* species showed similar mortality curves, mortality beeing highest in $L_2$ and $L_3$. The mean duration of development from $L_1$ to adult stage (days (±SE)) was similar in both species (*P. cupreus* 75 (±0.89), *P. versicolor* 74 (±1.20)). Therefore, experiments 2 and 3 were carried out exclusively with *P. versicolor*. Due to a lack of *C. fossor* specimens no further experiments could be run with this species.

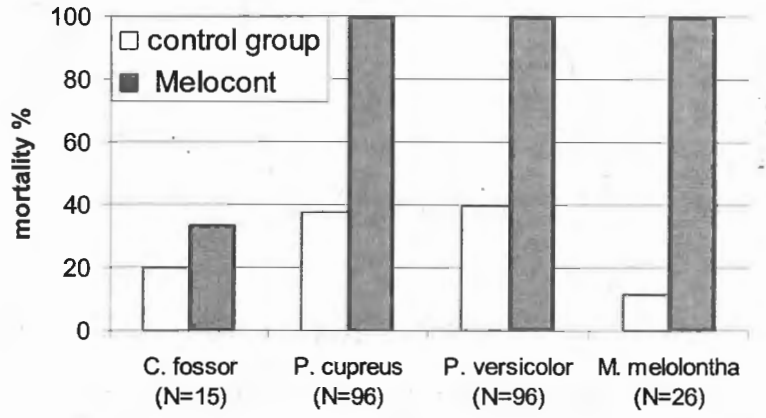


Figure 1. Mortality rates of the four beetle species treated with Melocont-Pilzgerste®. Significance of differences: *P. cupreus* $chi^2$=87.3, p<.001; *P. versicolor* $chi^2$=83.3, p<.001; *M. melolontha* $chi^2$=83.3, p<.001.

***Experiment with sterile barley***

The provision of sterilized barley significantly enhanced the mortality rate in the treated (85%) compared to the control group (2%; $chi^2$= 57.2, p<.001). Consequently, the reason for the high mortality in this and the experiment with M-PG was related to the addition of barley, which probably enhanced the growth of detrimental microorganisms. Negative effects of *B. brongniartii*, however, could have been masked by the addition of barley grain.

***Feeding experiment with* B. brongniartii *infested* M. melolontha *larvae***

The feeding of *B. brongniartii* infected pieces of *M. melolontha* larvae to third instar *P. versicolor* larvae had no negative effects on the larvae's development: mortality 7.7%/1.5%, developmental time [days (±SE)] 57.8 (±0.4)/57.7 (±0.5) and teneral's biomasses [ξ mg (±SE)] 46.5 (±0.61)/46.7 (±0.63) for treated/control group, respectively. This experiment was highly relevant for the field situation as *B. brongniartii* infected cadavers of *M. melolontha* may represent be the most important source of infection for endogaeic carabids under natural conditions.

***Conclusion***

Because of the low infection rates with *B. brongniartii* it is unlikely that the fungus is harmful to the tested carabid species under natural conditions as the fungal spore concentration in soil is much lower under field conditions. Furthermore, the infection via feeding and the contact

with infected cadavers of *M. melolontha,* respectively, might not be hazardous for *P. versicolor* larvae under natural conditions. However, negative effects of *B. brongniartii* could have been masked by the high mortality caused by the addition of barley grains. Therefore, further investigations are needed with spores of *B. brongniartii* to evaluate the exclusive effect of the fungus.

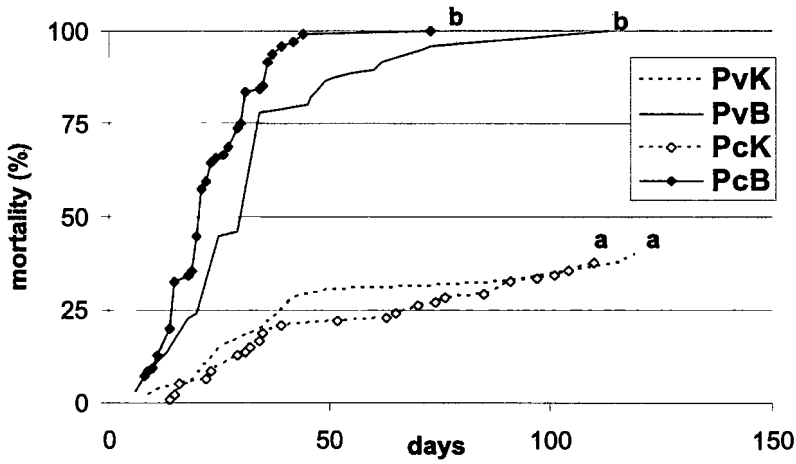


Figure 2. Mortality of *P. cupreus* (Pc) and *P. versicolor* (Pv) with (B) and without (K) Melocont-Pilzgerste®. Letters denote significant differences in survival rates (log-rank test $p<0.001$).

## Acknowledgements

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# Virulence of *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* on *Trialeurodes vaporariorum*

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**Abstract**: Infectivity of nine *Paecilomyces fumosoroseus* isolates and one *P. lilacinus* isolate was tested on *Trialeurodes vaporariorum*. Second instar nymphs on excised tomato leaflets were exposed to each isolate by applying 2mL spore suspension containing $10^7$ spore/mL. The tomato leaflets were incubated at 100%RH and 24°C for one week. Mortality was recorded on the 3rd and 6th day post-application. Five isolates of *P. fumosoroseus* caused significant mortality ranged from 29% to 44% after 3 days of incubation. All fungal isolates showed increasing infectivity as the incubation time was prolonged. At day 6 all *P. fumosoroseus* isolates caused highly significant mortality ranged from 62% to 98%. The most virulent isolates, 2658, 4408, 4411 and 4415, killed over 90% of the exposed second instars after 6 days of incubation. Several isolates of *P.fumosoroseus* showed potential as microbial control agents against *T.vaporariorum.*

**Key words**: *Paecilomyces fumosoroseus*, *Paecilomyces lilacinus*, Hyphomycetes, entomopathogenic fungus, *Trialeurodes vaporariorum*, Aleyrodidae

## Introduction

*Trialeurodes vaporariorum* Westwood (Aleyrodidae: Homoptera) is an important pest of various crops. Much of the crop loss in commercial glasshouses is due to the production of honeydew which encourages the growth of sooty moulds, such as *Cladosporium* and *Alternaria,* on the leaves. *T. vaporariorum* is also a vector of many plant viruses and some other pathogens. Different methods have been used for controlling *T. vaporariorum*. Physical control involves using insect screens, glasshouse ventilation, insect suction devices, insect glue, electromagnetic energy and photoperiodism (Berlinger & Lebiush-Mordechi, 1996). Chemical control is used extensively but it has been associated with resistance problems, ecological disturbances and high production costs (Horowitz & Ishaaya, 1996). In terms of biological control, predators such as species of Anthocoridae, Miridae, Chrysopidae, Coccinellidae, Diptera and Phystoseiidae have been observed attacking *T. vaporariorum* (Gerling, 1990). Whitefly parasitoids are found in the genera *Amitus*, *Azotus*, *Calse*, *Encarsia*, *Eretmocerus* and *Euderomphale* (Gerling, 1990). Fungi seem to be the only entomopathogens which could be used to control greenhouse white fly (GWF) because they are able to penetrate insects' protective coverings. Fransen (1990) and Lacey *et al.* (1996) reviewed the use of entomopathogenic fungi against whiteflies and found that the fungal pathogens of GWF included species allocated to the genera *Aschersonia, Beauveria, Verticillium* and *Paecilomyces*. After the outbreaks of *Bemisia* species in the USA and the Middle East intensive surveys of the natural enemies of whiteflies showed that *P. fumosoroseus* was the only fungus which can cause epizootics in field (Lacey *et al.*, 1996).

A selective control method for *T. vaporariorum* in addition to current control methods is desirable. *P. fumosoroseus* offers a perspective because of its unique characters; harmless to

mammals, birds and beneficial arthropods. In this study the use of *P. fumosoroseus* and *P. lilacinus* against *T. vaporariorum* on tomatoes grown in glasshouses was evaluated.

## Material and methods

A culture of *Trialeurodes vaporariorum* was obtained from the Close House Research Station. Plants with four to six partially expanded leaflets were used for producing the required stages of GWF at 24±1°C and a 16h light / 8h dark regime. About 40 adult GWFs were transferred into a mesh bag covering a tomato leaflet. After 24h of egg laying period the adult whiteflies were removed from the leaflet which was then incubated until the insects had reached the required development stage.

Fungal cultures (Table 1) were obtained from the U.S. Department of Agricultural Research Service, Entomopathogenic Fungal Culture Collection (ARSEF). They were grown on Sabouraud dextrose yeast medium at 24±1°C under a 16h light/8h dark regime. Conidia were harvested from 3 week-old cultures into sterile distilled water (SDW) containing 0.02% Tween 80. The density of suspension was then assessed using an improved Neubauer haemocytometer under x400 magnification and subsequently adjusted to the required concentration. Two millilitres of spore suspension, containing $1x10^7$ spores/mL, was applied to the underside of each leaflet using a Potter spray tower set at 517mmHg with a 0.7mm in diameter fine droplet spray nozzle. Four tomato leaflets were sprayed with each isolate and four tomato leaflets were used as control. Each control leaflet was treated with 2mL SDW containing 0.02% Tween 80. Each leaflet was then placed in a separate Petri dish and incubated at 24±1°C, at about 100% relative humidity and under a 16h light /8h dark regime for 7 days. The nymphs were examined individually and their conditions were recorded on the 3rd and the 6th day following the spore application.

The mortality data were normalised using arcsine transformation (Anscombe transformation) (Anscombe, 1948, cited by Zar, 1999). Transformed data at day 3 and day 6 were analysed separately using single-factor analysis of variance procedure (ANOVA) ($\alpha$=0.05) and Tukey test ($\alpha$=0.05) (Minitab, 1993).

Table 1. Details about the origin of the employed fungus cultures obtained from ARSEF

| Culture number | Fungal species | Geographical location | Insect host | Host plant |
|---|---|---|---|---|
| 2658 | *P. fumosoroseus* | Florida/USA | *T. vaporariorum* | Not specified |
| 3458 | *P. fumosoroseus* | Not specified | *Myzus persicae* | *Jasmine* |
| 4205 | *P. fumosoroseus* | Kuala Terla/Malaysia | *T. vaporariorum* | *Sonchus* |
| 4400 | *P. fumosoroseus* | Kuala Terla/Malaysia | *T. vaporariorum* | *Solanum melongena* |
| 4401 | *P. fumosoroseus* | Kuala Terla/Malaysia | *T. vaporariorum* | *S. melongana* |
| 4406, 4408, | *P. fumosoroseus* | Kuala Terla/Malaysia | *T. vaporariorum* | *Sonchus* |
| 4411, 4415 | *P. fumosoroseus* | Kuala Terla/Malaysia | *T. vaporariorum* | *Sonchus* |
| 3846 | *P. lilacinus* | Rampur/India | *T. vaporariorum* | *Sonchus* |

## Results and discussion

The method employed did not appear to be damaging GWF nymphs because very few of the control animals died during the 6d incubation period (Fig. 1). The experiment showed that all

*P. fumosoroseus* isolates tested were able to kill the nymphs. In addition, one culture of *P. lilacinus* was also shown to be pathogenic on GWF nymphs. The data obtained for several *Paecilomyces* isolates agree with the results of Vidal *et al.* (1997) and Wraight *et al.* (2000).

After 3 days of incubation, mortality varied significantly between the isolates from 11,7% (*P. lilacinus*) to 51,3% (*Pfr*4408) ($F=7,47$, $df=10,33$, $P<0.005$) (Fig. 1). Five isolates, 2658, 4400, 4408, 4411 and 4415, caused significant mortality relative to control. All isolates of *Paecilomyces* increased in their effectiveness as the incubation time was extended, which indicated a positive relationships between incubation time and pathogenicity of the isolates (Fig. 1). Similar findings were reported by Bolckmans *et al.* (1995) during 14d of incubation and by Vidal *et al.* (1997) during 10d of incubation period after *P. fumosoroseus* treatment to GWFs and *Bemisia tabaci* respectively. The mortalities produced by *P. fumosoroseus* isolates after 6d incubation were also significantly different from one to another and from the control ($F=34,80$, $df=10,33$, $P<0.005$). Only that of *P. lilacinus* was not significantly different from the control. *P. fumosoroseus* isolate 4400 caused 97,8% mortality while only 24,6% was killed by *P. lilacinus*. Over 90% of the initial GWF population were killed by isolates 4400, 4408, 4411 and 4415 after 6d of incubation. The results show that there are strong interspecific and intraspecific variations between fungal isolates tested. Similarly, previous studies have demonstrated such variations in entomopathogenic fungi (Hall, 1985; Ferron *et al.*, 1991; Hayden *et al.*, 1992).

While isolates 4415 and 4406 showed extensive sporulation on cadavers of GWF after 6d of incubation the remaining isolates sporulated only poorly. The visual changes on the insect caused by *P.lilacinus* were similar to those caused by isolates of *P. fumosoroseus,* but *P.lilacinus* appeared to sporulate better than some of *P. fumosoroseus* isolates following the death of GWF nymphs. All the isolates of *P. fumosoroseus*, except for 2658, showed a high degree of uniformity in terms of variation in effectiveness between replicate leaves (Fig. 1).

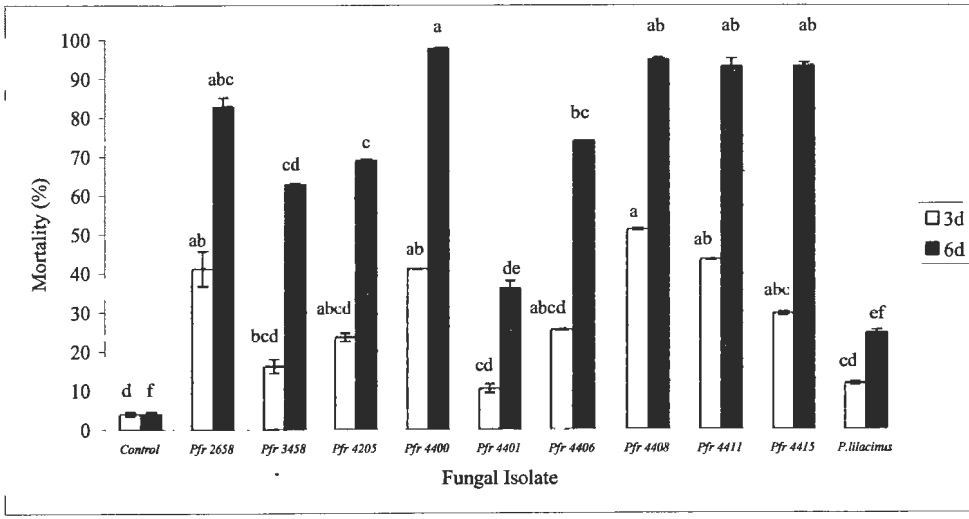


Figure 1. Mortality of second stage *Trialeurodes vaporariorum* nymphs caused by *Paecilomyces fumosoroseus* and *P. lilacinus* isolates as assessed after 3 days and 6 days at 24±1°C. Bars (mean±SEM) with different letters are significantly different from each other ($P<0.05$; Tukey test). See text for details.

The results of this study showed that all the tested isolates of *P. fumosoroseus* were able to kill whiteflies under the experimental conditions employed. The time taken to achieve a kill was often as little as 3d, but after 6d a large proportion of the population was usually affected.

## Acknowledgements

We thank Dr Colin H. Dickinson and Dr Brian J. Selman for their generous advice and useful discussions. *P. fumosoroseus* isolates were provided from the Agricultural Research Service Collection of Entomopathogenic Fungus Cultures (USA) by Dr Richard A. Humber.

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# Growth, persistence and virulence of strains of the entomopathogenic fungus *Beauveria brongniartii* in different pedoclimatic conditions of the Aosta Valley (Northwest Italy)

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**Abstract**: The aim of the present research was to select strains of the entomopathogenic fungus *Beauveria brongniartii* on the basis of growth and persitence capacity in the particular pedoclimatic conditions of the Aosta Valley, so to estimate their adaptability to the environment where there is the intention to carry out a project of microbiological control of *Melolontha melolontha* L. The virulence level towards *M. melolontha* manifested by the strains under study has been evaluated too. A conidial mixture of 5 strains (C2, F, K2, N3, W2) of *B. brongniartii* was spread in experimental fields, characterized by different pedoclimatic conditions, at Jovençan, Quart and Saint-Pierre. Soil growth and persistence of the 5 strains were periodically estimated in the months following the inoculation by microbic isolation from soil samples and genetical characterization by RAPD-PCR of the *B. brongniartii* strains obtained.

At the same time, the virulence level against *M. melolontha* of the strains studied was assayed: the presence of infected larvae was verified and the infection-responsible isolates were characterized and identified by RAPD-PCR.

On the grounds of the results obtained until now, in the peculiar pedoclimatic conditions of the Aosta Valley that do not favour the development of this fungus we conclude by affirming that strain F is likely to be the most suitable of the strains studied for the successful microbiological control of *M. Melolontha*.

**Key words**: *Beauveria brongniartii*, *Melolontha melolontha*, growth, persistence, virulence

## Introduction

In the last few years our Department has undertaken various studies (Ozino, 1995; Ozino *et al.*, 1998; Piatti *et al.*, 1998) to select strains of the entomopathogenic fungus *Beauveria brongniartii* suitable for succesful microbiological control of *Melolontha melolontha* in the particular pedoclimatic conditions of the Aosta Valley (Northwest Italy) where the cockchafer causes heavy damages to orchards and meadows.

The natural presence of *B. brongniartii* in this region is very low; the pedoclimatic conditions of the Aosta Valley do not favour the development of this fungus because of the relatively low average precipitation (Bondaz *et al.*, 1991; Cravanzola *et al.*, 1995). Therefore, it became very important to select strains of *B. brongniartii* able to adapt theirselves to the ground where they have to act.

The aim of the present research was exactly to select strains of *B. brongniartii* on the basis of the virulence level showed towards *M. melolontha* larvae present in the soil and considering the growth and persistence capacity in areas of Aosta Valley characterized by different pedoclimatic conditions.

## Materials and methods

Six orchards at Jovençan with a surface area of 625 to 1700 $m^2$ each and an infestation level of 7 to 61 larvae/$m^2$ were chosen for the research.

In May 2000 a conidial mixture of 5 strains of the entomopathogenic fungus *B. brongniartii*, previously grown on rye kernels, was spread in the experimental fields, with a total conidial load of 1.0 x $10^9$ conidia/$m^2$ (1.0 x $10^5$ conidia/g d.w.), with 2.0 x $10^8$ conidia/$m^2$ from each strain.

The 5 strains (C2, F, K2, N3, W2) were chosen for their high virulence level, obtained in laboratory infection assays, among 58 strains isolated from infected larvae of *M. melolontha* in the Aosta Valley (Piatti *et al.*, 1998).

The inoculation of the conidial mixture was preceded by an estimate of any *B. brongniartii* naturally present in the 6 experimental fields, by the classical methods of isolation of microorganisms from soil samples.

After inoculation, growth and persistence of the 5 strains in the fields were evaluated for two years and the virulence level against *M. melolontha* larvae in the soil after 2 years.

Microbic isolation from soil samples and from infected larvae, collected in the experimental fields, was performed on Sabouraud Dextrose Agar medium selective by chloramphenicol and actidione (Veen & Ferron, 1966). The *B. brongniartii* colonies obtained were submitted to DNA extraction as described in the Rogers & Bendich protocol (1988), modified by Henrion *et al.* (1994).

Extracted DNA was submitted to genetic characterization with RAPD-PCR analysis as previously described (Piatti *et al.*, 1998; Dolci *et al.*, 2002) in order to determine suitable molecular markers to identify and track, after inoculation in the ground, the 5 strains used.

Thermal cycling was performed as previously described (Cravanzola *et al.* 1997) in a Perkin-Elmer Cetus thermal cycler (model GeneAmp PCR System 2400).

Amplification products were separated by electrophoresis in 1,2% agarose gels using TAE buffer and visualized with ethidium bromide.

The research carried on with the aim of verifying the adaptation of the 5 strains in the soil of pedoclimatic zones of the Aosta Valley different from that of Jovençan. At Saint-Pierre and Quart, 2 orchards were chosen and another field, as reference, at Jovençan, with an infestation level of 5 to 40 larvae/$m^2$. The same protocol was used.

The conidial mixture of the 5 strains was spread in June 2002 and growth, persistence and virulence were valued in the months following the inoculation (July, September, October and November 2002).

## Results and discussion

The fungus was not isolated from the experimental fields which had been checked for the natural presence of any *B. brongniartii* before inoculation.

The results obtained in the 6 experimental fields at Jovençan regarding growth and persistence of the 5 strains of *B. brongniartii* considered, are shown in Figure 1.

Strains F, C2, W2 had a quite constant progress during the period considered; in particular, strain F showed the highest values of conidial load and at 2 years from inoculation it has a good persistence.

On the contrary, strain K2 showed a considerable difficulty in soil adaptation in the first months after inoculation while strain N3 was not able to develop in the fields considered.

With regard to virulence a general decrease of 12% to 42% of cockchafer infestation level was detected in the 6 fields; among the isolates obtained from 50 larvae collected and

infected by *B. brongniartii*, 62% were strain F, 31% strain C2 and 7% strain W2; strains K2 and N3 were never isolated.

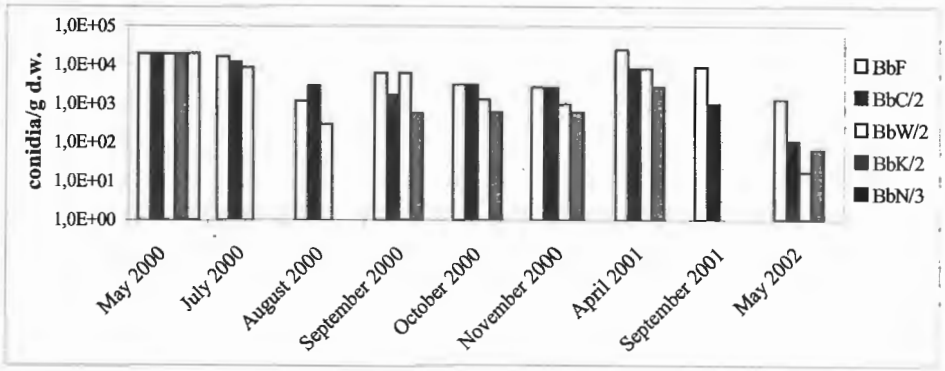


Figure 1. Conidial load (conidia/g d.w.) of the 5 *Beauveria brongniartii* strains present in the 6 fields taken as a whole in the period May 2000-May 2002.

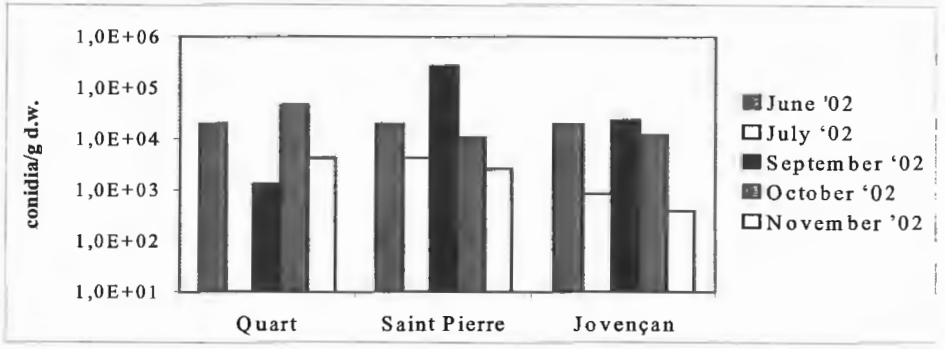


Figure 2. Conidial load (conidia/g d.w.) of *Beauveria brongniartii* present in the 3 fields in the period June 2002-November 2002.

On the basis of the results obtained at Jovençan we can affirme that strain F had the best soil adaptation in the area considered; strains C2 and W2 showed a fairly good adaptation too although their behaviour was more modest and less costant than that of strain F. Strain F also manifested the highest virulence level among the 5 strains.

The preliminary data obtained in the 3 orchards at Quart, Saint Pierre and Jovençan in the first months post-inoculation confirme the results obtained in the previous research. *B. brongniartii* has been able to adapt and grow in the soil, in fact it has been isolated in each field with a fairly good total conidial load (Fig. 2).

In particular, strain F has been isolated from the soil of the 3 fields considered with the highest values of conidial load and frequency (65% at Quart, 61% at Saint Pierre and 67% at Jovençan). The behaviour of the other strains was quite similar to that noticed in the previous studies.

With regard to virulence, a decrease of cockchafer infestation level of 73%, 20% and 45% has been detected respectively in the fields of Quart, Saint Pierre and Jovençan.

It must be pointed out that among the larvae infected by *B. brongniartii* found in the soil, and according to the RAPD-PCR charcterization, strain F isolates again prevale over the others.

On the basis of the results obtained from the 2 year research at Jovençan and considering the preliminary dates obtained in the new areas, we can affirme that strain F is likely to be the most suitable of the strains studied for the successful microbiological control of *M. melolontha* in the Aosta Valley.

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# Biocontrol of *Melolontha melolontha*: The fate of a *Beauveria brongniartii* biocontrol strain in the field

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**Abstract**: The filamentous soil fungus *Beauveria brongniartii* is used as a biocontrol agent (BCA) to control the soil dwelling larvae of the European cockchafer (*Melolontha melolontha*). The goal of this study is to investigate persistence and competitiveness of a *B. brongniartii* strain applied to the field and to pursue its behavior in a *B. brongniartii*-free environment as well as in an environment that contained indigenous *B. brongniartii* populations.

In fall 1999 we initiated two field trials by applying the *B. brongniartii* BCA at *M. melolontha* infested areas at Jenaz (eastern Switzerland) and Matten (central Switzerland) to provide the experimental base for our studies. The trial site at Jenaz contained a diverse indigenous population of *B. brongniartii* whereas the trial site at Matten was free of *B. brongniartii* at the start of the experiment. Furthermore, we have developed microsatellite markers as a genotyping tool to monitor the genetic diversity of *B. brongniartii* populations and to identify applied *B. brongniartii* BCA strains within indigenous *B. brongniartii* populations.

The BCA strain was re-isolated in parts of the treated area at Jenaz during the first year of the trial which confirmed its presence in the indigenous population. Seven to twelve percent of the isolates analyzed displayed the genotype of the BCA strain. At Matten the BCA strain established in the *B. brongniartii* free environment. The population density steadily increased during the first year of the trial and reached the same order of magnitude as the natural *B. brongniartii* density found at Jenaz. The density decreased by a factor of ten during the third year. One month after application the BCA strain was detected at low densities in adjacent untreated control plots at Matten, demonstrating the potential for short distance dissemination of the BCA.

**Key words**: BCA, monitoring, persistence, dissemination, genotypes

## Introduction

The fungus *Beauveria brongniartii* (Sacc.) Petch (Deuteromycota: Hyphomycetes) is a well established biocontrol agent (BCA) to control the larvae of the European cockchafer (*Melolontha melolontha)* in grasslands and orchards (Keller, 1992; Zelger, 1996). A product (Beauveria-Schweizer®, E. Schweizer Seeds Ltd, Switzerland) based on fungus colonized barley kernels (FCBK) is registered and commercially available in Switzerland since 1991. The FCBK product is applied directly into the soils of *M. melolontha* infested sites by use of an adapted seed drilling machine.

Establishment of specific detection methods is an important requirement in the process of developing a new BCA (Bidochka, 2001). Such methods are necessary to monitor efficacy and host specificity of a particular biocontrol strain or organism, and they allow quality control during production of the BCA. We recently have isolated microsatellite markers in *B. brongniartii* to provide a fast and reliable method for strain characterization and identification (Enkerli *et al.*, 2001). With the development of this detection tool it has now become possible to investigate the behavior of the BCA in the environment (Enkerli *et al.*, 2004) and its possible impact on soil ecology, e.g. persistence of a BCA strain in the soil, its dissemination

upon application or potential interactions between the BCA and indigenous populations of the same species or other microorganisms. Here we report on the initiation of two field trials in Switzerland that will provide the experimental base to address these questions in detail. We present data obtained during the first three years of these field trials.

## Materials and methods

In fall 1999 field trials were established at two different locations in Switzerland, one at Jenaz (eastern Switzerland) and one at Matten (central Switzerland) (Kessler *et al.*, 2004). The *M. melolontha* density at both locations was between 50 and 100 larvae per $m^2$ at the start of the field trials. At each site three different *B. brongniartii* application procedures were performed: application in spring (2000), fall (1999), and fall (1999) & spring (2000). Both test sites were divided into 16 adjacent plots (plot arrangement 4 x 4) of 20 x 20 m. Each application procedure was repeated in four plots. Four untreated plots served as control plots. Treatments consisted of applications of forty to fifty kilograms per hectare of the FCBK product (Beauveria-Schweizer, E. Schweizer Seeds Ltd., Thun, Switzerland). At the start of the field trials the test site at Matten was free of *B. brongniartii* whereas at Jenaz there was an indigenous *B. brongniartii* population present at a density of $1x10^3$ to $7x10^3$ colony forming units (CFU) per g dry soil.

Soil samples were obtained from a depth of 5 to 20 cm using a cylindrical soil sampler with an inner diameter of 5.5 cm. For each sampling period 10 samples per plot were collected. The selective medium (SM) method (Kessler *et al.*, 2003; Strasser *et al.*, 1996) and the *Galleria* bait method (GBM) (Zimmermann, 1986; Kessler *et al.*, 2003) were used to determine *B. brongniartii* density in the soil and to collect isolates. For the SM method equal amounts of the 10 soil samples per plot were mixed and CFU per g dry soil determined on SM plates (5 replications per soil mix). Average *B. brong*niartii densities were calculated from densities of the 4 plots per application procedure. The GBM was applied to each soil sample. A culture collection was established containing isolates obtained with the selective medium method (maximum 1 isolate per SM plate) and the GBM (maximum 1 isolate per soil sample) from different plots and time points from both trial sites up to spring 2001. Obtained *B. brongniartii* isolates were single spore isolated and maintained on SM.

Genotypes of isolates were determined by analyzing 6 microsatellite loci. DNA isolation as well as amplification and analysis of the microsatellite markers were performed as described by Enkerli *et al.* (2001).

## Results and discussion

The average *B. brongniartii* density at the trial site in Jenaz was in the same range ($1x10^3$ to $8x10^3$ CFU/g dry weight) for all application procedures during the time monitored (Fig. 1). Densities were not different from the natural *B. brongniartii* densities detected before application of the FCBK. Non of the application procedures resulted in a density increase as compared to the untreated control plots.

At Matten there was no indigenous *B. brongniartii* population prior to the application of the FCBKs (Fig. 2). The *B. brongniartii* density in the control plots remained at zero during the time monitored. In contrast, the *B. brongniartii* density increased in the plots of all application procedures after application of the FCBK. The maximum density was reached with all application procedures in April 01 at a comparable level of $1x10^3$ to $3x10^3$ CFU/g dry soil. The fastest density increase was detected for the fall & spring application procedure. *B. brongniartii* densities decreased in all treated plots by a factor of ten within the third year of the trial. The maximal densities detected at the trial site Matten reached the same order of magnitude as the density of the natural population detected at Jenaz.

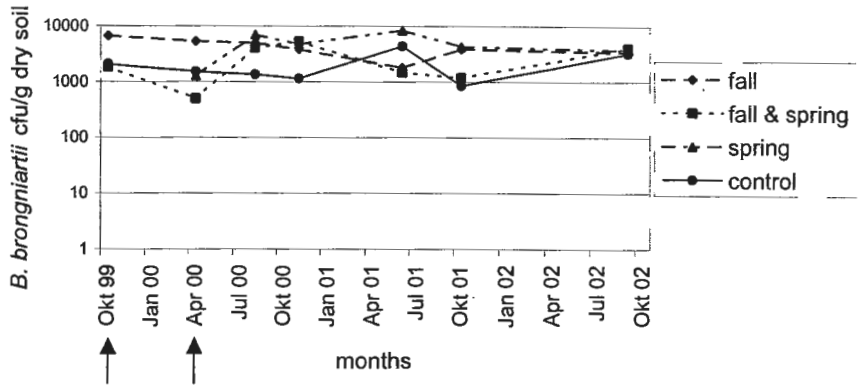


Figure 1. Average *B. brongniartii* density in the plots of the 3 application procedures and the control plots at trial site Jenaz. Application time points are indicated with arrows.

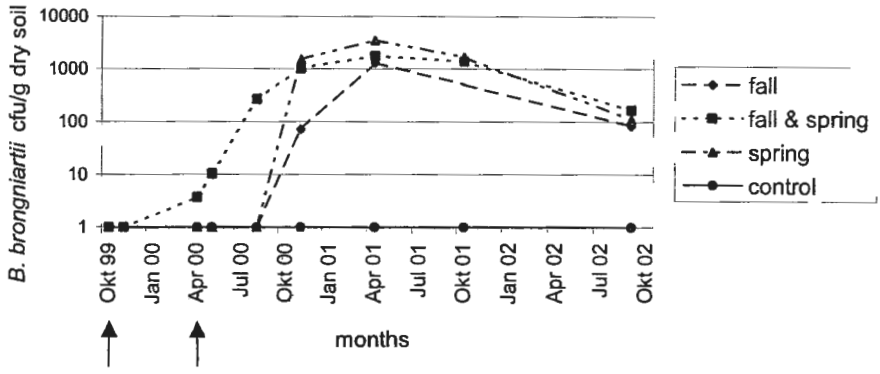


Figure 2. Average *B. brongniartii* density in the plots of the 3 application procedures and the control plots at trial site Matten. Application time points are indicated with arrows.

For the trial site Jenaz, the genotypes of a total of 153 isolates that were collected up to spring 01 and originated from either the spring application procedure (94) or the control (59) were investigated. One month after application non of the investigated isolates from the spring application displayed a genotype identical to the genotype of the applied strain. Three month and 1 year after application the genotype of 7.3% and 12.5% of the isolates were identical to the genotype of the applied strain. These isolates originated from only 2 of the 4 plots treated in spring 00. All isolates originating from the untreated control plots displayed a genotype different from the genotype of the applied BCA strain. Monitoring of the density and presence of genotypes will continue during the next five years to investigate whether the BCA strain will establish within the indigenous *B. brongniartii* population and disseminate into adjacent untreated control plots. This field trial should also allow to determine whether application of a single BCA strain has effects on the indigenous population structure.

At trial site Matten, thirty-two isolates were collected from plots treated with either one of the 3 application procedures up to spring 01. Genotype analysis of these isolates revealed that they all were identical to the genotype of the applied BCA strain. This finding confirmed

that the fungal densities detected in the treated plots represent the applied BCA strain. Three months after application 3 isolates with a genotype identical to the BCA strain were detected in 2 out of the 4 untreated control plots. The isolates were obtained with the GBM, whereas with the SM method no *B. brongniartii* isolates were detected in the control plots, suggesting a higher sensitivity of the GBM. A similar observation has been reported by Kessler et al. (submitted). The finding that the applied BCA strain could be identified in 2 control plots 3 months after application of this strain in adjacent plots suggested short distance dissemination of the fungus. Various mechanisms could be responsible for short distance dissemination: e.g. mycelial growth, transport by water, movement of infected larvae, or other soil dwelling organisms such as earth worms that have been in contact with *B. brongniartii* conidia (Hozzank *et al.*, 2003). Monitoring of fungal densities and genotypes at trial site Matten will continue to confirm our findings and provide further information on the short distance dissemination potential of this fungus.

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# A GLP/GEP based field study on *Beauveria brongniartii* with respect to Commission Directive 2001/36/EC

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**Abstract**: Entomopathogenic fungi have significant potential for the development of biological control agents (BCAs) against subterranean insect pests. However, a responsible risk-assessment of fungal biological control agents is necessary from an ecological perspective. This should include investigations on the environmental persistence of BCAs, the study of the significance of secreted major metabolites, and their possible hazards to human and animal health. These data are required for a successful registration in the European Union.

*Beauveria brongniartii* (Ascomycota, anamorph of Clavicipitales) is already used against *Melolontha melolontha* larvae (Coleoptera, Scarabaeidae) in Austria, Italy, Switzerland and France. Due to the experiences gained in these countries for almost 20 years *B. brongniartii* is an ideal model organism for the evaluation of existing guidelines and the setting up of standard procedures. This will help to generate data relevant for risk assessment and registration.

In the context of the EU-RTD-project RAFBCA (QLK1-2001-01391) an extensive three year field trial to study potential phytotoxic effects of *Beauveria brongniartii* has been started on the facilities of the GLP/GEP-certified ATC-Agro Trial Center GmbH in Austria. For the trial layout the guidelines of the EPPO, the UN FAO, the US EPA, and the European Union were screened and applied as required in the implementation of EU-commission directive 91/414/EEC.

**Key words**: *Beauveria brongniartii*, risk assessment, field trials, GLP, full notification

## Introduction

Much has been investigated in the field of mycotoxins produced on feed and fodder (Sinha & Bhatnagar, 1998). However ecological studies on the formation, significance and fate of secondary metabolites produced by fungi in the environment are scarce. There is strong evidence that fungi produce antibiotics under natural conditions in the soil, fulfilling a function in antagonism and in sporogenesis (Dasu & Panda, 1999; Demain, 1980; Gottlieb, 1976). Moreover, recent research has been focused on the environmental input and potential hazard of antibiotics, which reach the soil environment due to their use in human medicine and animal husbandry (Schlenker & Muller, 2001; Tolls, 2001).

In order to perform a responsible risk assessment of fungal biological pest control agents investigations on the environmental enrichment and the significance of secondary metabolites released by entomopathogenic fungi are needed. Therefore, it is the aim of this study to obtain information on the temporal-spatial distribution of the metabolites in the final product as well as in the applied environment. Field trials are conducted at the Agro Trial Center in Gerhaus (Lower Austria) to gather data for the risk assessment of *B. brongniartii* and its relevant metabolite oosporein. Studies are performed according to GLP and GEP guidelines. Both GLP, GEP qualifications are given to Kwizda – AGRO Division.

## Material and methods

***In-situ effects of Melocont®-Pilzgerste on crops and fruits***

A field trial was set up compliant with EPPO standards, OECD, EPA and FAO guidelines, as far as these protocols seemed to be applicable. A full randomised block design (EPPO, 1999) with 4 treatments and one control was used. This randomised block design layout was repeated in 4 trial segments in order to grow four cultures at the same time.

The four treatments include one reference insecticide (Agritox®, Kwizda GmbH) and three applications of Melocont®-Pilzgerste, i.e. the recommended rate of 50 kg/ha, the double (100 kg/ha) and the fivefold (250 kg/ha) of the recommended rate, to a depth of 8-10 cm. The control was applied with sterile barley. Two of the four trial segments were re-applied with the same treatment one year after the 1st treatment to simulate a repeated application.

The trial is laid out for three seasons with crop rotations and was initiated in April 2002. Cultures grown in the study are potatoes, sugar beet, maize, strawberries, winter barley, field bean, winter wheat, chinese cabbage, lettuce, winter canola, carrots, spinach and pumpkins. Samples for residue analysis are taken to detect and quantify relevant metabolites if they enter the food chain. Samples remain frozen until the validation of methods has been completed. Growth and harvest of the cultures are assessed following the recommendations stated in the EPPO standards (EPPO, 1999) to detect potential phytotoxic effects of the BCA.

***Isolation of* Beauveria brongniartii *from soil***

Prior to application of Melocont®-Pilzgerste (May 2002) soil samples were taken to determine pre-treatment levels of *B. brongniartii* in the soil. Post-application samples were taken in September 2002 and April 2003. Further sampling is scheduled for fall 2003 and spring 2004.

Soil samples were taken in 2 layers to a depth of 30 cm by using a sampling auger. Soil samples from each layer were mixed, air-dried, and sieved through a 2 mm sieve. Ten gram sub-samples from each depth (three replicates) were suspended in 40 mL 0.1 % (w/v) Tween80, shaken at 150 rpm for 30 min, and then treated in an ultrasonic bath for 30 s. *Beauveria*-selective agar plates (Strasser *et al.*, 1996) inoculated with 50 µL of these soil suspensions and dilutions thereof, respectively, were incubated for 14 days at 25 °C and 60 % RH (four replicates per sub sample). Colonies of *B. brongniartii* are given as cfu per gram soil dry weight.

## Results and discussion

***Isolation of* Beauveria brongniartii *from soil***

No relevant abundance of *Beauveria* was detected prior to the application of Melocont®. Results of re-isolation of *Beauveria brongniartii* after application are shown in Figure 1.

A significant statistical correlation (Spearman correlation coefficient 0.672, $p<0.001$) between applied amount of Melocont® and observed *Beauveria* densities is observable in the pooled data. Some of the control plots applied with Agritox® or sterile barley kernels also showed low numbers of *Beauveria* in the second year of the study. This effect does not occur in the strawberry fields, the only trial segment not treated with machinery after the application of Melocont®. Therefore a likely explanation for this effect is the transfer of inoculum to control plots by agricultural equipment.

The *Beauveria* density remained stable or increased slightly during the first winter after application. A distinct tendency to an increasing *Beauveria* density is observable especially in the deeper soil layer, suggesting a transfer from top soil to deeper layers by percolation and activity of soil fauna (e.g. earthworms) over time.

The fact that *Beauveria brongniartii*-concentrations in the soil are high (above the recommended effective amount of $3.5 \times 10^3$ cfu $g^{-1}$ dry wt) and stable is of significant value for the interpretation of harvest data.

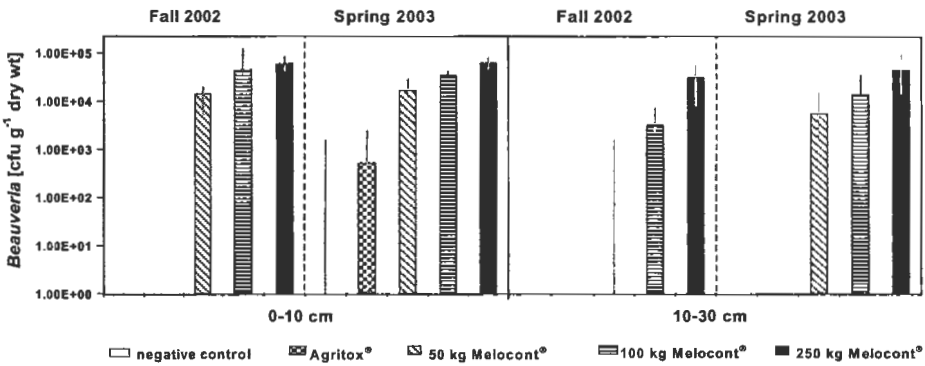


Figure 1. Summary of data obtained during re-isolation of *B. brongniartii.* Data were pooled over the 4 different cultures for this graph. Each bar represents 64 soil cores. Median values shown, error bars give $Q_{25}$ and $Q_{75}$, respectively.

***Phytotoxicity assessment***

With respect to the assessment criteria neither culture showed any signs of a negative or positive reaction that could be related to the application of the biocontrol agent or the reference insecticide. No phytotoxic effects were observed. Oneway ANOVA analysis of yields from the single plots revealed no significant differences in any of the cultures and harvest fractions neither in the first nor in the second season (Figs 2 & 3) . A second application of Melocont® to two of the trial segments also didn't produce any effects in terms of phytotoxic reactions.

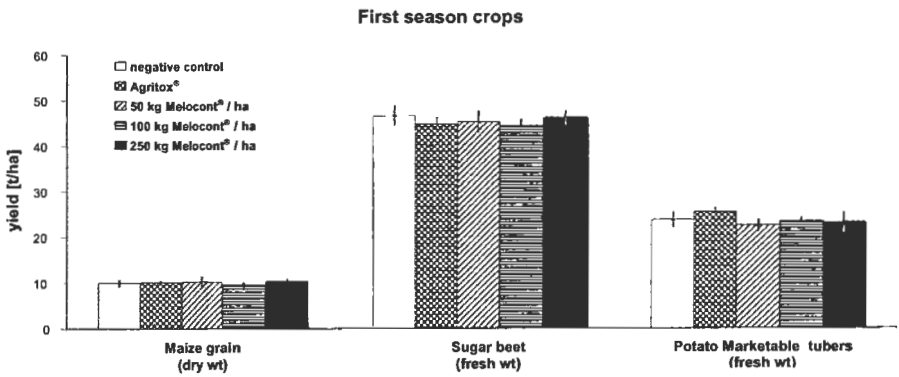


Figure 2. Overview of harvest data from the first field season after the application of Melocont®-Pilzgerste. Each bar represents the mean value of all replicates (n=4). Error bars show standard deviations.

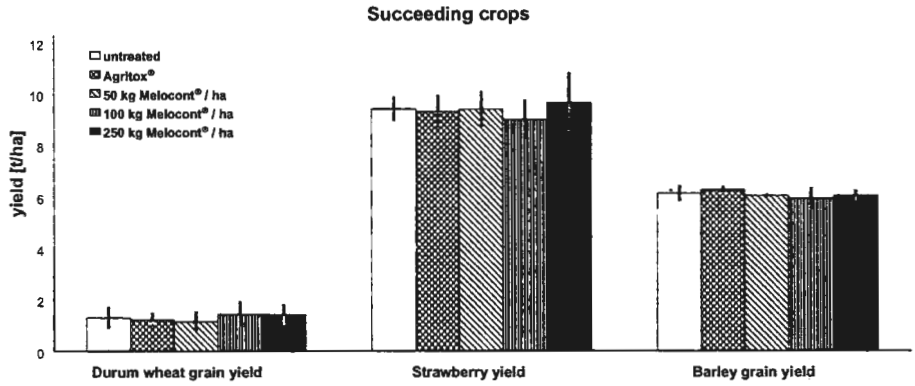


Figure 3. Yield data from succeeding crops approx. one year after the first application of Melocont®-Pilzgerste. Mean values shown (n=4). Error bars indicate standard deviations.

Also, the assessment of harvest quality (e.g. starch content in potato, sugar content in beet, thousand seed weight of grain) did not reveal any differences between treated plots and controls. Recapitulating the results of two full crop rotations in four fields it is stated that the soil application of *Beauveria brongniartii* had no effects on the grown cultures. More crop rotations should give further confirmation of the safety of the BCA in this respect.

It is expected that these field trials will provide important data for the registration of *B. brongniartii* in EU member states. They will help establish standard protocols for assessment of fungal BCAs and experience gathered in these studies will help review existing guidelines for risk assessment of fungal biocontrol agents with respect to their applicability and relevance.

## Acknowledgements

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# Entomopathogenic bacteria

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# Selection of resistant European Corn Borer (*Ostrinia nubilalis*) to *Bt*-corn and preliminary studies for the biochemical characterization

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**Abstract:** One possibility to control the European Corn Borer is the cultivation of *Bt*-corn. However, this can result in the development of resistant pest populations. To control this, larvae were collected in *Bt*-corn fields and in $F_2$ generation tested for resistance to the respective *Bt*-toxin. As preliminary studies for the biochemical characterization of possible mechanisms for resistance, studies on proteinase activities were conducted in the midgut of 5$^{th}$ instars of a susceptible German ECB population. The digestive proteinases trypsin, chymotrypsin, elastase and aminopeptidase could be identified.

**Key words**: European Corn Borer, *Ostrinia nubilalis*, *Bt*-corn, resistance, midgut, proteinases, trypsin, chymotrypsin, elastase, aminopeptidase

## Introduction

The European Corn Borer (ECB, *Ostrinia nubilalis*) is one of the economical most important pests in corn (*Zea mays* L.) in the USA and Europe. As an effective control technology, transgenic corn expressing a truncated Cry1Ab toxin from *Bacillus thuringiensis* has been developed. The resulting *Bt*-corn produces its own protective pesticide, which is highly insecticidal to ECB larvae. However, widespread cultivation of *Bt*-corn could result in the development of resistant pest populations.

In general, the potential of insect resistance to toxins of *Bacillus thuringiensis* can be located at any step of the toxic pathway: ingestion, pH-dependent solubilization, proteolytic processing; binding to specific receptors, membrane integration; pore formation; cell lysis and finally insect death (Ferré & van Rie, 2002).

In other pest-*Bt*-toxin-systems, two main mechanisms of resistance to *Bt*-toxins, one proteinase-mediated and the other receptor-mediated, have been identified (Oppert *et al.*, 1997, McGaughey & Oppert, 1998). Proteinase-mediated resistance mechanisms includes alterations in the midgut enzymatic activity such as degradation and inactivation of the toxin. Receptor-mediated resistance mechanisms includes resistance to *Bt*-toxins correlated with alterated toxin binding to midgut receptors. A change in physiological parameters e.g. pH can influence resistance as well.

## Materials and methods

Photometrical studies with pure midgut sap of 5$^{th}$ instars larvae were conducted using chromogenic substrates and specific inhibitors [N-benzoyl-L-arg-p-nitroanilide (BApNA) and soybean-trypsin-inhibitor (SBTI) for trypsin-like proteinases, N-succinyl-ala-ala-phe-p-nitroanilide (SAAFpNA) and N-tosyl-L-phe chloromethylketone (TPCK) for chymotrypsin-

like proteinases, N-succinyl-ala-ala-pro-leu-p-nitroanilide (SAAPLpNA) and elastatinal for elastase-like proteinases as well as leu-p-nitroanilide (LpNA) and bestatin for amino-peptidase.

## Results and discussion

***Screening for resistance***

The cultivation of *Bt*-corn could result in the development of resistant ECB pest populations. To control this, in 2001 70 larvae have been collected in about 150,000 *Bt*-corn plants (Event MON810). Biotests with 1st instars larvae in $F_2$ generation revealed no resistant individuals yet. The 805 larvae of 760,000 *Bt*-corn plants collected in 2002 will be tested in summer 2003.

***Preliminary studies for the biochemical characterization***

To establish preliminary reference systems for the characterization of potential available resistant individuals, studies on proteinase activities and on receptor binding in the midgut of susceptible 5th instars larvae were carried out.

***Studies on proteinase activities***

Houseman & Chin (1995) identified the digestive proteinases trypsin, chymotrypsin, elastase and aminopeptidase in the midgut of a Canadian population of *Ostrinia nubilalis*. To compare these results with German ECB and to establish a reference system for the identification of qualitative and quantitative changes in proteinase-activities (e.g. amount, molecular weight, substrate specifity) of resistant larvae, photometrical studies were carried out.

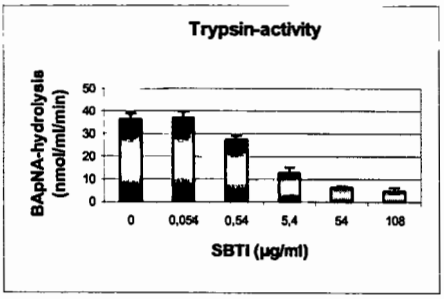


Figure 1. Trypsin-activity in midgut sap of susceptible 5th instar larvae.

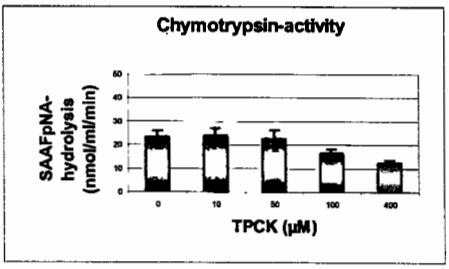


Figure 2. Chymotrypsin-activity in midgut sap of susceptible 5th instar larvae.

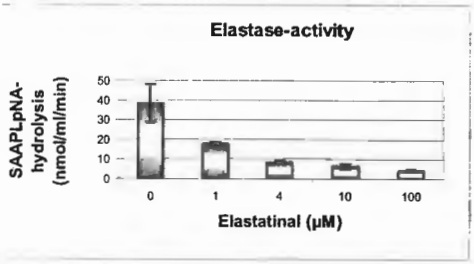


Figure 3. Elastase-activity in midgut sap of susceptible 5th instar larvae.

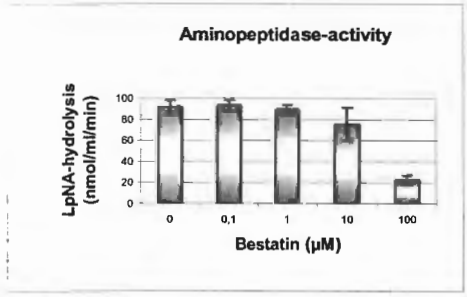


Figure 4. Aminopeptidase-activity in midgut sap of susceptible 5th instar larvae.

Similar to the above described results, in the midgut lumen of German susceptible 5th instar larvae the digestive proteinases trypsin (Fig. 1), chymotrypsin (Fig. 2), elastase (Fig. 3) and aminopeptidase (Fig. 4) could be identified. Besides, it is to point out, that the activity of the aminopeptidase was about twice higher compared to the other proteinases.

***Studies on receptor binding***

For binding analyses of the midgut receptors, intact brush border membrane vesicles (BBMV) have been isolated (Wolfersberger *et al.*, 1987). To proof the toxin binding and to characterize the rezeptor, ligand-blot was carried out. Binding was demonstrated using biotin labeled toxin that was detected with streptavidin-horseradish-peroxidase-conjugat and the ECL western-blotting system.

***Studies on pH-value***

The pH measurements were conducted in pure larval midgut sap of ECB 5th instar larvae. In larvae fed on corn leaves before sample preparation, a pH of 7.5 was measured, in hungry ones it was pH 7.2, and in those reared on artificial diet pH 7.3.

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# Insect resistance to *Bacillus thuringiensis*

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**Abstract:** *Bacillus thuringiensis* genes coding for insecticidal proteins (*cry* genes or *Bt* genes) have been transferred to agronomically important crops (such as corn and cotton) and this has conferred to these crops (*Bt*-crops) resistance to their most important insect pests. Although no case of field resistance to *Bt*-crops has been reported so far, the potential of insects to evolve resistance to insecticides is well known, and it is considered to be one of the threats posed to genetically modified insecticidal plants. Many insect pests have evolved, under laboratory conditions, resistance to *B. thuringiensis* formulated products. However, the diamondback moth (*Plutella xylostella*) is the only insect species that has evolved resistance to *B. thuringiensis* in the field, and this has even occurred several times independently. We have been working for over 10 years with the diamondback moth as a model system to test the potential of insects to become resistant to Cry toxins and we have paid especial interest in the mechanisms conferring resistance to multiple toxins.

**Key words**: Cry proteins, *Plutella xylostella*, diamondback moth, binding sites, mechanism of resistance.

## Introduction

The bacterium *Bacillus thuringiensis* synthesizes insecticidal proteins that aggregate to form parasporal crystals (Schnepf *et al.*, 1998). After ingestion, these proteins (Cry proteins) dissolve and are activated in the larval midgut. A key step in their mode of action is binding to specific sites in the brush border membrane of the larval midgut, which is followed by pore formation, cell lysis, and death. Because Cry proteins kill certain pests, yet have limited effects on most non-target species, sprays of *B. thuringiensis* have been used to control pests for decades. More recently, some *cry* genes have been transferred to agronomically important crops, and this has conferred to these crops (*Bt*-crops) resistance to their most important insect pests. In 2002, around 9.9 million hectares were planted to *Bt*-corn and 4.6 million hectares to *Bt*-cotton (James, 2002).

The main threat to the long-term success of this approach is evolution of resistance by pests. Although no case of evolution of insect resistance to Bt crops in the field has not been reported, strains of more than a dozen species of insects have evolved resistance to *B. thuringiensis* toxins in laboratory selection experiments (Ferré & Van Rie, 2002). So far, the diamondback moth (*Plutella xylostella*) is the only insect species that has evolved resistance to *B. thuringiensis* commercial formulations in the field, and this has even occurred several times independently.

The diamondback moth provides us with the unique opportunity to study mechanisms of resistance that have been selected in the field, in opposition to those studies with populations that have been obtained by selection in the laboratory. Our group has been working for over 10 years with the diamondback moth as a model system to test the potential of insects to become resistant to *Bt*-toxins. Besides the interest to determine the contribution of the alteration of binding sites in resistance, we have paid especial attention to the mechanisms conferring resistance to multiple toxins.

## Materials and methods

***Description of resistant strains***

In collaboration with other research groups, we have studied 8 populations of diamondback moth that had evolved resistance in the field. Two of these populations were from the Philippines (BL and PHI), two from Hawaii (NO-QA and NO-95C), two from Malaysia (SERD3 and 1AcSEL-MEL), and two from eastern United States (PEN form Pennsylvania, and Cry1C-Sel from South Carolina) (Ferré & Van Rie, 2002).

The two colonies from the Philippines were collected from the same area but separated three years apart. Both were originally highly resistant to Cry1Ab, but not to Dipel® (a formulation based on *B. thuringiensis* var. *kurstaki*) or to any other toxin tested, including the structurally related Cry1Aa and Cry1Ac, and Cry1F and Cry1J. NO-QA and PEN had the same resistant phenotype, both had been selected with Cry1A toxins and developed resistance to these toxins and cross-resistance to Cry1F and Cry1J. SERD3 was only tested with commercial products and was resistant to Dipel® and Florbac® (a formulation based on *B. thuringiensis* var. *aizawai*). 1Ac-SEL-MEL was selected with Cry1Ac in the laboratory and attained high levels of resistance to this toxin and also high levels of cross-resistance to Cry1Ab.

The NO-95C and Cry1C-Sel strains were selected with Cry1C and became resistant to this toxin. In both cases cross-resistance to Cry1A toxins was originally present.

***Binding studies***

Binding studies were performed using $^{125}$I-labeled *Bt*- toxins and brush-border membrane vesicles (BBMV) as described elsewhere (Herrero *et al.*, 2001). By competing the labeled toxin with the same unlabeled toxin (homologous competition) for binding to the BBMV, the binding affinity ($K_d$) and the concentration of binding sites ($R_t$) can be calculated. By competing the labeled toxin with different unlabeled toxins (heterologous competition) one can obtain information on whether different toxins bind to the same binding site.

## Results and discussion

***Binding site model in the diamondback moth and its use at predicting cross-resistance***

Using seven different toxins and competition experiments, an integrative binding site model has been proposed for the diamondback moth (Ballester *et al.*, 1999; Ferré & Van Rie, 2002). The model proposes the occurrence of four different binding sites for these seven toxins. There are three binding sites (binding sites 2, 3, and 4) recognised by just one type of toxin and another binding site (binding site 1) shared by five toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, and Cry1J). Cry1Aa binds to two different binding sites (1 and 2). Cry1B and Cry1C each recognise one binding site not shared with other toxins (binding sites 3 and 4, respectively). Cry1Ab and Cry1Ac just bind to the common site (binding site 1). Cry1F and Cry1J also bind to this site, but since they were not tested with label, the possibility of binding to other additional sites has not been excluded.

The predictive value of this model regarding cross-resistance is obvious. A major modification of the common binding site could affect binding of the five toxins and, consequently, this would confer resistance against at least four of them (the possible exception being Cry1Aa, which still would bind to the non-shared site; it has been speculated that binding to this site might not be effective in producing toxicity). In addition, a minor alteration of the binding site could also confer resistance to just one or a few toxins, by affecting binding of these without affecting binding of the others. Therefore, sharing a binding site indicates potential for development of cross-resistance by the mechanism of

binding site alteration, though it must be kept in mind that there are examples of cross-resistance for which binding to BBMV was not altered (Gould *et al.*, 1992).

***Mechanisms of resistance to Bt-toxins in the diamondback moth***

Studies with 8 resistant colonies have shown that Cry1A resistance is due, at least in part, to a reduction of binding (Ferré & Van Rie, 2002). Specifically, Cry1A-resistant insects fall into two categories depending on the type of binding site alteration. These have been called Type I and Type II, depending on whether just Cry1Ab binding is affected or if the alteration affects binding of other Cry1A toxins, respectively. The alteration found in the Cry1A-resistant colonies was of the Type I for PHI and SERD3 (and probably also that of BL), and Type II for NO-QA, PEN, and 1AcSEL-MEL. Cross-resistance to Cry1F and Cry1J in NO-QA is well explained by this model, since binding of these two toxins is presumably prevented in these two resistant colonies.

It is interesting to note that in the two cases of resistance to Cry1C no major alteration of the binding of this toxin was observed. Although the resistance ratio in the NO-95C was relatively low (19-fold for the activated toxin and 48-fold for the protoxin), the resistance ratio in Cry1C-Sel was extremely high (63,100-fold).

## Acknowledgements

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# Oral toxicity of *Photorhabdus temperata* against *Frankliniella occidentalis* and *Thrips tabaci*

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**Abstract**: The oral toxicity of excretion products of several *Photorhabdus* and *Xenorhabdus* strains was tested on two thrips species: *Frankliniella occidentalis* and *Thrips tabaci*. Out of 46 *Photorhabdus* isolates and 6 *Xenorhabdus* isolates only 6 North American *P. temperata* isolates were toxic to the thrips species. After 7 days of drinking from *P. temperata* supernatant a mortality of 90% could be reached. Possibilities of using *P. temperata* in the control of thrips will be discussed.

**Key words**: *Photorhabdus, Xenorhabdus, Frankliniella occidentalis, Thrips tabaci*, entomopathogenic toxins, oral toxicity, biological control

## Introduction

Thrips species like *Frankliniella occidentalis* (western flower thrips) and *Thrips tabaci* (onion thrips) are a serious pest on ornamental and vegetable crops (Lewis, 1997). Growers frequently use insecticides to control thrips in their crops, but increased tolerance or even resistance to the chemical compounds will necessitate them to search for other control methods (Brødsgaard, 1994).

Recently Bowen *et al.* (1998) discovered insecticidal toxins from the bacterium *Photorhabdus*. These toxins are excreted by the bacterium and are orally toxic to several insect species.

The aim of this study is to test whether toxins from *Photorhabdus* and, closely related, *Xenorhabdus* strains show oral toxicity to thrips species.

## Material and methods

### *Maintenance of insect and bacterial cultures*

A rearing of *F. occidentalis* was maintained on potted, flowering chrysanthemum plants, of the susceptible cultivar 'Sunny Regan' in a greenhouse at 25°C, 70% RH and 16 hours light. A rearing of *T. tabaci* was maintained in jars with pieces of leek leaves at 20°C and 16 hours light.

Table 1 lists the origin of the *Photorhabdus* and *Xenorhabdus* strains used in this study. All *P. temperata* PWX strains were kindly provided to us by Dr. A. Fodor and Dr. E. Szalas. Other strains were isolated from the haemocoel of *Galleria mellonella* larvae infected by nematodes. All strains where maintained on Lab Lemco agar (Oxoid) at 25°C in the dark.

### *Bioassays*

The oral toxicity of *Photorhabdus* and *Xenorhabdus* strains was tested in a small Murai cage (Loomans & Murai, 1997). Ten thrips were placed in a small Perspex Murai cage (Ø 40 mm, h 30 mm) with a small amount of pollen as extra food source. The cage was closed with a double layer of parafilm with the test solution between the layers (±200 µL). Each treatment consisted of five replicates.

In the tests with *F. occidentalis* every two or three days the parafilm sandwich was renewed and mortality and egg production was checked. Incubation at 25°C and 16 hrs light.

In the tests with *T. tabaci* the trips were checked for mortality after two days and then transferred to a piece of leek on water agar (1.5%) in a small Petri dish (5cm). The dish was closed using clingfilm. Incubation at 20°C and 16 hrs light.

Table 1. Origin of *Photorhabdus* and *Xenorhabdus* strains used in this study and their oral toxicity to *F. occidentalis*

| **Bacterial strain** | **Associated nematode species** | **Original place of isolation** | **Oral toxicity to thrips[1]** |
|---|---|---|---|
| *P. temperata* : PE87.3, PF85, Pjun, PB87.1, PW79, Pfr, PH92.1, PL81, PHi93, PA93 | *H. megidis* | The Netherlands | – |
| *P. temperata* PNB87, PNH1, PH94 | *H. megidis* | The Netherlands | –+ |
| *P. temperata* : PS94 | *H. megidis* | Belgium | – |
| *P. temperata* : P211 | *H. megidis* | UK | – |
| *P. temperata* : PPB | *H. megidis* | Poland | – |
| *P. temperata* : PK6, PSH | *H. megidis* | Germany | – |
| *P. temperata* : PK122, PM145 | *H. megidis* | Ireland | – |
| *P. temperata* : PK3 | *H. megidis* | Norway | – |
| *P. temperata* Pmeg | *H. megidis* | Canada | ++ |
| *P. temperata* : PWX1, PWX2, PWX3, PWX5, PWX6, PWX8, PWX8hyper | ?[2] | Wisconsin, USA | – |
| *P. temperata* : PWX9, PWX9hyper, PWX10, PWX11, PWX12 | ? | Wisconsin, USA | ++ |
| *P. temperata* : PWX13 | ? | Wisconsin, USA | –+ |
| *P. temperata* : PWX15 | ? | Wisconsin, USA | – |
| *P. temperata* : Pbac | *H. bacteriophora* | Australia | – |
| *P. luminescens* : Pmol | *H. bacteriophora* | Russia | – |
| *P. luminescens* : P2 | *H. bacteriophora* | Israel | –+ |
| *P. luminescens* : P4 | *H. bacteriophora* | Israel | – |
| *P. luminescens* : PP88 | *H. bacteriophora* | USA | – |
| *P. luminescens* : Psie | *H. bacteriophora* | Poland | – |
| *P. luminescens* : P23 | *H. bacteriophora* | Italy | – |
| *P. luminescens* : PNJ | *H. bacteriophora* | New Jersey, USA | – |
| *P. luminescens* : PDa1 | *H. bacteriophora* | Germany | – |
| *Photorhabdus* sp. | *H. marelatus* | | – |
| *X. poinarii* | *S. glaseri* #236 | | – |
| *X. nematophilus* | *S. carpocapsae* 703 | | – |
| *X. nematophilus* | *S. carpocapsae* Biosys | | – |
| *X. nematophilus* | *S. carpocapsae* mexicana N2 | | – |
| *X. nematophilus* | *S. affinis* | | – |
| *Xenorhabdus sp.* | *S. riobrave* | | –+ |

(1) – = no thrips mortality, –+ = mortality < 50%, ++ = mortality > 50% after 7 days

(2) ?= nematode species was not identified

All strains were tested once against *F. occidentalis*. The four most toxic strains (Figs. 1 and 2) were tested three times against both thrips species.

To prepare the test solution liquid cultures of the bacteria were prepared by inoculating a single colony into 30 mL nutrient broth (0.8% Lab Lemco Broth, Oxoid), shaken at 210 rpm for three days at 25°C in the dark. The cultures were centrifuged for 20 min at 4000 rpm. The supernatant was sterilised over a 0.2 µm filter and 0.4% Sugarbait (concentrated sugar solution, Brinkman) was added. As control solutions, water and Lab Lemco Broth (LLB), both with 0.4% Sugarbait, were used.

***Statistical analysis***

Binomial data are analyzed using a generalized linear model (GLM). The model is:

logit(p) = f(Replications, Treatment) (p = E(x/n) = %mortality).

This model was fitted using the statistical language GENSTAT. The reproduction rate (rr) was calculated as follows: rr= # eggs/# living thrips.

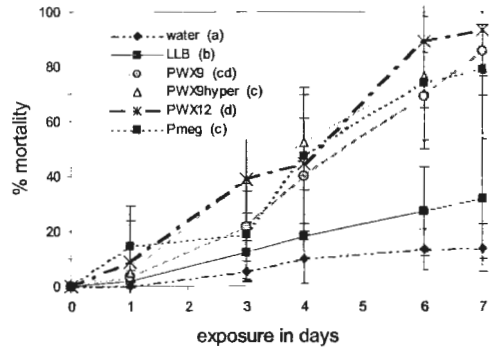


Figure 1. Oral toxicity of *Photorhabdus temperata* toxins to *Frankliniella occidentalis*. Thrips where exposed to the toxin for 7 days. (a): values with the same letter do not differ significantly after 7 days.

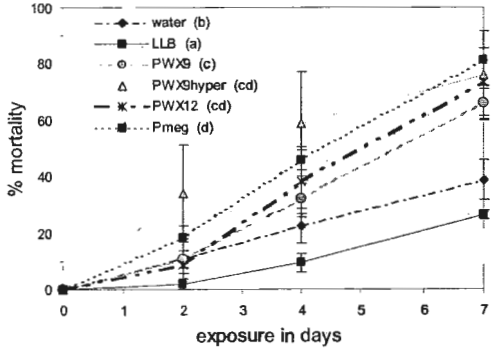


Figure 2. Oral toxicity of *Photorhabdus temperata* toxins to *Thrips tabaci*. Thrips where exposed to the toxin for 2 days and then transferred to leak leaves. (a): values with the same letter do not differ significantly after 7 days.

## Results

*F. occidentalis* survives very well in a Murai cage with sugar-water. The parafilm sandwich serves as an artificial leaf from which the thrips suck. *T. tabaci*, however, could not survive for 7 days on only sugar-water (results not shown). Therefore these thrips were transferred to a piece of leek after 2 days drinking from the toxin solution.

46 *Photorhabdus* strains and 6 *Xenorhabdus* strains where tested for toxicity against *F. occidentalis* (Table 1). Only toxins from 6 *P. temperata* strains caused high mortality of *F. occidentalis*, 79 to 93% after 7 days (Table 1, Fig. 1). Five other *P. temperata* strains, one *P. luminescens* strain and one *Xenorhabdus* sp. strain caused only low mortality (less than 50%).

The strains that caused high mortality against *F. occidentalis* also worked well against *T. tabaci* (Figure 2). Even after only two days of drinking from the toxin solution 66 to 81% of the thrips died after 7 days.

Egg production of *F. occidentalis* is also affected by the toxins. Already after 1 day a decrease in the reproduction rate (number of eggs per living thrips) can be seen (Fig. 3).

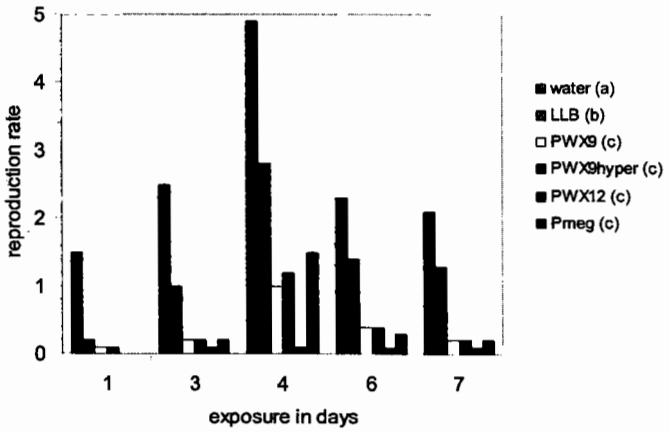


Figure 3. Effect of *Photorhabdus temperata* toxins on the reproduction rate of *Frankliniella occidentalis*. (a): values with the same letter do not differ significantly.

## Discussion

From all the *Photorhabdus* and *Xenorhabdus* strains tested only a few of the *P. temperata* strains from North America showed high oral toxicity to thrips. Although the purified toxin was not tested, thrips mortality is probably caused by toxins like the Tc toxins described by Bowen *et al.* (1998). These toxins are proteins, excreted by the bacteria. In our tests supernatant loses toxicity to thrips after heating to 70°C or addition of proteinase K (results not shown), suggesting that toxicity is caused by a protein. Why only the North American *P. temperata* strains and not the European *P. temperata* strains cause toxicity is not clear. It could be that the European strains do not carry (all) the toxin genes or that these genes are not expressed. Another possibility is that the strains do not produce enough toxins to kill thrips or

a different type of toxin that is not toxic to thrips or that the toxins are not excreted. *Xenorhabdus nematophilus* also produces toxins, partly similar to Tc toxins (ffrench-Constant & Bowen, 2000). However, the *X. nematophilus* strains tested here did not show toxicity to thrips.

In the tests the mortality of *T. tabaci* is less than that of *F. occidentalis* (66 to 82 versus 85 to 93% respectively after 7 days). However, *F. occidentalis* has been drinking from the toxins for 7 days while *T. tabaci* has only been drinking for 2 days, so this cannot be compared. Mortality of both thrips species could even be higher when purified toxin is used instead of the whole supernatant.

The reproduction rate of *F. occidentalis* is also influenced by the supernatant (Fig. 3). The reproduction rate is reduced by LLB medium without bacterial excretion products, so there is a factor in the medium itself that reduces egg production. However, medium with bacterial excretion products reduce egg production significantly more so there is also a bacterial factor involved.

This shows that the toxin has a double effect for the control of thrips: not only does it kill thrips directly but it also reduces the next generation of thrips by reducing the reproduction rate in a very early stage.

How can we use these oral toxins for the control of thrips? Since thrips is a sucking insect it might be difficult to let them take up toxins that work orally. Thrips are known to drink from drops of sugar-water or honey. This makes it possible to kill thrips with a crop treatment with large droplets of sugar-water with toxins. Another possibility is to spray a layer of toxins on the leaves. Each time the thrips puts its stylet in the leaf it has to go through this toxin layer. At this moment we are checking the possibilities of killing thrips by spraying *Photorhabdus* toxins on leaves as a film or in little drops of sugar-water.

Another possibility of using these toxins for thrips control is in transgenic plants. Like with *Bacillus thuringiensis* (Bt) toxins, it should be possible to create transgenic plants expressing *Photorhabdus* toxins. When thrips suck from these plants and take up the toxin they will be killed. In Europe most countries are not in favour of transgenic plants. However, in the USA where transgenic plants carrying the Bt toxin are used, this might be a viable alternative.

## Acknowledgements

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# Entomopathogenic Viruses

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# Future potential for biological control in Latvia: occurrence and natural variability of baculoviruses

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**Abstract**: Baculoviruses are potential agents for the control of different forest and orchard pests. The aim of the studies was to investigate occurrence and natural variability of baculoviruses in Latvia. Nucleopolyhedroviruses were isolated from 13 pest species. Granuloviruses were isolated from the fruit-tree pests *Cydia pomonella L.*, *Yponomeuta padella* L. and cabbage pests *Pieris brassicae* L. and *P. rapae* L An optimised method of virus DNA determination by the PCR was used for *in–situ* determination NPVs in different populations of insects (*Lepidoptera*). We recorded presence of NPV in declining populations of the European tent caterpillar *Malacosoma neustria* in western and central part of Latvia.

**Key words**: nucleopolyhedroviruses, granuloviruses, pests, occurrence

## Introduction

In nature, baculoviruses (Baculoviridae), cause diseases of insects and can control the population size of their hosts. Baculoviruses, including nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), are considered to be safe biological insecticides and have a great potential in pest control (Entwistle, 1997). It is necessary to investigate the occurrence of baculoviruses and to provide their remaining in biocenosis for prolonged period to limit the amount of plant pests with the help of this environmental potential. Knowledge on interaction between baculoviruses and host insect, that support decrease of plant pest population, helps to develop an effective strategy of biological control. New sensitive methods of pathogen detection are required for monitoring of occurrence and presence of viruses in the insect populations. The development of laboratory techniques that allow viruses to be identified at low concentrations and in individuals that are asymptotic is altering our understanding of occurrence of viruses in populations. Recently, Laboratory of Experimental Entomology of Institute of Biology, University of Latvia, either has been studying the NPVs occurrence and persistence. Therefore the aim of our studies was to extend knowledge of natural enemies of plant pests and investigate occurrence and natural variability of baculoviruses in Latvia.

The main tasks were: 1) to obtain new isolates of baculoviruses, 2) to implement and develop the methodology molecular identification of isolates, 3) to describe the natural occurrence of viruses in pest populations and the homology of population.

## Material and methods

### *Collection and rearing of insects*

To examine virus infections in natural insect populations, dead, infected and living insects were collected from natural habitats using standard methods. Living insects were reared in the laboratory- in isolators on fresh natural foods (branches of food plants) or on semi-synthetic media. The composition of the semi-synthetic media for rearing *Malacosoma neustria, Orgya*

*antiqua* and *Operophthera brumata* was described by Berzina & Zarins (1979) for *Bupalus piniarius* and *Lymantria monacha* by Zarins (2001).

***Virus isolation, purification and microscopy***

Virus material was obtained from field collected infected larvae or by subjecting asymptomatic larvae to following stress-factors: extreme temperatures (16 – 24 hours at +4 - + 6°C, then at + 28 – + 35°C); the food treated with 0.5% $ZnSO_4$, 1.0% $H3BO_3$, and 0.3% $NaNO_2$. Dead larvae were frozen with liquid nitrogen and homogenized in distilled water with 0.1 % SDS. The homogenate was then filtered through cheese-cloth. The filtrate was centrifuged at 6.000 g (NPV) and 11.000 (GV) for 40 min. Polyhedrae and GV capsules were purified by centrifugation on 30-66 % (w/v) linear sucrose gradient. Inclusion bodies bands were collected, checked by microscope and washed twice with distilled water. The virus pellet was resuspended in a small volume of de-ionized water and stored at -18° C (Evans & Shapiro, 1997).

Standard cytological methods (Adams & Bonami, 1991) were used for observation of baculoviruses. Cytological preparations were investigated by a light microscope (Amplival, Germany). For ultra structural examination, polyhedrae were dissolved with 1% NaOH for 5 min and negatively stained with 2% phosphotungstic deid. The preparations were observed in an electron microscope (JEM-100C, JEOL, Japan).

***Determination and identification of viral DNA by PCR***

Extraction of virus and host genomic DNA from individual insects was done according to Saville et *al.* (1997). We used nested PCR for determination of NPV. Designed primers correspond to the polyhedrin gene of Mn NPV Latvian isolate (Jankevica, 1999). The first set of primers amplified 985 bp, and the second set 708 bp of polyhedrin gene. The PCR reaction mixture contained the following components: insect or viral DNA - 1μL; 10x PCR buffer - 5μL; $MgCl_2$ - 5μL: BSA (1mg/mL) -4μL; dNTP (10mM) - 1μL; Primer1 (10pM/μL) - 2μL; Primer2 (10pM/μL) - 2μL; Taq polymerase 5U/μL (MBI Fermentas) -1μL; water to 50μL. The PCR conditions were: 30 cycles on GeneAmp PCR System 2400 (Perkin Elmer). First step 95°C -30 sec., second step 56°C - for first set of primers or 62°C for second set of primers 30 sec., third step 72°C -1 min. The final step of the reaction was followed by extension at 72°C for 7 min. At the end of the reaction the resulting amplification products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualised under short wave UV light, the size of DNA fragment was detected (Saville *et al.*, 1997).

## Results and discussion

Research on baculoviruses was carried out in Institute of Biology since 1965. NPV and GV were isolated from the most dangerous vegetable, fruit-tree and forest pests. Generally natural epizootic caused by NPVs have been observed in most areas were host populations reach high density. Epizootics caused by NPVs and GVs were observed rather rarely in Latvia, baculoviruses were isolated only from 19 insect species. Most of NPVs were isolated from Lepidoptera (Table 1). GVs were isolated from the fruit-tree pests *Cydia pomonella*, *Yponomeuta padella* and cabbage pests *Pieris brassicae* and *P. rapae* (Table 2).

Morphological characteristics of *M. neustria* NPV isolates showed that the dimensions of polyhedra were 850 to 1400 nm. Polyhedrae contained large number of rod-shaped multiple virions. Virions contained 1 to 7 nucleocapsids. NPV populations are not homologous. NPV isolated in different localities shows different virulence (Jankevica & Zarins, 1999). Latvian isolates of *M. neustria* NPV, *L. monacha* NPV, B. *piniarius* NPV, *C. pomonella* GV and *P. brassicae* GV have an high virulence and have a good potency in insect pest control.

Table 1. List of nucleopolyhedroviruses isolated from natural pest populations in Latvia since 1965.

| Host insect | | Year of first NPV isolation | Reference |
|---|---|---|---|
| **Order, family** | **Species** | | |
| LEPIDOPTERA | | | |
| Lasiocampidae | *Malacosoma neustria* L. | 1967 | Zarinsh & Kalnina, 1971 |
| | *Eriogaster lanestris* L. | 1997 | Jankevica, 1999 |
| Geometridae | *Operophthera brumata* L. | 1978 | Rituma, 1978 |
| | *Bupalus piniarius* L. | 1992 | Zarins, 2001 |
| Lymantriidae | *Orgyia antiqua* L. | 1977 | Rituma, 1977 |
| | *Lymantria monacha L* | 1993 | Zarins, 2001 |
| Yponomeutidae | *Yponomeuta cognatella* Hg. | 1973 | Simonova & Kazanskaja, 1977 |
| | *Y. evonymellus* L. | 1976 | |
| | *Y. malinellus* Zell. | 1968 | |
| Pieridae | *Pieris brassicae* L. | 2001 | Jankevica, unpublished |
| HYMENOPTERA | | | |
| Pamphiliidae | *Acantholyda posticalis* Mats. | 1968 | Zarins & Eglite, 1993 |
| | *Gilpinia pallida* Kl. | 1978 | Zarins & Eglite, 1993 |
| Diprionidae | *Neodiprion sertifer* (Geoffr.) | 1973 | Zarins & Eglite, 1993 |

Table 2. List of granuloviruses isolated from natural lepidopteran pest populations in Latvia since 1965.

| Host insect | | Year of first GV isolation | Reference |
|---|---|---|---|
| **Family** | **Species** | | |
| Noctuidae | *Agrotis segetum* L. | 2000 | Jankevica, unpublished |
| Geometridae | *Bupalus piniarius* L. | 1987 | Zarins & Eglite, 1993 |
| | *Cydia pomonella* L. | 1968 | Zarins & Eglite, 1993 |
| Pieridae | *Pieris brassicae* L. | 1965 | Zarins & Eglite, 1993 |
| | *P. rapae* L. | 1971 | Zarins & Eglite, 1993 |
| Yponomeutidae | *Yponomeuta padella* L. | 1989 | Zarins & Eglite, 1987 |

In recent years nested PCR are used for monitoring of occurrence and presence of pathogens in the insect populations. Using a sensitive PCR-based method NPVs were found in different stages of insect's development: adults, larvae and cocoons.

*M. neustria, E. lanestris* and *A. segetum* populations were observed in western and central part of Latvia. NPV was identified in all declining *M. neustria* populations. For example, 63% of examined *M. neustria* larvae collected in Grobina were infected by NPVs. We detected NPV in 75 - 100% of *M. neustria* adults collected in the Liepaja district. Results showed that collected *M. neustria* larvae and imago had latent or sublethal NPV-infection. Presence of NPV in observed *E. lanestris* populations was very low. Only 2 % of collected *E. lanestris* specimens were NPV positive. Results obtained by microscopy showed that 11 % of 3rd instar *P. brassicae* larvae were infected with NPV. We did not detected NPV in observed *A. segetum* populations.

The method for the detection of polyhedrin-specific DNA sequences using PCR has been proved to have good possibilities for the determination of presence of viruses in pest populations.

The priority of developed method will be the possibilities to detect pathogens *in situ*, in different developmental stages of insects, therefore forecasting of epizootics in pest populations will be possible.

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# Understanding the mechanism of increasing susceptibility of insects to baculovirus by fluorescent brightener

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**Abstract**: Fluorescent brighteners are applied to enhance the virulence of baculoviruses. In the present study, Fluorescent Brightener 28 was used to increase the susceptibility of noctuid larvae to different viruses. It was shown that this fluorescent brightener increases the susceptibility of *Agrotis segetum* (Lepidoptera: Noctuidae) to granulovirus more than to nucleopolyhedrovirus. Since there are indications that fluorescent brighteners influence the peritrophic membrane, histological investigations on this membrane were conducted with light microscopy. The peritrophic membrane of late *A. segetum* larvae is multilayered and lines the whole midgut. It functions as a mechanical barrier and ultrafilter to parasites and pathogens and as envelope for fecal pellets. The addition of fluorescent brightener to the diet of this noctuid dissolved the peritrophic membrane, which explains the increase in susceptibility to its viruses.

**Keywords**: Peritrophic membrane, fluorescent brightener, Noctuidae, *Agrotis segetum*, Nucleopolyhedrovirus, Granulovirus, mechanism of enhancement, midgut, synergism

## Introduction

Baculoviruses (nucleopolyhedroviruses and granuloviruses) are important biocontrol agents which can be used in plant protection for a safe, environmentally friendly, effective and specific pest control. The main problem in using a baculovirus is deterioration of its activity under field conditions (Tamez-Guerra *et al.,* 2000) as well as the low susceptibility of some target pest insects. Previous studies have reported the possibility to improve the efficacy of baculoviruses by increasing the susceptibility of the insects with enhancers (Shapiro & Vaughn, 1995; Dougherty *et al.,* 1996; El-Salamouny *et al.,* 1997; Okuno *et al.*, 2003).

Infection of insect larvae occurs by oral ingestion of the viral occlusion bodies (OBs). The OBs dissolve in the alkaline digestive juices of the gut, releasing enveloped virus particles. They must first pass the peritrophic membrane (PM) which lines the midgut before initiating an infection of the midgut epithelial cells (Granados & Lawler, 1981). Thus, the peritrophic membrane may serve as mechanical barrier against microorganisms invading the insect midgut cells (Derksen & Granados, 1988). Optical brighteners disturb the midgut defence system in insects. Feeding of *Trichoplusia ni* larvae with a single dose of 1 % Calcofluor resulted in complete inhibition of PM formation in the midgut. Cross sections of the midgut showed that *T. ni* larvae fed with Calcofluor processed no PM (Wang & Granados, 2000).

The aim of the present study was to understand the mechanism of increased susceptibility of *Agrotis segetum* by fluorescent brightener.

## Material and methods

***Test insects***

Neonate larvae of the turnip cutworm, *Agrotis segetum* were reared on semi-artificial diet based on beans, yeast, wheat germs, agar, vitamins, and antifungal and -bacterial substances, such as sorbic acid, Nipagine, wesson salt, and frisomycin (Hassani, 2000).

***Viruses***

Nucleopolyhedrovirus: *Agrotis segetum* nucleopolyhedrovirus (*Agse*NPV) and Granulovirus: *Agrotis segetum* granulovirus (*Agse*GV). Both originate from the culture collection of the Institute for Biological Control, BBA, Darmstadt, Germany.

***Synergistic additive***

Fluorescent Brightener 28 (FB 28) = Calcoflour White = Tinopal, UNPA-GX (Sigma).

***Bioassay***

Virus was mixed into the semi-synthetic diet in presence or absence of the tested additives, following the standard method for mixing *Cydia pomonella* granulovirus with the diet, at a diet-temperature below 40 °C (Huber, 1981). The mixture was placed in special bioassay plates containing 50 cells (LICEFA, Bad-Salzuflen, Germany). Fifty neonate larvae were used per each plate. In case of the enhancement tests, seven virus concentrations were tested with a spacing of $\sqrt{10}$. The bioassay plate was covered with a layer of tissue paper and a polyethylene sheet that was fixed with two rubber bands. The plates were incubated at 26 °C, 60-70 % RH and 16 hours light and 8 dark. Mortality due to infection was recorded every two days, up to 12 days. Three repetitions of each bioassay were made.

***Measurement of the larval weight***

The larvae of *A. segetum* were reared on diet with FB 28 added at concentrations of 0.05 % and 0.1 %. After 12 days the weight of 30 larvae each was recorded individually using a digital balance. Three replicates for each FB 28 concentration in addition to the untreated control were made for comparison.

***Light microscopy: dissection and isolation of the peritrophic membrane***

For isolation of the peritrophic membrane (PM), fifth instar larvae of *A. segetum* were dissected in ringer solution with the use of a binocular. Pieces of the PM were transferred to glass slides, stained by aqueous solution of Congo red 1 % (w/v), covered with a cover-slip and examined with the light microscope.

***Light microscopical sections***

For serial sections of the midgut as well as of the whole larvae, the fixation and embedding process were similar to that described by Kleespies *et al.* (2001). First, third and fifth instar larvae were fixed with Dubosq-Brazil's alcoholic Bouin's and embedded in Histosec (Merck, Darmstadt, Germany). Serial sections at 9 µm were prepared of third and fifth instars and 5 µm of first instar larvae. The same procedure was used for larvae fed on diet with FB 28 at concentrations of 0.1 % (12 days) and 1 % (24 hrs). The sections were stained with Heidenhain's iron haematoxylin and counterstained with erythrosine or with Giemsa's stain (both from Merck, Darmstadt, Germany). A Leica DMRB photomicroscope (Leica, Bensheim, Germany) with phase contrast and bright field equipment was used to examine the histological sections.

## Results

***Comparative enhancement tests of GV and NPV infections***

*A. segetum* granulovirus and *A. segetum* polyhedrovirus were tested against *A. segetum* larvae in presence or absence of two concentrations of FB 28. The data showed that a reasonable enhancement effect was obtained for both, GV and NPV. In case of using *Agse*GV against its homologous host *A. segetum*, the $LC_{50}$ value was $2.62 \times 10^6$ G/ml. It decreased significantly by addition of FB 28 to $1.69 \times 10^4$ and $3.43 \times 10^3$ G/ml for concentrations of 0.05 % and 0.1 %, respectively (Fig. 1).

For *Agse*NPV against *A. segetum* larvae, the $LC_{50}$ value was $8.3 \times 10^3$ PIB's/ml diet. It decreased to $5.93 \times 10^2$ and $2.35 \times 10^2$ PIB's/ml diet, for the same fluorescent brightener concentrations, as described for *Agse*GV, respectively (Fig. 1).

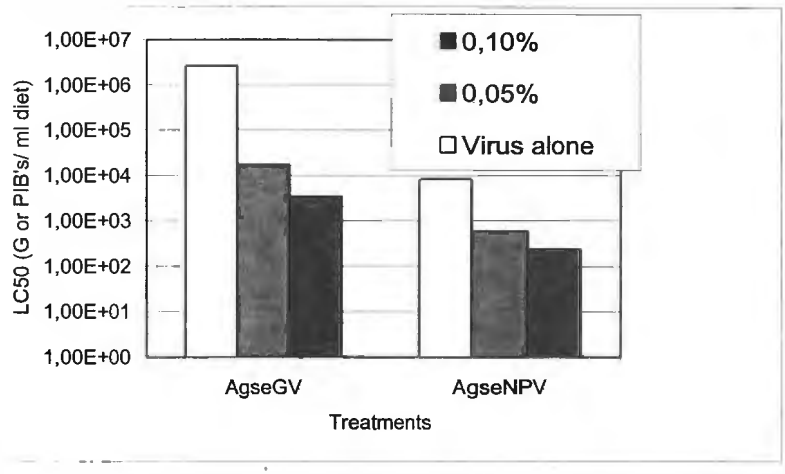


Figure 1. Synergistic effect of Fluorescent Brightener 28 on the susceptibility of *Agrotis segetum* neonate larvae to two homologous baculoviruses.

***Effect of Fluorescent Brightener 28 on the weight of* A. segetum *larvae***

A significant decrease of weight was noticed for larvae treated with 0.1 % FB 28. The average weight of the untreated larvae was 1.65 g. In contrast, larvae fed on diet with 0.1 % FB 28 showed an average weight of 0.72 g. 0.05 % FB 28 had no significant influence on the larval weight.

***Histological studies on the peritrophic membrane***

In order to answer the question, why the susceptibility of *A. segetum* can be increased by FB 28, we studied the structure of the PM of treated and untreated insects by light microscopy: The isolation of the PM was performed successfully in untreated *A. segetum* fifth instar larvae. It was stained with Congo red and examined by light microscope. It was shown that it is multi-layered.

Serial longitudinal sections of the isolated midgut of fifth instars, as well as of complete third and first instar larvae of *A. segetum* showed that the membranes of the third and fifth instars are multilayered (3-5 layers) and that they line the whole midgut (Fig. 2a). It was clearly shown that there are more layers in the anterior part of the midgut and these layers are

thicker than those of the posterior part. In first instar larvae, the membrane consists only of one layer but still lines the whole midgut.

***Effect of Fluorescent Brightener 28 on the peritrophic membrane of* A. segetum *larvae***

Light microscope sections of *A. segetum* larvae reared on a diet with 0.1 % FB 28 for 12 days revealed a dramatic degradation of nearly the whole peritrophic membrane in all exposed instars (1st, 3rd and 5th) (Fig. 2b). Comparable results were obtained with same larval instars fed for 24 hrs on a diet with 1% FB 28. Only in the fifth instar, small rests of the membrane could be observed at the beginning of the midgut.

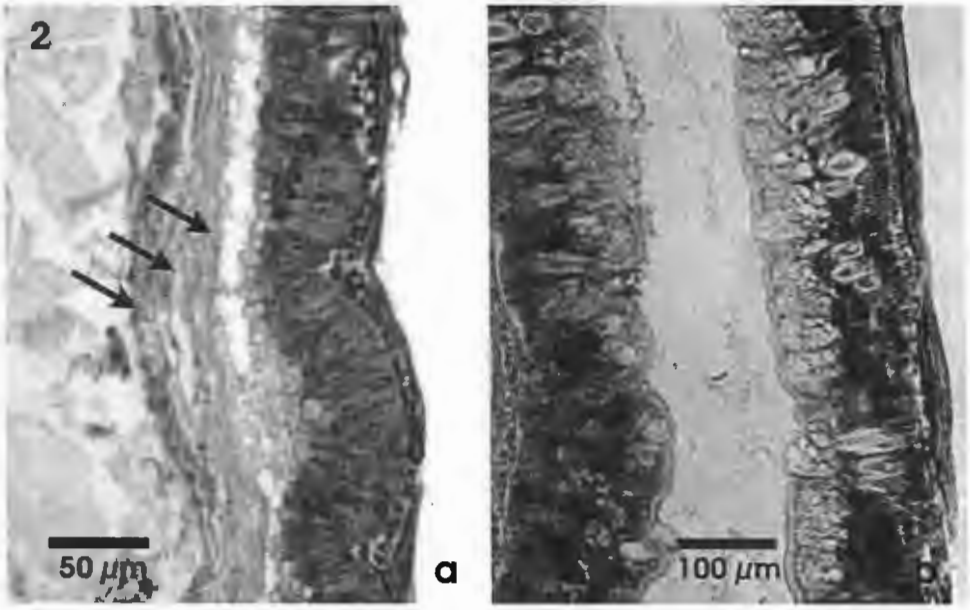


Figure 2. Effect of Fluorescent Brightener 28 on the peritrophic membrane of *A. segetum* 5th instar larvae; (2a) without FB 28, the arrows mark the multilayered PM; (2b) treatment with FB 28 (0.1 %), note the complete degradation of the PM.

## Discussion

The present study showed that the addition of Fluorescent Brightener 28 to the *A. segetum* polyhedrovirus as well as to the granulovirus increased the susceptibility of the larvae to both viruses. In general, the possibility of increasing the susceptibility of lepidopterous larvae by addition of fluorescent brighteners agreed with the findings of Shapiro & Vaughn (1996), and El-Salamouny *et al.* (1997). Shapiro & Robertson (1992) postulated that fluorescent brightener may open gaps in the midgut. The present work confirmed by histological studies that the increase of the susceptibility of *A. segetum* to baculoviruses is due to the degeneration of the peritrophic membrane, which is considered to be the first defense barrier against the virus particles trying to invade the midgut epithelial cells. These findings are conform to those of Wang & Granados (2000) and Okuno *et al.* (2003), who received similar results with *T. ni* and *Spodoptera litura*, respectively.

The small size of the larvae treated with FB 28 could be also explained by the degradation of the peritrophic membrane, since the digestion and food consumption is disturbed.

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# Competition in insect larvae between wild-type baculovirus (*Spodoptera exigua* nucleopolyhedrovirus) and a marked recombinant with enhanced speed of action

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**Abstract**: Baculoviruses are effective biocontrol agents of pest insects. These pathogens are specific and have minimal environmental impact. However, they are slow-acting compared to chemical insecticides. Recombinant baculoviruses have been generated with improved insecticidal properties. A major concern, however, is the fitness of such recombinants relative to wild type baculoviruses. In this contribution we have studied the comparative fitness of a recombinant *Spodoptera exigua* nucleopolyhedrovirus (SeMNPV-XD1) and wild type SeMNPV (US1), when serially passaged in 2nd instar beet armyworm (*S. exigua*) larvae in mixed infections. SeXD1 has an improved speed of action compared to SeUS1 and is marked by the absence of the ecdysteoid UDP-glycosyl transferase *(egt)* gene and the presence of the Green Fluorescent Protein (*GFP*) gene. The relative proportion of each virus upon successive passages was determined by plaque-assays of budded virus and by polymerase chain reaction. The analysis shows that SeUS1 outcompetes SeXD1 after few passages in *S. exigua* larvae. A mathematical model was developed to predict the competitive advantage of wild-type SeUS1 over the recombinant SeXD1.

**Key words**: *Spodoptera exigua*, SeMNPV, recombinants, competitive fitness, biological control

## Introduction

The beet armyworm, *Spodoptera exigua*, is a lepidopteran insect species causing extensive economic losses in many cultivated crops throughout the temperate and subtropical regions of the Northern hemisphere and in greenhouses. Control of *S. exigua* for a long time has depended almost exclusively on chemical insecticides, therefore the insect developed resistance to many commonly used chemical insecticides. Baculoviruses are naturally occurring insect pathogens and ideal biological agents to control pest insects (Moscardi, 1999). *S. exigua* multicapsid nucleopolyhedrovirus (SeMNPV) is an attractive bio-insecticide, since the virus is monospecific to the beet army worm and highly virulent in comparison to other baculoviruses (Smits *et al.*, 1994). A major drawback is their slow speed of action relative to chemical insecticides. Improvement of the biological properties of SeMNPV has been achieved either by strain selection (Muñoz *et al.*, 2000) or by genetic engineering strategies (Dai *et al.*, 2000). Genetic approaches have been used predominantly to increase speed of action (Black *et al.*, 1997). The question is whether baculovirus recombinants can cause any harm to the environment. A further concern is the survival of recombinants in the environment and relates to the question of baculovirus fitness in general (Bull *et al.*, 2003). We have addressed this question by setting up a model system to study competitive fitness of baculoviruses. Mixed infections with different ratios of wild type and recombinant SeMNPV were carried out in *S. exigua* larvae (Fig. 1). The relative proportions of each virus were measured over successive passages in the insect and a preliminary model to predict the competitive fitness was developed.

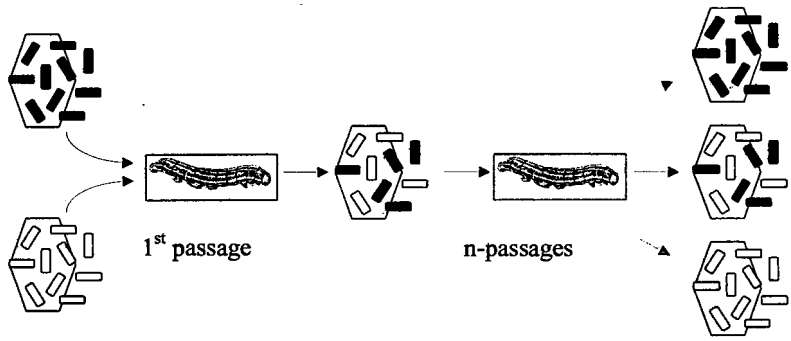


Figure 1. Mixed infection of *S. exigua* larvae with both wild type SeUS1 (black) and recombinant SeXD1 (white) SeMNPV genotype. Upon successive passaging in insects three types of progeny genotypes are possible: the recombinant will dominate, co-exist with the wild type virus or disappear.

## Materials and methods

The wild-type SeMNPV, strain US1 (SeUS1), was donated by dr. B.A. Federici (UC Riverside, USA) and was propagated in laboratory-reared 4th instar *S. exigua* larvae. SeMNPV recombinant XD1 (SeXD1) was isolated by Dai *et al.* (2000) and contained GFP as a marker in lieu of the p10 gene and had a deletion of 10.6 kb, from nucleotide 18513 to 29106, encompassing fourteen open reading frames (ORFs 15-28) (Fig. 2). Deleted genes include the *egt*, *chitinase*, *cathepsin* and several other genes with unknown function. SeXD1 was isolated after recombination of SeMNPV and a plasmid construct carrying the GFP gene followed by alternate infection in Se301 insect cells (plaque-purification) and in *S. exigua* larvae (oral infection and budded virus isolation from hemolymph) to secure bioactivity *in vivo* and *in vitro*. SeXD1 had improved a significantly lower $ST_{50}$ value (-25%) than SeUS1, but a similar $LD_{50}$ value (Dai *et al.*, 2000). The *S. exigua* cell line Se301 was a gift from Dr. T. Kawarabata (Institute of Biological Control, Kyushu University, Japan).

Infections of 4th instar *S. exigua* larvae were carried out using diet plugs contaminated with polyhedra in different ratios of SeUS1 and recombinant SeXD1 (5:1, 1:1 and 1:5). The concentration of polyhedra was determined by counting in a hemocytometer. In all cases, larvae were infected with SeMNPV polyhedra at a dose of $10^6$ polyhedra/larvae (25 times the $LD_{50}$ value for this instar (Bianchi *et al.* (2001)), with at least 30 larvae for each mix. The polyhedra from 10 larvae were used to infect healthy *S. exigua* larvae through six successive larval passages.

Budded virus was isolated from the hemolymph of infected larvae and an assay (plaque assay) was carried out to count foci (plaques) with polyhedra (SeUS1 + SeXD1) and plaques showing GFP as well (SeXD1 only). Viral DNA was isolated from polyhedra and a polymerase chain reaction (PCR) was performed using primers specific for p10 (SeUS1) and primers specific for GFP (SeXD1). The primers will amplify a 517 base pair (p10) and a 998 base pair fragment (GFP) upon agarose gel electrophoresis.

The relative proportions of SeUS1 and SeXD1, based on the plaque assays, in samples, which had been successively passaged in *S. exigua* larvae, were used as input for a model describing the competitive fitness of these viruses. For mixed infections the model of Godfrey *et al.* (1997) was used for data fitting and for calculation of the replication advantage (W).

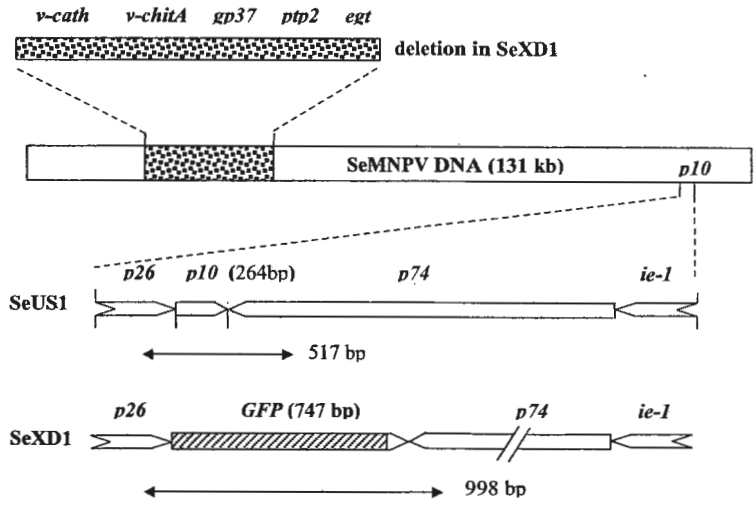


Figure 2. Schematic representation of wild type SeMNPV (SeUS1) and recombinant SeMNPV (SeXD1). Deletion (*egt*) and insertion (GFP) are indicated in SeXD1, as well as the specific PCR fragments for both viruses.

## Results

***Virus mixes and insect passages***

Fourth instar *S. exigua* larvae were orally infected with polyhedra of SeUS1 and SeXD1 in different ratios. Polyhedra of both viruses were produced in insects (*in vivo*) prior to serial passaging. Most of the larvae were successfully infected using contaminated diet containing enough polyhedra to cause 100% mortality (a final dose of $10^6$ polyhedra/larva): larvae normally died after about 6 days. Larvae infected with SeXD1 alone or with the virus mixture containing SeXD1 and SeUS1 in a 5:1 ratio, normally turned black after death, and showed limited liquefaction. The latter may be due to lack of chitinase and cathepsin in SeXD1 (Dai *et al.*, 2000; Hauxwell *et al.* 1999). After every passage the polyhedra were extracted from larval cadavers, purified and the concentration determined. These solutions were used for further successive passage in *S. exigua* larvae at $10^6$ polyhedra/larva.

***Plaque-assay***

Hemolymph samples were collected at 48 hours after infection and the concentration of infectious (budded) virus was calculated as Tissue Culture Infective Dose 50% value / ml. The relative proportion of SeUS1 and SeXD1 plaques was recorded 48h post infection. In the mix with a ratio of 1:1 SeXD1 disappears after 6 successive passages, in the mix with a ratio of 1:5 (SeXD1 : SeXD1) after 12 passages and with a final ratio of 5:1 (SeUS1: SeXD1) after 9 successive passaging. Although the kinetics are somewhat erratic, the results clearly show that the proportion of SeXD1 decreases for all mixes and that SeXD1 finally disappears. In contrast, the proportion of wild type SeUS1 virus reached 100%.

***PCR analysis of viruses***

To monitor the presence of the various viruses in the sample at each passage, a semi-quantitative PCR was set up and the results were analyzed by agarose gel electrophoresis. For example for the mix with a ratio of 1:1 (SeUS1 : SeXD1) a p10-specific PCR product (517 bp) was present in

all passages in roughly equal amounts. However, a SeXD1-specific PCR product (998 bp) gradually disappeared from after 4 passages. PCR analysis thus confirmed the general trend of the plaque-assay experiments, indicating that the SeUS1 finally overtakes SeXD1 in a competitive situation.

***Modelling***

A simulation model was used to describe the competition between two baculoviruses with distinctive fitness. Such a study may assist in the feasibility of using recombinant baculoviruses for inoculative control of insects in the field without perturbing the natural population of viruses. In a first model, we assumed that the ratio between wildtype (SeUS1) and recombinant (SeXD1) baculovirus evolves geometrically (Godfray *et al.*, 1997; de Wit, 1960), where

$$\frac{z(k)}{1-z(k)} = \frac{z(0)}{1-z(0)} w^k$$

In this equation, $z(k)$ is the proportion of wildtype baculovirus after the $k^{th}$ passage and $z(0)$ the initial value of $z$. The proportion of recombinant is $1\text{-}z(k)$. The average value of $w$ fitting our data is 1.9, indicating that after each passage the ratio of wildtype to recombinant baculoviruses has increased by a factor of 1.9, signalling wildtype dominance upon passaging in insects.

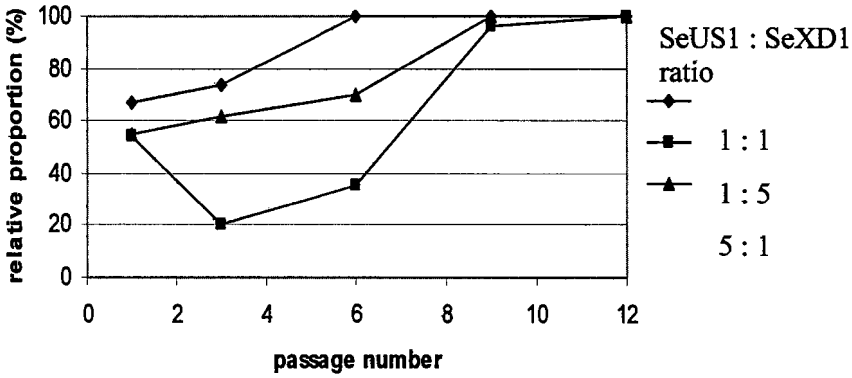


Figure 3. Relative proportion of SeUS1 versus SeXD1 after serial passage in 4th instar *S. exigua* larvae as measured after plaque-assay experiments.

## Discussion

The introduction of recombinant baculoviruses in the environment may result in their uncontrolled spread and in replacement of wild type baculoviruses. Information on the parameters, which influence baculovirus behavior in the field, may allow predictions on the behavior of recombinant baculoviruses in the environment (Vlak *et al.*, 1995). Preliminary results from a study on the competition between wild type SeMNPV (SeUS1) and recombinant SeMNPV (SeXD1) with improved speed of kill in mixed infections of *S. exigua* larvae in variable ratios clearly show the dominance of the wild type virus after several successive passages. It is not known yet what is the real cause for SeUS1 dominance, since

the virulence of both viruses in terms of $LD_{50}$ is very similar (Dai *et al.*, 2000). The missing genes are probably not essential for virus virulence *in vivo* (Heldens *et al.*, 1998). So, an alternative explanation must be sought. The enhanced speed of action of SeXD1, due to the lack of the *egt* gene, may result in reduced budded virus production or spread in the larva as compared to SeUS1, or in incomplete packaging of occluded virons in polyhedra. The results from these preliminary experiments suggests that also in the field wild type SeMNPV will get dominance over time over the recombinant virus when virus mixtures are applied. These results are a good starting point for the field evaluation of recombinant baculoviruses in general and SeMNPV in particular.

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# Other entomopathogens

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*IOBC/wprs Bulletin Vol. 28(3) 2005*
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# Morphology, pathology and phylogeny of *Cystosporogenes legeri*, a microsporidium of the European grape vine moth, *Lobesia botrana* Den. et Schiff.

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**Abstract**: A microsporidium was recently isolated from a laboratory stock of the European grape vine moth, *Lobesia botrana* Den. et Schiff. (Lepidoptera, Tortricidae). Light and electron microscopic investigations of infected insects showed that gross pathology, morphology and ultrastructure of the microsporidium are similar to those described earlier for *Pleistophora legeri*. Based on comprehensive morphological, ultrastructural and molecular studies a taxonomic designation to *Cystosporogenes legeri* nov. comb. was proposed. The presence of spores in first instar larvae, in the maturing gonads of females and males and in eggs makes it obvious that the parasite is transmitted transovarially.

**Key words**: *Lobesia botrana*, European grape vine moth, Microsporidia, *Cystosporogenes legeri* nov. comb., *Cystosporogenes operophterae*, *Pleistophora legeri*, transmission, phylogeny

## Introduction

Paillot (1941) reported microsporidial infections in samples of the grape vine moth, *Eupoecilia* (=*Clysia*) *ambiguella* Hbn. derived from field populations at Saint-Genis-Laval (France). He described the microsporidium as *Mesnilia legeri*. Lipa (1981) studied the same microsporidium in the European grape vine moth, *Lobesia botrana*, and transferred it to the genus *Pleistophora*, as *Plistophora legeri* (Paillot) comb. nov. Since the definition of the genus *Pleistophora* was too broad, Canning *et al.* (1985) created new genera including *Cystosporogenes*. This genus is characterized by: (1) the development of all stages in a vesicle bounded by an envelope of uncertain origin, and (2) being uninucleate (haplophase) throughout all stages of development.

Recently, we have isolated a microsporidium from the European grape vine moth, *L. botrana* and *E. ambiguella*. Based on comprehensive morphological, ultrastructural, and molecular investigations we proposed to change its taxonomic designation to *Cystosporogenes legeri* nov. comb. (Kleespies *et al.*, 2003). In this study we re-examined the phylogenetic relationship of *Cystosporogenes legeri* using a broader data set of microsporidia and further investigations on its transmission were done.

## Material and methods

### *Rearing conditions of the host*

The laboratory stock of the European grape vine moth, *L. botrana*, has been maintained for many years at the State Education and Research Center for Agriculture, Viticulture and Horticulture (SLFA) in Neustadt/Weinstrasse. Adult insects are kept in cages at room

temperature where they lay eggs on plastic sheets. These egg sheets are transferred to small plastic boxes containing artificial medium. Larvae are kept at 27 °C, 70 % relative humidity and a day : night period of 16 : 8 hours until pupation.

***Histopathological techniques***

Pathogenesis and life cycle studies were conducted with early instar larvae of *L. botrana* from the microsporidian-infected laboratory stock. Wet mount preparations of dissected organs, tissues and eggs were examined by phase contrast microscopy. Giemsa smears were prepared for assessment of vegetative stages. In addition, individuals of first and third instars as well as of adults were fixed with Duboscq-Brazil's alcoholic Bouin's and embedded in Histosec (Merck, Darmstadt, Germany). Six to nine µm sections were cut, stained with Heidenhain's iron hematoxylin and counterstained with erythrosin. Fresh spores and polar filaments that extruded in squash preparations were measured using phase contrast microscopy. For ultrastructural studies, infected tissues were fixed overnight at 4 °C in 3.0 % glutaraldehyde in Veronal buffer (pH 7.2) and postfixed in 2.0 % osmium tetroxide in the same buffer for 5 h. After dehydration, tissues were embedded in methacrylate. Thin sections were obtained with a Leica Ultracut S microtome and stained using uranyl acetate and lead citrate. Sections were observed and photographed using a Zeiss EM 902 electron microscope (Zeiss, Oberkochen, Germany).

***Isolation, purification and molecular analyses***

Microsporidian spores were purified from infected 4$^{th}$ instar larvae. Details of the purification, DNA isolation, amplification of the 16 S rDNA, and DNA sequencing are described in Kleespies *et al.* (2003).

***Phylogenetic analysis***

A BLAST search was performed to identify 16 S rDNA sequences similar to that of our isolate. The sequences were aligned using Clustal W, and phylogenetic analyses were accomplished using maximum parsimony and neighbour joining methods (PAUP 4.0, Swofford, 2001).

## Results and discussion

***Morphology and life cycle: light microscopy***

Studies of the microsporidian life cycle in Giemsa-stained preparations showed the first stage to be a uninucleate schizont, which was followed by spheroid schizonts with 2, 3, 4 or more nuclei. The nuclei were always single, never diplokaryotic. Commonly, schizonts became elongate and then produced multinucleate chains with large, lightly staining nuclei. These chains of schizonts underwent multiple fission or plasmotomy. The first stages of the sporulation process were multinucleate sporogonial plasmodia. These were spheroid with small densely staining nuclei. During sporogony cytoplasmic cleavage into many uninucleate daughter cells was observed. Each sporophorous vesicle eventually contained 8, 16, or more uninuclear sporoblasts. Spores were often observed in multiples of 8 (8, 16, and 32). The unfixed mature spores were bean-shaped and uninucleate, measuring 2.72 (± 0.26) x 1.63 (± 0.16) µm (n = 100). The mean length of the extruded polar filament was 39.2 µm (n = 50).

***Pathology: light microscopy***

The epithelial cells of the larval midgut were most heavily infected. The infection was also seen in other organs and tissues such as fat body (Fig. 1A), hypodermis, ganglia, gonads, and in eggs (Fig. 1B). Finding Microsporidia in the maturing gonads as well as in the eggs, and in first instar larvae makes it obvious that the parasite is transmitted transovarially.

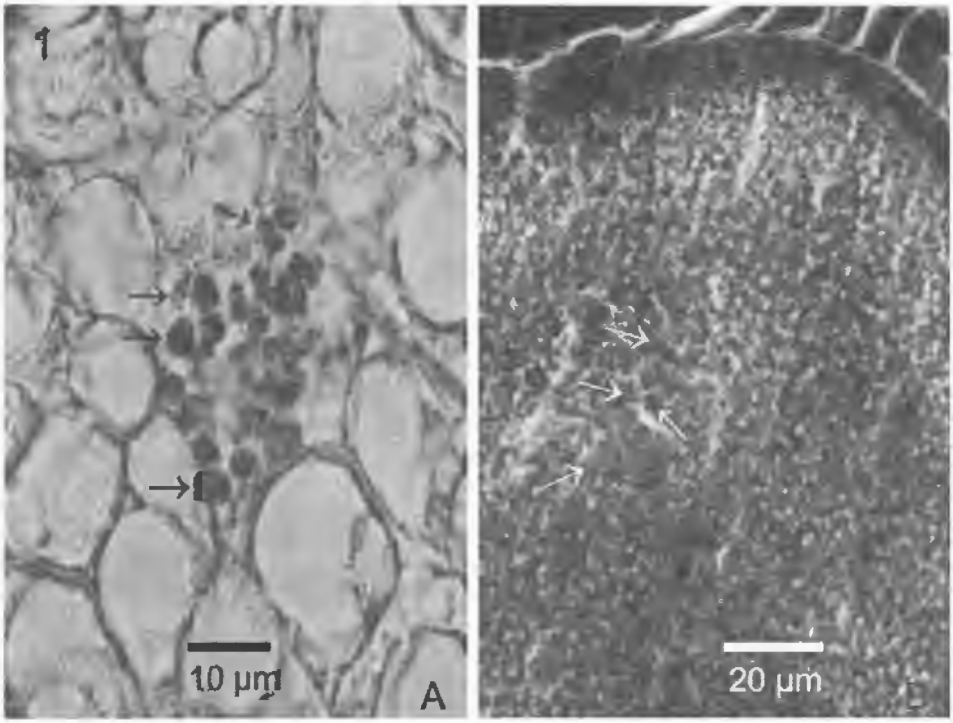


Figure 1. Sections of *L. botrana*, infected with the microsporidium, stained with Heidenhain's iron hematoxylin. A: Third instar larva, spores in sporophorous vesicles (arrows) in cells of the fat body; B: adult female, spores in sporophorous vesicles (arrows) in an egg.

***Morphology and life cycle: electron microscopy***

The earliest stages were spherical to oval shaped haplokaryotic schizonts. These were mono-, bi-, tri-, tetranucleate, or even multinucleate and contained numerous free ribosomes. External to the plasma membrane, schizonts were surrounded by a membrane-like envelope. Chains of schizonts were also observed in some sections. The division of the schizonts occurred by binary or multiple fission. The envelope encircling the schizonts is of uncertain origin and divides with the schizonts.

The earliest sporogonic stages were multinucleate plasmodia which divided by cytoplasmic cleavage to form separate uninucleate cells. Sporonts, sporoblasts and spores also occurred in an outer envelope that was very fragile and therefore frequently broke down during spore formation.

The detailed fine structure, for the most part, conforms to the typical microsporidian spore except that the straight anterior (manubrial) part of the polar filament is enclosed by a binary polaroplast. The outer, cup-like part of the polaroplast is composed of a densely packed system of lamellae that extends further into the spore, finally surrounding more than half of the nucleus (Figs. 2A - B). The inner part of the polaroplast consists of a more loosely arranged system of lamellae. In the mature spore the coils of the isofilar polar filament range from seven to eight. (Fig. 2A). These coils are arranged in a single row along the inside periphery of the spore. The nucleus itself is surrounded by several layers of rough endoplasmic reticulum.

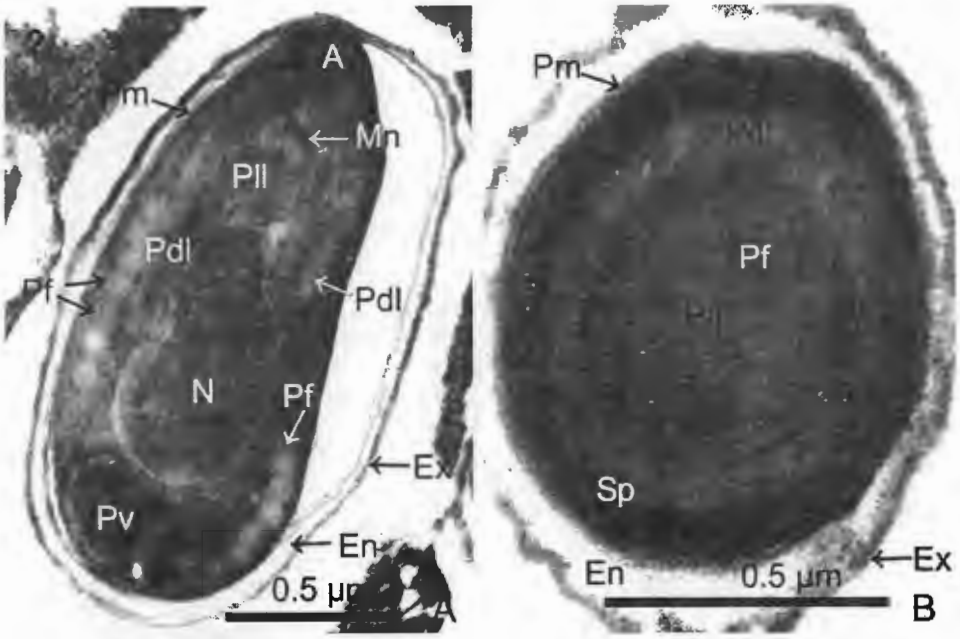


Figure 2. Electron micrographs of the microsporidium-spore. A: Longitudinal section of a spore showing seven turns of the polar filament (Pf), a single nucleus (N), and the polaroplast with the inner loose lamellar part (Pll) and the outer dense lamellar part (Pdl), (A) anchor, (En) endospore, (Ex) exospore, (Mn) straight anterior (manubrial) part of the polar filament, (Pm) plasma membrane, (Pv) posterior vacuole; B: transverse section of a spore through the polaroplast with the two different zones, (En) endospore, (Ex) exospore, (Pf) polar filament, (Pdl) dense lamellar part of the polaroplast, (Pll) loose lamellar part of the polaroplast, (Pm) plasma membrane.

***Phylogenetic analysis***

In order to get a better understanding of the phylogenetic position of *C. legeri* nov. comb., the phylogeny of a 1246 bp fragment of the small subunit rDNA gene was re-examined. Sequences with the 15 highest BLAST scores were aligned with Clustal W. The small subunit rDNA sequences of *Nosema carpocapsae* and *N. bombi* were included as outgroups. These analyses confirmed that the nucleotide sequence of our microsporidium was most similar (95.7% identity) to that of *C. operophterae*, differing in 25 nucleotide positions. The divergence between the two sequences was comparable to that within the "*Endoreticulatus*" group, which contains *E. schubergi, E. bombycis* and two *Pleistophora* sp., and shows sequence identities of 96.7 – 98.6 %. The phylogenetic analyses were carried out using maximum parsimony (MP) and neighbour joining distance (NJ) methods. A consensus tree based on 500 bootstrap replicates for MP and NJ is given in Fig. 3. In all analyses our isolate clustered with *C. operophterae*, indicating a close relationship between the two organisms. The monophyletic clustering of the two proposed *Cystosporogenes* species with *V. corneae, V. corneum* and the *Endoreticulatus* group is well supported by both MP (98%) and NJ distance (99%). Although the relationship within the *Endoreticulatus* group is less well resolved, the formation of the *Endoreticulatus* group is also well supported by all methods used in these analyses.

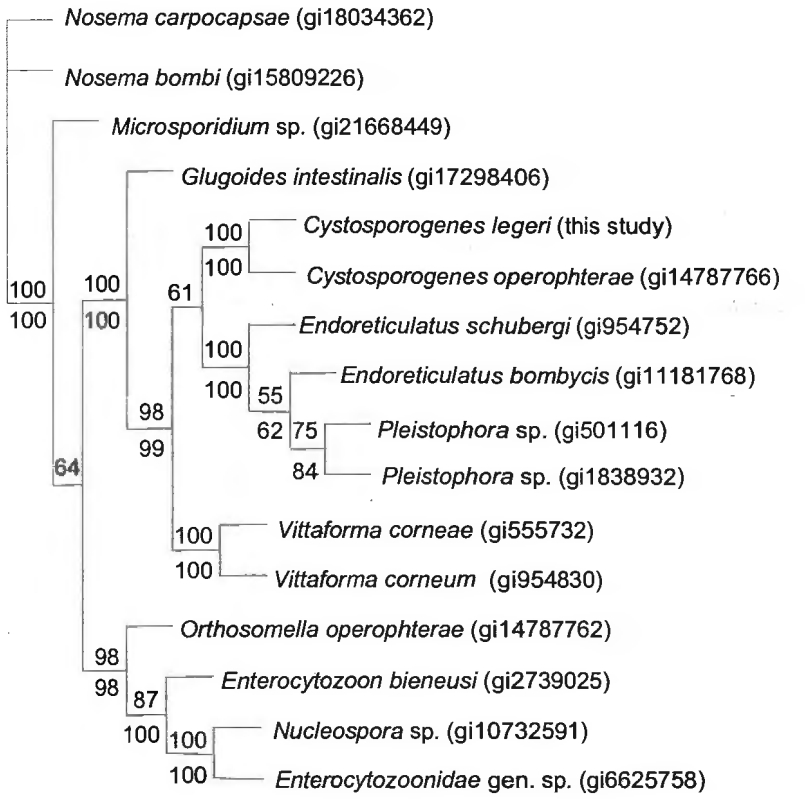


Figure. 3. Phylogenetic tree of 16 S rDNA sequence data of 16 species of Microsporidia. The tree shows the majority rule consensus tree of bootstrap analysis applying maximum parsimony (500 replicates) and neighbour joining distance (500 replicates) algorithms. Numbers on the tree branches indicate the percentage of bootstrap replicates which supported the given topology by MP (above lines) and NJ (below lines). *N. carpocapsae* and *N. bombi* were included as outgroups.

## Acknowledgements

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# Occurrence of pathogens in bark beetles (Coleoptera, Scolytidae) from Alpine pine (*Pinus cembra* L.)

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**Abstract:** Log sections, branches and twigs from bark beetle infested *Pinus cembra* trap logs were collected in a managed *P. cembra* forest in the central Alps of Austria (Lienz, East Tyrol). The material was incubated in breeding chambers in the insectary of the Institute, and emerging bark beetles were removed daily, determined, dissected and checked for infections with pathogens under a light microscope. In *Ips amitinus* Eichh., four different pathogenic protozoan species could be observed – Rhizopoda, Amoebina: *Malamoeba scolyti*, Apicomplexa, Eugregarinida: *Gregarina typographi*, Apicomplexa, Neogregarinida: *Mattesia* sp. and Microspora: *Chytridiopsis typographi*. Another *Gregarina* sp. (Apicomplexa, Eugregarinida) was found in *Pityogenes conjunctus* Reitt. which was the only pathogen in this beetle species. No pathogens could be diagnosed in *Hylurgops glabratus* Zett., *Tomicus piniperda* L., *Polygraphus grandiclava* L. and *Dryocoetes autographus* Ratz.

## Introduction

In the Austrian Alps *Pinus cembra* is a very important tree species, because it is very well adapted to the rough climate in high altitude, occurring up to the timber line. In some areas of the Alps bark dwelling insects can cause severe problems to *P. cembra* and *Larix decidua*. Since the last few years a bark beetle outbreak in some *P. cembra* stands in east Tyrol resulted in attack of living trees and lead to a dramatically increase of tree mortality. The main problem with the forests in these areas is that they have multifunctional values, *P. cembra* has not only an economical value (wood production), but also an ecological (protection of landscape against erosion) and a social value (prevention of villages in the valleys against avalanches and torrents). Control of bark beetles and of other bark and wood boring insects is still an unsolved problem, mostly restricted to removal of currently infested trees. This practice is not possible in all cases, especially in cliffy areas it is usually not easy or impossible to salvage these trees.

The occurrence and epizootiology of pathogens in bark beetles is one of the least studied aspects in their population dynamics. Recent studies brought evidence of several new pathogen species in bark beetles from Norway spruce (*Picea abies*) (Wegensteiner *et al.*, 1996; Haidler, 1998; Händel, 2001; Händel *et al.*, 2003; Haidler *et al.*, 2003), Scots Pine (*Pinus sylvestris*) (Kohlmayr, 2001; Zitterer, 2002; Kohlmayr *et al.*, 2003) and white fir (*Abies alba*) (Weiser *et al.*, 1995). No reports exist about the pathogen complex in bark beetles breeding on Alpine pine (*P. cembra*), this fact was the reason to initiate a first preliminary study.

## Material and methods

Log sections, branches and twigs from bark beetle infested *P. cembra* trap trees were cut off in July 2001 in a managed *P. cembra* forest in the central Alps of Austria (Oberleibnig, Lienz,

East Tyrol) at three sites, in 1800m (site no. 1), 1900m (site no. 2) and 1980m (site no. 3) altitude.

The collected material was incubated (separately for each sampling site) in breeding chambers in the insectary of the Institute at 24°C (± 2°C) and at long day conditions (L:D = 16:8). All emerging bark beetles were removed daily, determined and counted. Some of them were dissected according to the method described in Wegensteiner et al. (1996), and were checked for the occurrence of pathogens under a light microscope (Nikon E 800, at magnifications 20x to 1000x).

## Results and discussion

In total, 6097 bark beetle specimens emerged from the incubated material. The absolutely dominant species was *Ips amitinus* Eichh. (5957 beetles), whereas *Pityogenes conjunctus* Reitt., *Hylurgops glabratus* Zett., *Tomicus piniperda* L., *Polygraphus grandiclava* L. and *Dryocoetes autographus* Ratz. emerged only in very small numbers.

In 548 dissected *I. amitinus*, four different entomopathogenic protozoan species could be observed: Rhizopoda, Amoebina: *Malamoeba scolyti* in the cells of the Malpighian tubules and of the midgut epithelium (size of cysts: 2.4-4.8 µm x 4.8-7.2 µm), Apicomplexa, Eugregarinida: *Gregarina typographi* in the midgut lumen (size of gamonts: 53-67 µm x 82-115 µm), Apicomplexa, Neogregarinida: *Mattesia* sp. free in the haemolymphe and in the adipose tissue (size of spores: 6.5-7 µm x 15-19 µm), and Microspora: *Chytridiopsis typographi* in the cells of the midgut epithelium (diameter of pansporoblasts: 12-14 µm). It was interesting to find conspicuous differences in pathogen spectrum and infection rates in the beetles from the three sites. *G. typographi* was found in the beetles from all three sites, and overall prevalence was highest for this species (18.1%), but prevalence was noticeable lower in the beetles from site no. 3. *C. typographi* was found only in the beetles from two sites, and in lower prevalence (11.2%-13.9%). *M. scolyti* and *Mattesia* sp. were found in beetles from one site only. Pathogen diversity was highest in the beetles from the (two lower) sites no. 1 and no. 2 (Table 1).

Table 1. Prevalence of different pathogen species in *I. amitinus* (in %) from the three sites (site no.1, 2 and 3), number of inspected beetles (n) and total numbers respectively average infection rates of the four pathogen species

| Site n° | n | *Malamoeba scolyti* | *Gregarina typographi* | *Mattesia* sp. | *Chytridiopsis typographi* |
|---|---|---|---|---|---|
| 1 | 188 | 0.5 | 18.6 | — | 11.2 |
| 2 | 231 | — | 26.0 | 0.9 | 13.9 |
| 3 | 129 | — | 3.1 | — | — |
| Total | 548 | 0.2 | 18.1 | 0.4 | 9.7 |

*P. conjunctus* was found emerging from branches and twigs from the sites no. 1 and 2. *Gregarina* sp. (Apicomplexa, Eugregarinida) (size of gamonts: 58 x 96 µm) was the only pathogen which could be observed in the midgut lumen of *P. conjunctus* (n = 12), the prevalence of this pathogen was relatively high (33.3% to 44.4%), but number of inspected beetles is very low (Table 2). This *Gregarina* sp., very similar to *G. typographi* in size and shape, is the first pathogen described from this bark beetle species.

Table 2. Prevalence of *Gregarina sp.* in *P. conjunctus* (in %) from the sites no. 1 and no. 2 (n = number of inspected beetles)

| Site n° | n | *Gregarina* sp. |
|---|---|---|
| 1 | 9 | 44.4 |
| 2 | 3 | 33.3 |
| Total | 12 | 41.7 |

The pathogen complex of the examined *I. amitinus* from *P. cembra*, was not very diverse, compared to results of this bark beetle species from Alpine Norway spruce stands in the studies of Haidler (1998) and Händel (2001). Totally new is the record of *M. scolyti* in *I. amitinus*, it is noticeable that cyst size is smaller than cysts of the type host, *D. autographus* (Purrini, 1978; Purrini, 1980). The evidence of spores of the neogregarinidan species *Mattesia* sp. in *I. amitinus* from *P. cembra* is also new. Until now, *Mattesia* sp. was known to infect the bark beetle hosts *Ips typographus* (Wegensteiner & Weiser, 1996a), *Pityogenes chalcographus* (Händel, 2001; Händel *et al.*, 2003) and *Ips acuminatus* (Zitterer, 2002). Spore size in *I. amitinus* is comparable to the size in other bark beetle species. The prevalence of this pathogen species was quite low in *I. amitinus*, similar to the results in all other bark beetle hosts. The occurrence of *G. typographi* and *Ch. typographi* in *I. amitinus* is known from former investigations (Haidler, 1998; Händel, 2001; Händel *et al.*, 2003; Haidler *et al.*, 2003).

Surprisingly, no pathogens were found in *H. glabratus* and *D. autographus*, two species which attack normally Norway spruce, and in *T. piniperda* and *P. grandiclava* (breeding only in *Pinus* spp.). Several pathogens were described from *H. glabratus*, *D. autographus* and *T. piniperda* (Haidler, 1998; Händel, 2001; Kohlmayr, 2001; Händel *et al.*, 2003; Haidler et al., 2003; Kohlmayr et al., 2003). To date, no pathogens were reported on *P. grandiclava*. In any case, the numbers of emerged respectively of inspected beetles were too low (altogether only 9 individuals) to get representative data on pathogen occurrence in these beetle species attacking *P. cembra*.

Until now, it is not clear if phloem quality of host trees might limit the occurrence and prevalence of pathogens in bark beetles. For Norway spruce it is known that quality of phloem changes during the seasons (Moritz & Führer, 1988); Wegensteiner & Weiser (1996b) and Händel (2001) argued that this most probably influences prevalence and incidence of pathogens.

However, a more representative number of beetles should be examined during further investigations, to get more reliable data on the pathogen complex of all the bark beetle species breeding on *P. cembra*.

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# Laboratory evaluation of *Malamoeba scolyti* Purrini (Rhizopoda, Amoebidae) in different bark beetle hosts (Coleoptera, Scolytidae)

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**Abstract**: Different bark beetle species (*Dryocoetes autographus, Tomicus piniperda, Hylurgops palliatus, Hylastes ater, Polygraphus poligraphus, Pityogenes chalcographus, Pityogenes calcaratus, Ips typographus, Ips sexdentatus, Ips laricis*) were found to be sensitive to *Malamoeba scolyti* infections in the laboratory. Infection rates were very different depending on number of cysts offered in drinking water or on phloem chips.

**Key words:** *Malamoeba scolyti, Dryocoetes autographus, Tomicus piniperda, Hylurgops palliatus, Hylastes ater, Polygraphus poligraphus, Pityogenes chalcographus, Pityogenes calcaratus, Ips typographus, Ips sexdentatus, Ips laricis*, artificial infection, host spectrum, bark beetles, Scolytidae.

## Introduction

Protozoa causing diseases in bark beetles have been subject of several investigations, but the number of specific publications is still limited (ref. *in* Mills, 1983; ref. *in* Bathon, 1991; ref. *in* Wegensteiner *et al.*, 1996). Purrini (1978) was the first to describe a pathogen of the genus *Malamoeba* from the bark beetle *Dryocoetes autographus*. Later Purrini (1980) classified this new species, concerning observations in *D. autographus* and in *Hylurgops palliatus* as *Malamoeba scolyti* n.sp. First tests to infect *Pityogenes chalcographus* with *M. scolyti* were partly successful (Purrini & Führer, 1979). Further research on the abundance and distribution of *M. scolyti* in *D. autographus* from six localities in northern and north-western Germany revealed that this pathogen regularly occurred in adults of this bark beetle species in all examined populations (Kirchhoff & Führer, 1985). These results stimulated some additional research work on experimental infection analysis and on the life-cycle of *M. scolyti* in *D. autographus*. Artificial infection experiments including histological studies revealed new and more detailed knowledge regarding the life-cycle and the development of *M. scolyti* in *D. autographus* (Kirchhoff & Führer, 1990). Subsequent research, published in this paper, focused on physiological and ecological host spectrum of *M. scolyti*.

## Materials and methods

Infection experiments with *M. scolyti* were started in the laboratory with *D. autographus* young beetles which descended from an infection-free field population. They were reared in the insectary on water-soaked log sections of Norway spruce (*Picea abies*) at 24°C (± 1°C), 98% relative humidity (Zwölfer, 1932) and at long day conditions (L:D = 18:6). An *in vivo* culture of *M. scolyti* from homogenized midguts and Malpighian tubules of infected adult

*D. autographus* was conducted by continuously infection of these beetles, which guaranteed permanent fresh *Malamoeba* cysts. Beetles to be infected had starved for 12 to 24 hours just before start of the experiments. Afterwards the artificial infection was performed by offering either cysts in Aqua dest., tap water or in insect Ringer (via drinking), or by offering cyst-contaminated spruce phloem chips (via eating) for 24 hours (Purrini & Führer, 1979). After this period all beetles obtained fresh phloem chips and remained in hygrostatic vessels at 20°C (± 1°C), 92% relative humidity (Zwölfer, 1932) and without light. Further infection experiments were conducted with nine different bark beetle species: *Tomicus piniperda, Hylurgops palliatus, Hylastes ater, Polygraphus poligraphus, Pityogenes chalcographus, Pityogenes calcaratus, Ips typographus, Ips sexdentatus, Ips laricis*. *P. chalcographus* and *P. calcaratus* originated from infection-free laboratory stocks, all the other beetles were gathered from infection-free field populations. They were fed with spruce or pine phloem chips with regard to their food preference. The midgut and the Malpighian tubules were checked post infection at day of death; after four weeks all remaining beetles were dissected. Beetles that had died within a one or two days period post-infection, were excluded from analysis as well as dead beetles with *Beauveria bassiana*-infection.

## Results and discussion

Artificial infection of *D. autographus* with *M. scolyti* in the laboratory was successful, eating *M. scolyti* cyst-inoculated phloem chips led to higher infection rates (50.0% to 86.2%) than drinking *M. scolyti* cyst suspension, even offering a high dosage (0.0% to 20.0%)(Table 1). Infection rates increased in correlation with number of inoculated cysts on phloem chips, no significant differences were found in using cysts from grinded guts or from faeces. These experiments show also, that the phloem chips + Aqua dest. cyst suspension (100 000 cysts/ml) resulted in a higher infection rate (81.1%) than the same cyst concentration in tap water (50.0%) ($p < 0.001$). Suspending the cysts in Ringer solution (400 000 cysts/ml) was less successful (55.2% infection) in comparison with the same concentration in Aqua dest. (86.2% infection) ($p < 0.001$). Offering a suspension of less than 50 000 cysts/ml (A. dest.) via drinking caused no infection at all, but the same cyst concentration offered via inoculated phloem chips caused infection in 61.7% of the beetles (Table 1).

Table 1: Infection of *D. autographus* with *M. scolyti*, offering different cyst concentrations on phloem chips (ph.chips) by use of tap water, insect Ringer solution (Ringer), Aqua dest. (A.dest.) or using cysts from excrements (ex.), or offering different cyst concentrations in drinking water (A.dest-drink).

| Variant | No. of cyst | No. of beetles | Infection rate |
|---|---|---|---|
| ph.chips + tap water | 100 000 | 40 | 50.0 % |
| ph.chips + Ringer | 400 000 | 58 | 55.2 % |
| ph.chips + A.dest. | <50 000 | 107 | 61.7 % |
| ph.chips + A.dest. | 75 000 | 148 | 75.0 % |
| ph.chips + A.dest. | 100 000 | 90 | 81.1 % |
| ph.chips + A.dest. | 400 000 | 159 | 86.2 % |
| ex.+A.dest.+ph.chips | <50 000 | 28 | 64.3 % |
| A.dest.-drink | 350 000 | 20 | 20.0 % |
| A.dest.-drink | <50 000 | 20 | 0.0 % |

All other beetle species were successfully infected via drinking *M. scolyti* cyst suspensions. Infection rates were high in *H. ater* and (except tap water) in *H. palliatus* (up to 90.0%) in correlation with higher inoculation dosages, compared to *T. piniperda* and *P. poligraphus* (Table 2). Infection rates were lower in all other species, relatively high in *I. typographus* (tap water) and *I. sexdentatus,* when offering low cyst dosages, but no infection could be found in *I. typographus* which had nematodes in the midgut lumen; use of Ringer had no positive effect (Table 2). Purrini & Führer (1979) found similar infection rates in *P. chalcographus*, but in contrast, presence of Nematodes in the haemocoel promoted *M. scolyti* infection rates.

Table 2: Infection of different bark beetle species (in %) after drinking different suspension liquids with different concentrations of *M. scolyti* cysts (n = number of inspected beetles)

| Bark beetle species | Variant | No. of cysts | n | Infection rate |
|---|---|---|---|---|
| *Hylurgops palliatus* | tap water | 100 000 | 60 | 15.0 |
| | A. dest. | 300 000 | 75 | 58.7 |
| | A. dest. | 650 000 | 10 | 90.0 |
| *Hylastes ater* | A. dest. | 750 000 | 10 | 50.0 |
| *Polygraphus poligraphus* | A. dest. | 100 000 | 20 | 10.0 |
| *Tomicus piniperda* | A. dest. | 100 000 | 60 | 23.3 |
| | A. dest. | 100 000 | 26 | 35.0 |
| *Pityogenes chalcographus* | A. dest. | 500 000 | 70 | 20.0 |
| | A. dest. | 650 000 | 80 | 6.3 |
| *Pityogenes bidentatus* | A. dest. | 100 000 | 25 | 28.0 |
| *Pityogenes calcaratus* | A. dest. | 600 000 | 40 | 30.0 |
| | A. dest. | 800 000 | 60 | 28.3 |
| *Ips typographus**1 | tap water | 50 000 | 50 | 28.0 |
| | A. dest. | 300 000 | 100 | 15.0 |
| *Ips typographus**2 | A. dest. | 50 000 - 150 000 | 325 | 0.0 |
| *Ips laricis* | A. dest. | 150 000 | 16 | 31.3 |
| | Ringer | 450 000 | 60 | 22.0 |
| *Ips acuminatus* | A. dest. | 750 000 | 30 | 23.3 |
| *Ips sexdentatus* | A. dest. | 75 000 | 10 | 30.0 |
| | A. dest. | 100 000 | 50 | 16.0 |
| | A. dest. | 350 000 | 41 | 14.6 |

*1 without nematodes in the midgut; *2 with nematodes in the midgut

Check of field beetles approved the physiological host range of *M. scolyti* also as ecological host range for most of the lab-tested beetle species. *M. scolyti* was found in field populations of *Hylurgops glabratus* (Haidler, 1998), in *H. palliatus* and *Hylastes cunicularius* (Händel, 2001), in *I. typographus* (Wegensteiner, 1994; Wegensteiner *et al.*, 1996; Haidler, 1998; Händel, 2001), in *Ips amitinus* (Händel & Wegensteiner, 2005) and in *I. acuminatus* (Zitterer, 2002). Similar to artificial infection, *M. scolyti* infection rates were always lower in all these field collected beetles compared to the prevalence of *M. scolyti* in *D. autographus* (Haidler, 1998; Händel, 2001). However, a broad host spectrum is a promising requirement for successful introduction and for persistence of pathogens in a local bark beetle community.

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# Mixing entomopathogens

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# Preliminary survey on the occurrence of entomopathogenic nematodes and fungi in Albanian soils

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**Abstract**: The natural occurrence of entomopathogenic nematodes and fungi was investigated in Albanian soils using the *Galleria* baiting technique, during the period September 2000 - June 2001. Various biotopes (woodlands, orchards, seacoast, grasslands, field and uncultivated lands) were sampled in 69 different sites. Entomopathogenic fungal strains were obtained from 16 sites (23%). There were 2 entomopathogenic species: *Beauveria bassiana* (6%) and *Metarhizium anisopliae* (3%). Strains of *Penicillium* sp. (1%), *Aspergillus* sp. (1%) and *Fusarium* sp. (12%) were also isolated but these common species in soils are considered to have no entomopathogenic potential. Entomopathogenic nematodes, i.e. a strain of *Steinernema carpocapsae*, were obtained from just one soil sample. This preliminary survey is the first report on the occurrence of entomopathogenic fungi and nematodes in Albania.

**Key words**: *Steinernema carpocapsae, Beauveria bassiana, Metarhizium anisopliae*, occurrence

## Introduction

Entomopathogenic fungi and nematodes play an important role in controlling insect populations in nature and have great potential as biocontrol agents for use in microbial pest control. Until now, no data were available on their occurrence and importance in soils from Albania. This study is a preliminary contribution to the knowledge of the natural occurrence of these beneficial organisms in Albanian soils.

## Material and methods

The natural occurrence of entomopathogenic nematodes and fungi was investigated in Albanian soils using the *Galleria* baiting technique (Bedding & Akhurts, 1975; Zimmermann, 1986). Surveys were carried out during the period September 2000 - June 2001. Various biotopes (woodlands, orchards, seacoast, grasslands, field and uncultivated lands) were sampled in 69 different sites in 4 administrative regions of Albania (Tiranë, Durrës, Shkodër and Vlorë) were sampled with different biotopes: woodland, orchard, seacoast, grassland, field and uncultivated lands (Fig. 1).

Approximately 10 kg of soil were collected for each site by pooling 5-6 samples of ~2 Kg taken at depths of 20-30 cm. The soil was transported in sterile plastic bags to the laboratory. Nematodes were checked using 5 final instars of *Galleria mellonella* as bait insects placed in a long-handled tea infuser in the middle of each sample; then the samples were moistened and incubated at 25 °C for 7-10 days. The isolation of fungi was performed according to Zimmermann (1986).

Infected wax moth larvae from each sample were surface-sterilized by keeping them for 3 min in 1% sodium hypochlorite and rinsing them in distilled water. Then, the larvae were

incubated at 25 °C in Petri dishes with moistened filter paper until the presence of pathogens could be assessed.

## Results and discussion

Parasitic fungi were obtained from 16 sites of 69 locations (23%). They were found to be distributed from 20 to 180 m a.s.l. and were collected in all habitats. The soil pH ranged from 7.2 to 8.4 and organic content from 0.27 to 3.8%.

There were two entomopathogenic species isolated: *Beauveria bassiana* (Balsamo) Vuillemin (6%) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (3%). *Penicillium* sp. (1%), *Aspergillus* sp. (1%) and *Fusarium* sp. (12%) were also isolated. The three latter species are opportunistic and saprophytic common fungi in soils and are considered as having no entomopathogenic potential (Table 1; Fig. 2).

Entomopathogenic nematodes were obtained from just one soil sample, from which a *Steinernema carpocapsae* strain was isolated.

This preliminary survey is the first report on the occurrence of entomopathogenic fungi and nematodes in Albania. The data are related to a few localities and provide indicative informations only. Further investigations are needed to provide more exhaustive information.

The various isolates of *B. bassiana*, *M. anisopliae* and *S. carpocapsae* are currently under study to determine their peculiarities and ecological characteristics.

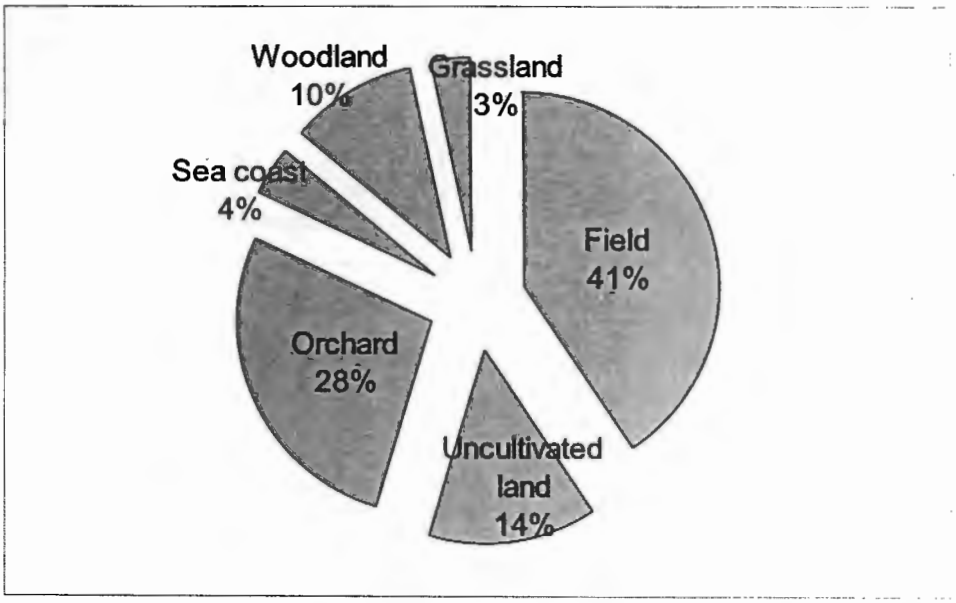


Figure 1. Different types of biotopes investigated (%) in Albania for the occurrence of entomopathogenic fungi and nematodes in soils.

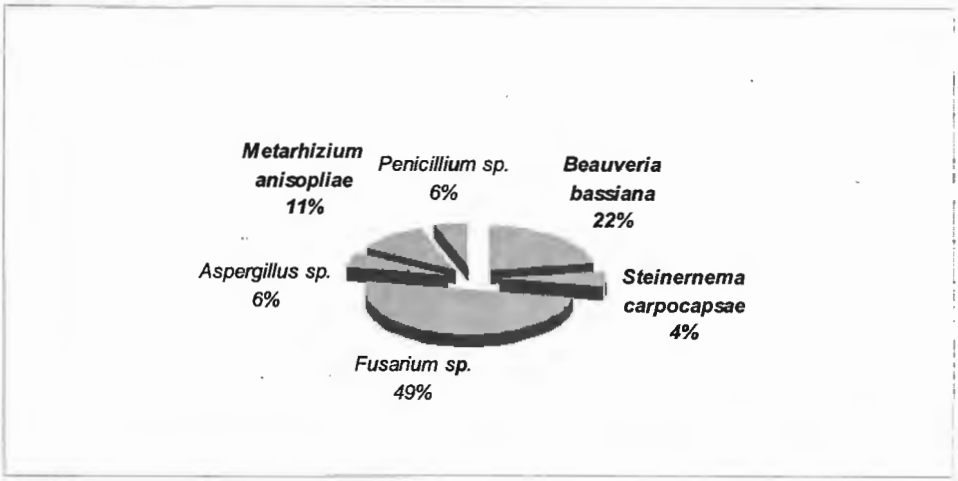


Figure 2. Respective percentage of the entomopathogenic fungi and nematodes isolated from various Albanian soils in a preliminary survey.

Table 1. Entomopathogenic fungi and nematodes isolated from various Albanian soils: localities, corresponding altitude,date of sampling,type of soil.

| N° strain | Locality | Altitude (m a.s.l.) | Date of collection | Habitat | Strain (EPN or EPF) | Soil texture |
|---|---|---|---|---|---|---|
| Alb-2 | Tiranë | 180 | Sept. 00 | Field | *Penicillium*/*Aspergillus* | Clay |
| Alb-5 | Durrës | 50 | Oct. 00 | Field | *Steinernema carpocapsae* | Loamy sand |
| Alb-7 | Qerek | 180 | Oct. 00 | Orchard | *Beauveria bassiana* | Silt |
| Alb-8 | Krujë | 40 | Oct. 00 | Field | *B. bassiana* | Sand |
| Alb-9 | Krujë | 40 | Oct. 00 | Field | *B. bassiana* | Sand |
| Alb-12 | Jaruja | 120 | Oct. 00 | Orchard | *Fusarium* sp. | Silt loam |
| Alb-13 | Qerek | 180 | Oct. 00 | Field | *Fusarium* sp. | Silt |
| Alb-14 | Lundra | 180 | Oct. 00 | Orchard | *Beauveria bassiana* | Silt |
| Alb-17 | Lundra | 180 | March 01 | Orchard | *Fusarium* sp. | Silt loam |
| Alb-18 | Vlorë | 20 | March 01 | Orchard | *Fusarium* sp. | Sand |
| Alb-23 | Vlorë | 20 | May 01 | Sea cost | *Metarhizium anisopliae* | Sand |
| Alb-25 | Jonufer | 30 | May 01 | Orchard | *Fusarium* sp. | Sand |
| Alb-27 | Quinam | 300 | May 01 | Woodland | *Fusarium* sp. | Silt |
| Alb-29 | Jonufer | 30 | May 01 | Orchard | *Fusarium* sp. | Loamy sand |
| Alb-32 | Qerek | 180 | May 01 | Field | *Metarhizium anisopliae* | Silt |
| Alb-34 | Krujë | 40 | May 01 | Woodland | *Fusarium* sp. | Loamy sand |
| Alb-38 | Durrës | 50 | May 01 | Field | *Fusarium* sp. | Sand |

## Acknowledgements

The authors express their gratitude to Gisbert Zimmermann (Federal Biological Research Centre for Agriculture and Forestry, Darmstadt, Germany) for the identification of entomopathogenic fungal species.

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# Laboratory evaluation of microbial control products on the Mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae)

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**Abstract**: The efficacy of several microbial control products on the Mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera, Pyralidae) was investigated. The products, that have been tested by mixing with (or spraying on) the diet of the pest, were as follows: Agree WP (*Bacillus thuringiensis* subsp. *kurstaki* / subsp. *aizawai*), Dipel 16.000 WP (*B.t.* subsp. *kurstaki*), Dipel 32.000 WP (*B.t.* subsp. *kurstaki*), Bactospeine WP (*B.t.* subsp. *kurstaki*), Xentari WG (*B.t.* subsp. *aizawai*), BMP 123 WP (*B.t.* encapsulated d-entotoxin), Botanigard ES (*Beauveria bassiana* strain GHA 11.3%), Naturalis (*B. bassiana* strain JW-1, 11.3%), Spod-X (*Spodoptera exigua* multicapsid nuclear polyhedrosis virus – SeMNPV), Mamestrin (*Mamestra brassicae* MNPV), Madex (*Cydia pomonella* granulosis virus – CpGV) and GemStar (*Helicoverpa zea* single capsid nuclear polyhedrosis virus – HzSNPV). The efficacy of Agree, Bactospeine and Dipel (both formulations) was > 96% at the recommended doses, whereas the efficacy of BMP, Xentari, Botanigard and Naturalis was 38.2, 23.7, 77.6 and 61.4% respectively. The efficacy of the viral insecticides was 11.9-31.4% for Spod-X and less than 6.0% for the other products (Madex, Mamestrin, GemStar).

**Key words**: *Ephestia kuehniella*, *Bacillus thuringiensis*, *Beauveria bassiana*, virus, microbial control.

## Introduction

Grains stored and processed in mills are subject to infestation by insect and mite pests. Especially, flour production can be disturbed by the presence of the Mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera, Pyralidae). For many years control of *E. kuehniella* in mills has been carried out by means of methyl bromide fumigations. However, methyl bromide is an ozone depleting material and it is to be phased out in industrial countries within the coming years. Although *E. kuehniella* can be controlled by a number of pesticides, no method exists that can completely replace methyl bromide in mills (UNEP, 1995). There is therefore a need to find alternative control measures.

In this context we started a research programme to determine the potentialities of several commercial microbial insecticides for the effective control of the Mediterranean flour moth. The aim of this contribution is to present the preliminary results gained from laboratory evaluation.

## Material and methods

The tested products contained bacteria, viruses or fungi. The products based on the active endotoxin (protein) of *Bacillus thuringiensis* were applied in the form of wettable powder at different proportions. The products containing fungus (i.e. *Beauveria bassiana*) or viruses (several baculoviruses) were applied in the form of emulsion.

Two treatments were carried out. The products that tested in the first treatment were:

1. Bactospeine WP (*B. thuringiensis* subsp. *kurstaki*, 16.000 I.U./mg),
2. Agree WP (strain GC-91 *B. t.* subsp. *kurstaki* / subsp. *aizawai*, 25.000 I.U./mg.),
3. Xentari WG (*B.t.* subsp. *aizawai*, 15.000 I.U./mg),
4. Dipel 16.000 WP (*B.t.* subsp. *kurstaki*, 16.000 I.U./mg),
5. Dipel 32.000 WP (*B.t.* subsp. *kurstaki*, 32.000 I.U./mg),
6. BMP 123 WP (*B.t.* encapsulated d-entotoxin, 32.000 I.U./mg),
7. Botanigard ES (*Beauveria bassiana* strain GHA 11,3%),
8. Naturalis (*B. bassiana* strain JW-1, 11,3%),
9. Spod-X (*Spodoptera exigua* multicapsid nucleopolyhedrovirus – SeMNPV).

In the second treatment, the insecticidal baculoviruses products tested were as follows:

1. Spod-X (*Spodoptera exigua* multicapsid nucleopolyhedrovirus – SeMNPV),
2. Mamestrin (*Mamestra brassicae* multicapsid nucleopolyhedrovirus – MbNPV),
3. Madex (*Cydia pomonella* granulosis virus – CpGV),
4. GemStar (*Helicoverpa zea* single capsid nucleopolyhedrovirus – HzSNPV).

For each product, three trials were carried out using plastic cylindrical dishes 2 cm height and 9 cm width in laboratory conditions (temperature: 26±1°C, relative humidity: 60±2% and photoperiod: 16 hours light / 8 hours dark). In the first treatment, the products were mixed in three doses with (or sprayed on) flour (A: recommended dose; B: 1/2 of the recommended dose, and C: 1/4 of the recommended dose), whereas only one dose, the recommended one, were applied in the second treatment. Each trial was carried out by adding 3x20 second-instar *E. kuehniella* larvae to the mixture diet. The dishes were observed daily for 10 days, and the number of dead larvae recorded.

The efficacy data were calculated using Abbott's formula (Kurstak, 1982). Mortalities after 10 days were compared through the Tukey-Kramer (HSD) test (Sokal & Rolf, 1995) using the statistical package JMP (Shall *et al.*, 2001).

## Results and discussion

***First treatment***

The mortality of *E. kuehniella* larvae and efficacy of the microbial products are presented in Tables 1 and 2. Treatments with Dipell 32.000, Dipel 16.000 and Agree whatever the dose, and treatment with Bactospeine at the recommended dose were effective against *E. kuehniella* (efficacy > 90%). The doses A and B of Botanigard had efficacy 77.6 and 67.2 respectively. Less effective were the following formulations: Botanigard dose C, Bactospeine dose 2, BMP dose A and Naturalis, whatever the dose (efficacy: 38.2-63.8%). The least effective (9.1-31.4%) were Xentari and Spod-X, whatever the dose, as well BMP at doses B and C and Bactospeine at dose C. The efficacy of every product at the recommended dose is presented in Fig. 1.

***Second treatment***

In the second treatment the four viral preparations caused no significant mortality on the larvae compared to the control (Table 3).

Table 1. First treatment with microbial-based insecticides on *Ephestia kuehniella* larvae: HSD-test results.

| Bactospeine | | | | Agree | | | | Xentari | | | | Dipel 16.000 | | | | Dipel 32.000 | | | | BMP | | | | Botanigard | | | | Naturalis | | | | Spod-X | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | B | C | M | A | B | C | M | A | B | C | M | A | B | C | M | A | B | C | M | A | B | C | M | A | B | C | M | A | B | C | M | A | B | C | M |
| a | b | c | e | a | a | a | de | c | cd | cd | e | a | a | a | e | a | a | a | e | cb | c | cd | e | a | ab | b | e | b | b | bc | e | bc | bc | bc | de |

A: recommended dose, B: ½ of the recommended dose, C: ¼ of the recommended dose, M: Control
(different small letters represent differences among treatments at $P = 0.05$)

Table 2. First treatment: Efficacy of microbial-based insecticides on *Ephestia kuehniella* larvae.

| | Bactospeine | | | Agree | | | Xentari | | | Dipel 16.000 | | | Dipel 32.000 | | | BMP | | | Botanigard | | | Naturalis | | | Spod-X | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C |
| 1 | 3.3 | 0.0 | 0.0 | 3.3 | 1.7 | 1.7 | 0.0 | 0.0 | 0.0 | 1.7 | 3.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.7 | 1.7 | 0.0 | 5.0 | 6.7 | 3.3 | 0.0 | 0.0 | 0.0 |
| 2 | 3.3 | 0.0 | 0.0 | 10.0 | 5.0 | 1.7 | 3.4 | 0.0 | 0.0 | 10.7 | 3.6 | 1.8 | 11.9 | 0.0 | 0.0 | 1.7 | 3.4 | 1.7 | 3.3 | 5.0 | 1.7 | 5.2 | 8.6 | 5.2 | 0.0 | 1.7 | 1.7 |
| 3 | 6.7 | 6.7 | 3.3 | 35.0 | 30.0 | 10.0 | 6.8 | 1.7 | 1.7 | 43.6 | 23.6 | 25.5 | 59.3 | 30.5 | 0.0 | 0.0 | 7.1 | 0.0 | 8.3 | 5.0 | 6.7 | 10.3 | 19.0 | 10.3 | 3.4 | 3.4 | 3.4 |
| 4 | 20.0 | 15.0 | 8.3 | 67.8 | 55.9 | 22.0 | 8.5 | 3.4 | 1.7 | 70.9 | 52.7 | 49.1 | 86.4 | 67.8 | 52.5 | 0.0 | 7.3 | 0.0 | 18.6 | 8.5 | 15.3 | 26.3 | 26.3 | 24.6 | 10.5 | 8.8 | 7.0 |
| 5 | 33.3 | 23.3 | 13.3 | 84.7 | 67.8 | 37.3 | 13.6 | 6.8 | 1.7 | 87.3 | 72.7 | 58.2 | 100 | 89.8 | 72.9 | 3.6 | 10.9 | 1.8 | 55.2 | 37.9 | 39.7 | 29.8 | 31.6 | 28.1 | 5.7 | 9.4 | 1.9 |
| 6 | 48.3 | 28.3 | 18.3 | 94.8 | 84.5 | 48.3 | 15.3 | 10.2 | 6.8 | 90.9 | 80.0 | 65.5 | 100 | 89.8 | 84.7 | 9.1 | 14.5 | 1.8 | 63.8 | 46.6 | 48.3 | 31.6 | 31.6 | 29.8 | 25.0 | 15.4 | 5.8 |
| 7 | 65.0 | 35.0 | 23.3 | 94.7 | 91.2 | 61.4 | 15.3 | 10.2 | 8.5 | 98.2 | 90.9 | 85.5 | 100 | 100 | 91.1 | 18.2 | 14.5 | 1.8 | 67.2 | 53.4 | 48.3 | 38.6 | 36.8 | 31.6 | 25.0 | 19.2 | 15.4 |
| 8 | 76.7 | 38.3 | 23.3 | 100 | 96.5 | 77.2 | 16.9 | 13.6 | 10.2 | 100 | 92.7 | 90.9 | 100 | 100 | 100 | 20.0 | 14.5 | 3.6 | 67.2 | 58.6 | 51.7 | 47.4 | 43.9 | 33.3 | 28.8 | 25.0 | 19.2 |
| 9 | 85.0 | 38.3 | 25.0 | 100 | 96.3 | 87.0 | 18.6 | 15.3 | 13.6 | 100 | 96.4 | 100 | 100 | 100 | 100 | 21.8 | 14.5 | 5.5 | 70.7 | 63.8 | 56.9 | 57.9 | 54.4 | 42.1 | 28.8 | 28.8 | 25.0 |
| 10 | 96.7 | 55.0 | 26.7 | 100 | 100 | 90.6 | 23.7 | 16.9 | 15.3 | 100 | 100 | 100 | 100 | 100 | 100 | 38.2 | 20.0 | 9.1 | 77.6 | 67.2 | 63.8 | 61.4 | 56.1 | 45.6 | 31.4 | 27.5 | 23.5 |

A: recommended dose, B: ½ of the recommended dose, C: ¼ of the recommended dose

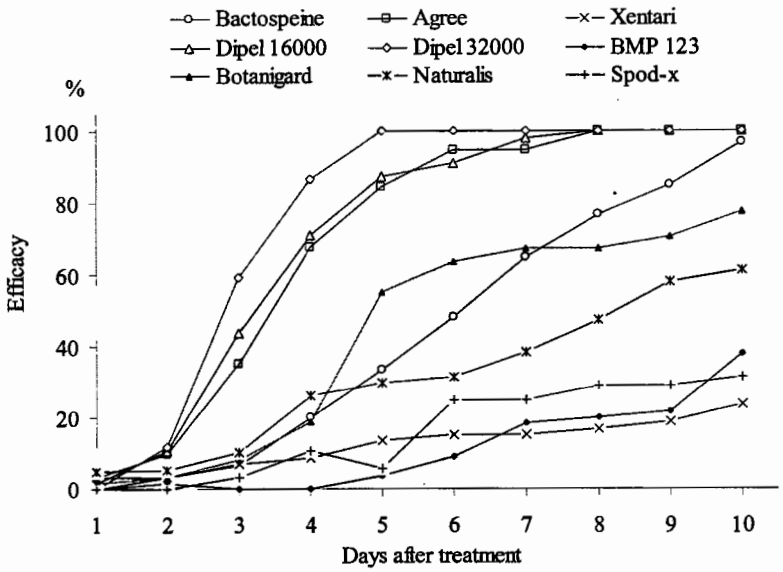


Figure 1. Efficacy of microbial-based insecticides on *Ephestia kuehniella* (recommended doses)

Table 3. Effect of four baculovirus-based products on larvae of *Ephestia kuehniella*.

| Product | Mortality after 10 days (%) | Efficacy (%) |
|---|---|---|
| Spod-X | 13.3a | 11.9 |
| Madex | 3.3a | 1.7 |
| Mamestrin | 6.7a | 5.1 |
| Gemstar | 3.3a | 1.7 |
| Control | 1.7a | |

Interestingly, the action of each biological product on *E. kuehniella* larvae was different, but the efficacy of the bacterial insecticides was obviously higher. *Beauveria bassiana*-based products appeared more active than the tested viral preparations. Further investigation is necessary in order to evaluate the full potential of microbial pesticides as biological control agents.

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# The potential of entomopathogenic fungi and nematodes against strawberry root weevil *Otiorhynchus ovatus* L. (Coleoptera, Curculionidae)

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**Abstract**: The sensitivity of *Otiorhynchus ovatus* adults to the selected strain of *Paecilomyces fumosoroseus,* previously isolated from this pest in the field conditions, was tested in the laboratory. The field experiment was carried out on strawberry plantation near Skierniewice. The fungus *P. fumosoroseus* was applied under strawberry plants as a water suspension after the harvest, in the end of July. At the same term the fungus *Beauveria bassiana* propagated on seeds of wheat was applied at the rate of 120 kg/ha with respect to a combination of entomopathogenic fungi, "Larvanem" based on entomopathogenic nematode *Heterorhabditis megidis* and insecticides Dursban 480 EC (chloropyrifos) and Mospilan 20 SP (acetamiprid) were used. In the laboratory experiment mortality caused by *P. fumosoroseus* depended on spore concentration and form of application. *P. fumosoroseus* applied as pipetted suspension controlled adults up to 52%. Mortality of 68% of adults specimens dipped in spore suspension $10^6$ $ml^{-1}$ of fungus was observed. In the field experiment both fungal strains showed good results in the control of *O. ovatus*. In combination with *P. fumosoroseus* and *B. bassiana* efficiency abtained 94,6% and 95,9% in relation to the control respectively and was comparable with the effect of insecticide. On both experimental fields very good effect against root weevils showed entomopathogenic nematodes. On the plots treated with "Larvanem" efficacy amounted 98,7% and 99,4% respectively and was higher than efficacy of fungi and insecticides as well.

**Key words**: entomopathogenic fungi, nematodes, strawberry root weevils, biological control

## Introduction

Soil pests are a problem on many cultivated plants in Poland and in other countries. On strawberry plantations localised in many regions of Poland, the strawberry root weevil *Otiorhynchus ovatus* is the most dangerous pest, but the black vine weevil *O. sulcatus* can also be found. Strawberry root weevil larvae usually damage mostly plants on older plantations. More than one hundred larvae of this pest were found on one strawberry plant. In Poland the most serious roots damage is caused by the later instar larvae in April and May or even in early June (Labanowska, 1994; Labanowska & Olszak 2003). If necessary, chloropyrifos or diazinon can be applied, either as a field preventive treatment before strawberry planting or after the harvest to control the pests.

Many researchers look for some new and more environmental friendly methods for the control of root weevils i.e. with use of entomopathogenic fungi (Zimmermann, 1981, 1982, 1984; Stenzel 1992; Vainio & Hokkanen 1993; Malinowski *et al.*, 2001) or entomopathogenic nematodes (Rutherford *et al.*, 1987; Vainio & Hokkanen 1993).

Entomopathogenic fungi are the most common pathogens isolated from larvae and adults of *Otiorhynchus* spp. (Zimmermann, 1981). They include *Beauveria bassiana*, *B. brongniartii*, *Metarhizium anisopliae*, *M. flavoviride*, *Paecilomyces farinosus*, *P. fumosoroseus* and *Verticillium lecanii*. Interestingly, *Paecilomyces fumosoroseus* is a restricting factor of *O. ovatus* population in field conditions in Poland (Mietkiewski *et al.*, 1992), with an average mortality of larvae and pupae caused by the fungus reaching 18%.

The aim of this study was to determine the potential of both fungi *P. fumosoroseus* and *B. bassiana* and nematodes as well against the strawberry root weevil *O. ovatus* in laboratory and field conditions.

## Material and methods

The sensitivity of *O. ovatus* adults to the selected strain of *P. fumosoroseus*, previously isolated from this pest in the field conditions, was tested in the laboratory. The specimens of pest were collected on an infested strawberry field in June 2001, then brought to the laboratory and placed in glass Petri dishes and treated with fungus spore suspension in two forms: a) pipetting to the dish in 1 ml of water on wet filter paper and b) dipping of adults for 15s. Each test consisted of 10 dishes with 10 specimens per dish. One group of beetles remained totally untreated as a control. Fresh leaves of strawberry were provided as a food every three days. Mortality was recorded daily.

The field experiment was carried out on strawberry plantation near Skierniewice. Complete blocks were the experiment design, each one was 60-200 sqm in size and containing 4 plots as replication. The fungus *P. fumosoroseus* was applied under strawberry plants as a water suspension after the harvest in the end of July. At the same term the fungus *B. bassiana* propagated on seeds of wheat was applied at the rate of 120 kg/ha with respect to a combination of entomopathogenic fungi, "Larvanem" based on entomopathogenic nematode *Heterorhabditis megidis* and insecticides Dursban 480 EC (chloropyrifos) and Mospilan 20 SP (acetamiprid) were used. Emulsified insecticides were used as plant and soil sprayings before bloom or after harvest and "Larvarem" only after harvest of strawberries.

The efficacy of the treatments was evaluated in June or early July by counting larvae, pupae and adults of the pest. Six plants per plot, i.e. 24 in each treatment, were removed and their roots and sieved soil were searched for pest presence. The results were analysed using analysis of variance. Means differences were evaluated with Duncan's multiple range "t" test at 0.05 significance level.

## Results and discussion

***Laboratory test***

In the laboratory experiment mortality of *O. ovatus* caused by *P. fumosoroseus* depended on spore concentration and form of application (Table 1). Fungus applied as a pipetted suspension with spores concentration of $10^5$ and $10^6$ $ml^{-1}$ caused the mortality of adults 32% and 52% respectively. Mortality of 68% of the beetles dipped in conidia suspension ($10^6$ $ml^{-1}$) was noted. On dead *O. ovatus* specimens apart from *P. fumosoroseus*, opportunistic or saprophytic fungi like: *Fusarium* sp. *Gliocladium sp.* and *Mucor sp.* were found.

Experiments in the laboratory to select virulent strains of fungi had been done earlier by several authors (Zimmermann & Simons 1986; Moorhouse *et al.*, 1990, Vainio & Hokkanen, 1993). In bioassays, *B. bassiana*, *M. anisopliae*, *M. flavoviride*, *P. farinosus* and *P. fumosoroseus* were tested, mainly against eggs or larvae. All stages of vine weevil were susceptible to these fungi; however, adults proved to be more resistant (Zimmermann, 1996).

***Field experiment***

In the field experiment both fungal species showed very good results in the control of root weevils. On the first plantation, fungus *B. bassiana* applied to the soil on seeds of wheat, attained 95,9% of efficacy and its effect was comparable with Dursban 480 EC (98,7%) and much better than Mospilan 20 SP (84,0%). Also *P. fumosoroseus* showed very good efficacy reducing pest population in 94,6% in relation to the untreated control (Tab.2). On the second plantation (Tab.3) efficacy of *B. bassiana* amounted 93,8% and was much better than Dursban 480 EC (58,3%) and Mospilan 20 SP (83,9%).

There are only few data on microbial control of *Otiorhynchus* spp. in outdoor or field experiments. Zimmermann & Simons (1986) reported a population reduction of about 30-50% in some outdoor experiments on strawberry and ornamentals after prophylactic treatment of *M. anisopliae.* The low levels of control may be due to suboptimal soil temperatures. Good results expressed in reduction up to 97,2% of *O. sulcatus* population caused by *M. anisopliae* in the field experiments on strawberries, were obtained by Moorhouse *et al.* (1990).

On both experimental fields very good effect against root weevils showed Larvanem based on the entomopathogenic nematode *Heterorhabditis megidis.* On the plots treated with Larvanem efficacy amounted 98,7% and 99,4% respectively and was higher than efficacy of entomopathogenic fungi and insecticides as well. Rutherford *et al.* (1987) studied the impact of entomopathogenic nematodes on *O. ovatus*, found, that *Steinernema feltie* F. was less effective against this pest than *Heterorhabditis* spp. Vainio & Hokkanen (1993) reported that *Otiorhynchus* larvae were highly susceptible to entomopathogenic *Steinernema* isolates in the laboratory. Some strains of *M. anisopliae* killed the pest larvae nearly as well as the nematodes but *B. bassiana* was less effective against root weevils than the other pathogens.

The persistence and activity of entomopathogenic fungi and nematodes in the soil depend on many environmental and soil factors such as temperature, moisture, soil substrate and also characteristic and number of host insects.

Results obtained in our experiment and also reported earlier by others authors demonstrated that an effective microbial control of weevils infested strawberry plants is possible, but still needs more ecological studies to understand the relation between pest and pathogens.

Table 1. Mortality (%) of *Otiorhynchus ovatus* adults caused by *Paecilomyces fumosoroseus* in laboratory experiments.

| **Factor of mortality** | **Concentration and form of application** | | | **Check (untreated)** |
|---|---|---|---|---|
| | $10^5$ (pipetting) | $10^6$ (pipetting) | $10^6$ (dipping) | |
| *Paecilomyces fumosoroseus* | 32.0 | 52.0 | 68.0 | - |
| *Fusarium* sp. | 1.0 | 6.0 | 4.0 | 1.0 |
| *Gliocladium* sp. | 9.0 | - | 4.0 | - |
| *Mucor* sp. | 11.0 | - | 2.0 | 2.0 |
| Unsporulated mycelium | 5.0 | 2.0 | - | 1.0 |
| Indefinited causes | 29.0 | 28.0 | 6.0 | 9.0 |
| Total dead specimens | 87.0 | 88.0 | 84.0 | 13.0 |
| Alive specimens | 13.0 | 12.0 | 16.0 | 87.0 |

Table 2. Efficacy of entomopathogenic fungi, insect parasitic nematodes and insecticides in the control of *Otiorhynchus ovatus* on strawberry plants in the field (I plantation).

| Combination | Rate | Average number of weevils per plant | % efficacy |
|---|---|---|---|
| Entomopathogenic fungi | | | |
| *Beauveria bassiana* | 120 kg/ha | 0.4 b* | 95.8 |
| *Paecilomyces fumosoroseus* | $10^6 ml^{-1}$ spores | 0.5 b | 94.6 |
| Nematodes | | | |
| Larvanem | $10^6$ larvae/$m^2$ | 0.1a | 98.7 |
| Insecticides | | | |
| Dursban 480 EC | 5.9 | 0.1 a | 98.7 |
| Mospilan 20 SP | 0.6 | 1.5 c | 84.0 |
| Control (untreated) | - | 9.3 d | - |

* means followed by the same letter do not differ significantly.

Table 3. Efficacy of *Beauveria bassiana,* nematodes and insecticides in the control of root weevils (Otiorhynchidae) on strawberry plants in the field (II plantation).

| Combination | Rate | Average number of weevils per plant | | | % efficacy |
|---|---|---|---|---|---|
| | | *O. ovatus* | *O. sulcatus* | *O. ovatus* + *O. sulcatus* | |
| Entomopathogenic fungus | | | | | |
| *Beauveria bassiana* | 120 kg/ha | 0.1 | 0.7 | 0.7 b* | 93.8 |
| Nematodes | $10^6$ | | | | |
| Larvanem | larvae/$m^2$ | 0.0 | 0.1 | 0.07 a | 99.4 |
| Insecticides | | | | | |
| Dursban 480 EC | 5.0 | 0.2 | 4.4 | 4.5 d | 58.3 |
| Mospilan 20 SP | 0.6 | 0.3 | 1.5 | 1.7 c | 83.9 |
| Control (untreated) | - | 4.8 | 3.9 | 10.7 e | - |

* means followed by the same letter do not differ significantly.

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# Evaluation of two microbial products and an insecticide for integrated thrips control in glasshouse Chrysanthemums

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**Abstract**: *Frankliniella occidentalis*, the Western Flower thrips, is a major pest species in Chrysanthemum. Lack of selective and effective pesticides for its control is one of the main causes for failure of introducing integrated pest management (IPM) programmes. In 2002 a glasshouse experiment with cut Chrysanthemum was carried out with the microbial pesticides Nemasys F (*Steinernema feltiae*, Becker Underwood) and Mycotal/Addit (*Verticillium lecanii*, Koppert BV). These microbial agents and a water control were applied weekly with a total of 9 applications. A chemical reference (Spinosad, Dow Agrosciences) was only applied twice, in the fourth and fifth week. To assess the effect on the main natural enemy of thrips, the thrips predator *Amblyseius cucumeris* (Thripex-plus, Koppert BV) was released in half of the plots.

Application of Nemasys F resulted in a highly considerable reduction (74%) of thrips in the Chrysanthemum crop. We found no evidence for a shift in sex ratio towards more male thrips due to Nemasys F. Mycotal/Addit gave rise to a less substantial reduction of thrips (38% after six weeks and 14% at harvest). One week after the second application of Spinosad 92% less thrips was found compared to water treated plots. At harvest, this was only 39% due to re-entry of thrips into the plots.

The predatory mites had a large effect on the thrips population (57% at harvest). When combined with the microbials this effect was enhanced up to 96% for Nemasys F and 90% for Mycotal/Addit. Spinosad had some negative effect on the predators directly after spraying, but six weeks after the last application, the predators were present in numbers comparable to the other plots, and contributed to the thrips control (90%).

**Key words**: chrysanthemum, *Frankliniella occidentalis*, glasshouse, *Steinernema feltiae*, *Verticillium lecanii*, Spinosad, microbial control, integrated pest management

## Introduction

The introduction of integrated pest management programmes in Chrysanthemum in the Netherlands has turned out to be very difficult. One of the main causes is thought to be the Western Flower thrips, *Frankliniella occidentalis,* and in particular the lack of selective and effective pesticides for its control. At present, in the soil-based cut Chrysanthemum cultivation only a few effective insecticides against thrips are permitted. Furthermore, most of these thripicides lack selectivity that is necessary for integration with natural enemies in integrated pest management (IPM) programmes. The aim of our research in cut Chrysanthemum is to develop a feasible IPM programme and therefore we evaluate microbial, botanical and synthetic pesticides and natural enemies.

In the experiment described below we compared the efficacy of two microbial pesticides and the insecticide "Spinosad" (Dow Agrosciences; Drinkall & Boogaard, 2001) against Western Flower thrips. A microbial pesticide on the base of the entomopathogenic nematode *Steinernema feltiae* is "Nemasys F" (Becker-Underwood), which is a product for foliar applications (Wardlow *et al.*, 2001). A second microbial pesticide is based on the entomopathogenic fungus *Verticillium lecanii*, "Mycotal" (Koppert BV). Mycotal is a

mycoinsecticide against whiteflies but has a side-effect on thrips (Helyer *et al.*, 1992; van der Schaaf *et al.*, 1991). It is advised to use Mycotal together with the wetter "Addit" (Website of Koppert) that is meant to improve the effectiveness of the fungus at less favourable periods of lower relative humidity. These two microbials and the insecticide were tested in a semi-practice situation with a cut Chrysanthemum cultivation that takes typically 9 - 12 weeks from planting through harvest. Also the effect of these products on the main natural enemy for thrips, the predatory mite *Amblyseius cucumeris,* was tested.

## Material and methods

***Experimental set up***

The experiment took place in May - July 2002 and was carried out in cut Chrysanthemum cultivars 'Euro' and "Windmill", in a glasshouse of 300 $m^2$ containing eight planting beds. Each bed was divided into eight plots of 2.5m x 1.0m with 160 plants each, resulting in 32 experimental plots with "Euro" and 32 plots with "Windmill".

One week after planting, larvae and adults of *Frankliniella occidentalis* were released in each plot, which resulted in an average of 1.5 thrips per plant in the second week after planting. The thrips originated from a culture on cut Chrysanthemum flowers.

***Treatments***

In four of the eight beds the predatory mite *Amblyseius cucumeris* was introduced once in the second week after planting (Thripex-plus, Koppert BV; one sachet/$m^2$). From the second week on, Nemasys F (*Steinernema feltiae,* Becker Underwood), Mycotal/Addit (*Verticillium lecanii*, Koppert BV) and control (water) treatments were applied weekly in eight plots with, and eight plots without predatory mites, with a total of nine applications. Nemasys F was sprayed in a concentration of $2.6*10^3$ IJs/mL, 125 mL/$m^2$ plus 0.03% wetting agent Agral. The crop was kept wet for at least two hours after spraying the nematodes. Mycotal/Addit was sprayed in a concentration of $10^7$ conidia/mL, 125 mL/$m^2$ and 0.25% Addit. The chemical reference (Spinosad, Dow Agrosciences) was only applied twice, in the fourth and fifth week (0.1%, 125 mlLm$^2$). In the control plots 125 mL/$m^2$ water was sprayed.

***Assessments and analyses***

Samples of the crop were taken for a pre-count in the second week, and subsequently in the fourth, sixth, and eleventh (harvest) week of the experiment, in order to assess the effect of the applications. From each plot the top 20 cm of 15 plants were cut and rinsed in 60% alcohol. The rinse fluid was subsequently sieved to count the Western Flower thrips and predatory mites present. For thrips the sex of the adults was determined.

Numbers of thrips and numbers of predatory mites were each statistically processed, using a three-way ANOVA with "cultivar", "presence/ absence of predatory mites" or "treatment" as factors. Since no significant interaction was found between the factors "cultivar" and "treatment" or "cultivar" and "presence/absence of predatory mites", the results for both cultivars were pooled.

## Results and discussion

***Four weeks after planting***

After three applications of Mycotal/Addit, Nemasys F or water and one day after the first application of the Spinosad, only Spinosad gave a significant lower number of thrips (Fig. 1A). At this time, the numbers of the predatory mite *Amblyseius cucumeris* emerging from the sachets were still very low (0.2 mite/plant) and no significant effect of the treatment on these numbers was found, although slightly fewer predatory mites were found in the Spinosad plots (see Fig. 2).

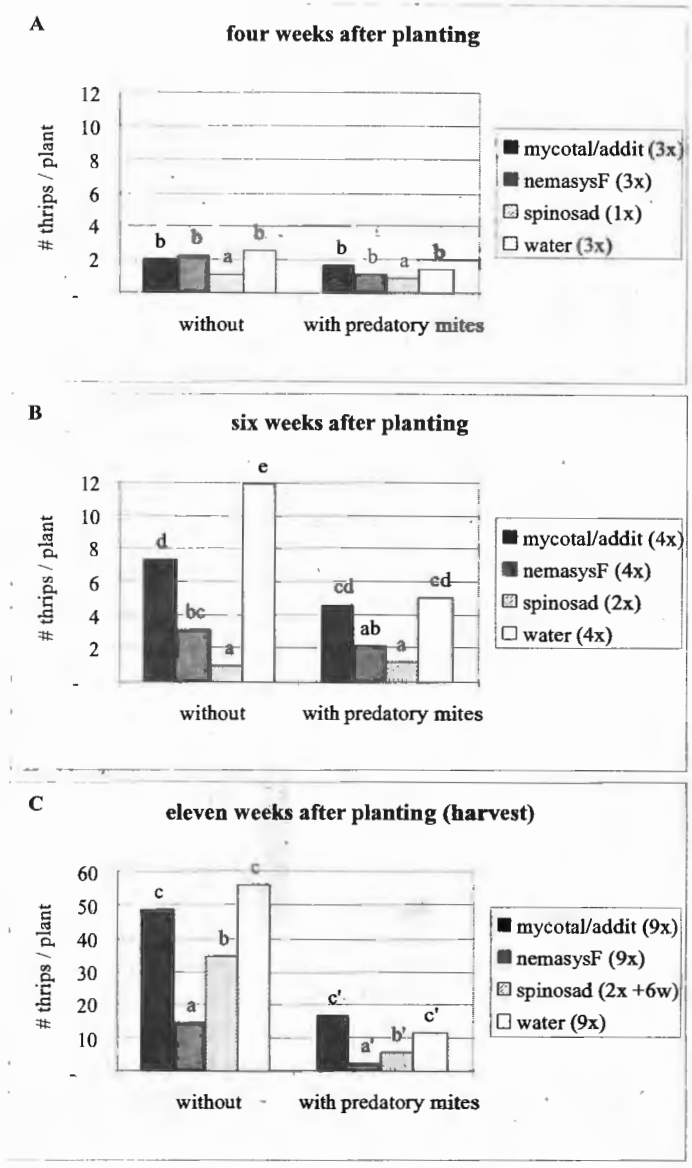


Figure 1. Numbers of *Frankliniella occidentalis* after treatment with Mycotal/Addit, Nemasys F, Spinosad or water, in the presence or absence of the predator mite *Amblyseius cucumeris*.

A) 4 weeks after planting, 1 day after 3rd application with Mycotal/Addit, Nemasys F or water, or 1 day after 1st application of Spinosad; a<b at P<0.001.

B) 6 weeks after planting, 6 days after 4th application with Mycotal/Addit, Nemasys F or water, or 6 days after 2nd application of Spinosad; a<b<c<d<e at P=0.016.

C) 11 weeks after planting, 4 days after 9th application with Mycotal/Addit, Nemasys F or water, or 6 weeks after 2nd application of Spinosad; a<b<c and a'<b'<c' at P<0.001. Numbers of thrips in presence of predatory mites < Numbers of thrips in absence of predatory mites (P<0.001).

*Six weeks after planting*

One week after four applications of Mycotal/Addit, Nemasys F and water and two chemical applications, large differences were evident between the treatments (Fig. 1B). In the plots without predatory mites, Mycotal/Addit had a moderate effect on the thrips population with 38% less thrips than in the water treated plots. In contrast, the effect of Nemasys F on the thrips population was considerable: 74% less thrips was found compared to water treatment. The treatments with Spinosad gave the best result: 92% less than in the water plots.

The presence of the predatory mites made a large difference in the water plots and accounted for 57% less thrips compared to the water plots without mites (Fig. 1B). An improvement of 10% (not statistically different) was found when the predatory mites were combined with Mycotal/Addit treatments. When comparing this combination to the control plots without predatory mites, 62% less thrips were found. The effect of the predatory mites was significantly improved with 58% when also Nemasys F treatments were applied. In comparison to water plots without predatory mites, the combination of these two treatments leaded to 82% less thrips. Treatment with Spinosad had the highest effect on thrips: 92% and 90% less than in the water treatment without and with predatory mites respectively. In these plots significantly lower numbers of predatory mites were present compared to the other treatments. Application of the microbial pesticides had a slight but not significant effect on the presence of predatory mites (see Fig. 2).

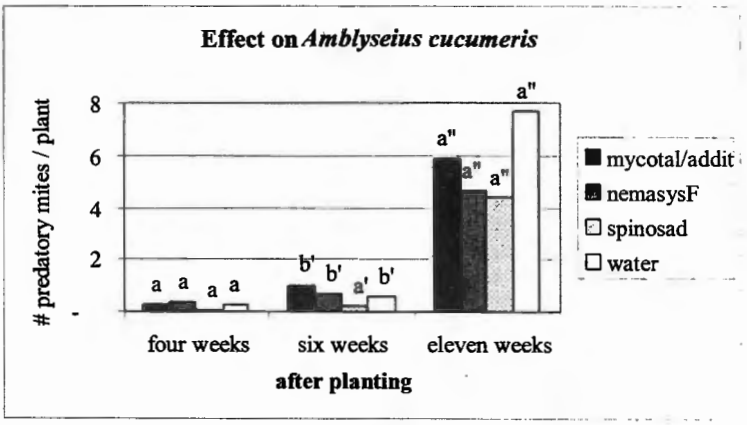


Figure 2. Numbers of *Amblyseius cucumeris* after treatments with Myctoal/Addit, Nemasys F, Spinosad or water, in plots where the predatory mite was released, at different times during experiment. At four and eleven weeks after planting: no significant differences between treatments; at six weeks after planting: a'<b' at P=0.014.

*At harvest*

At harvest, the plots with the microbials or water had received nine applications. In this final assessment the Nemasys F treatments resulted in the lowest numbers of thrips, both in the plots without (74% less than water) and with predatory mites (82% less than water; see Fig. 1C). The combined use of predatory mites and nematodes resulted in 96% less thrips compared to the water control without mites. It is expected that a treatment within an entire glasshouse would even increase the effectiveness of Nemasys F, since the plots with low thrips numbers were somewhat affected by neighbouring plots with higher numbers of thrips.

It was suggested by Wardlow *et al.*, (2001) that Nemasys F treatments results in a shift in sex ratio in favour of male thrips. However, our data do not support this. We found a shift in sex ratio towards *less* males with not only Nemasys F but also with all other treatments, including predatory mite introduction (data not shown). There seems to be a positive relation between thrips control rates and female biased sex ratios, suggesting that effective treatments had a larger effect on males than on female Western Flower thrips.

At the same time the Mycotal/Addit treatments resulted only in a slightly, but not statistically significant, better effect (14%) than the water treatments (Fig. 1C). In the plots with predatory mites even more thrips were found (though not a statistically significant difference). In earlier experiments in autumn 1996 and spring 1997, five applications of Mycotal (without Addit) reduced the number of thrips by 60% and 85% respectively (E. Beerling, unpublished results). Perhaps the climatic conditions in the current experiment were less favourable for development of the fungus. According to the producer, Addit improves effectiveness of the fungus at a lower relative humidity (Website of Koppert). In this experiment the relative humidity varied from 50% to 90% and the average daily temperature was 25 °C and at night 18 °C.

At harvest, the plots with Spinosad received their second and last treatment six weeks ago. Thrips re-entered the plots without predatory mites, which resulted in a much smaller difference (39%) between these and the water treated plots, compared to five weeks earlier. But in plots with predatory mites, the natural enemies were able to keep the thrips numbers low: 52% less thrips compared to water with, and 90% compared to water without mites (Fig. 1C).

There was a very significant difference in numbers of thrips between the plots with and without predatory mites (Fig. 1C). The numbers of mites were much higher than at earlier sampling dates (Fig. 2). There was no significant effect of the various treatments on the predatory mites, although the numbers in the water treated plots were highest. Hardly no predatory mites (<0.03 mites/plant; data not shown) were found in samples of plots were no mites were released.

With these results it was shown that Nemasys F and Mycotal/Addit, and to a lesser extend Spinosad, can be used safely in combination with the predatory mite *A. cucumeris*. The combined use of the microbials or chemical insecticide with the predators even caused a higher reduction of the Western Flower thrips population.

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# Natural enemies applied in biological pest control: pathogens in field and mass-reared populations

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**Abstract**: Pathogen-free natural enemies are important for obtaining high efficacy in biological pest control and reliable research data, as pathogens may affect the performance of their host or alter their reproduction and behaviour. In the present article we briefly summarize and up-date an earlier published literature review on pathogens, potential pathogens with unknown host effects, endosymbiotic *Wolbachia* spp., and unidentified diseases of natural enemies applied in biological pest control. Moreover, microscopic diagnostic studies of natural enemies applied in biological pest control are presented. As many as 151 references in literature including reports on viruses, bacteria, fungi, protozoa, and nematodes were found for 24 genera of natural enemies. The results are derived from field, laboratory, and mass-reared populations. During light and electron microscopic studies 18 unidentified potential pathogens and 6 determined pathogen species including viruses, bacteria, fungi, protozoa, and nematodes have been detected in 13 out of 15 investigated species of natural enemies. These results clearly demonstrate a potential threat of diseases in mass productions of beneficial arthropods and arthropods in general. Therefore, more research is needed to increase knowledge on potential pathogens and to develop feasible and reliable diagnostic methods for known pathogens.

**Key words**: biological control, natural enemies, disease, pathogen, review, viruses, protozoa, bacteria, fungi, nematodes

## Introduction

At present, about 125 natural enemies are reared for inundative and seasonal inoculative forms of biological control, with several species being applied throughout the world (van Lenteren, 2003). The success of a biological control programme is depending on many factors, including the quality of the beneficial arthropods that are used (van Lenteren, 2003). Diseases and infections with symbiotic bacteria may greatly affect the quality and the efficacy of mass-reared natural enemies in biological pest control.

Mass-reared arthropod 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 (Sikorowsky & Lawrence, 1994). Moreover, in mass-production of arthropods, climatic conditions may be more optimal for pathogens and horizontal pathogen transmission may be more effective than in natural situations (Sikorowsky & Lawrence, 1994). These factors may enhance disease incidence, the development of novel diseases and/or virulent pathotypes. Knowledge of diseases of natural enemies is thus indispensable for reliable biological pest control programmes.

In the present article we summarize data of an earlier published literature review on pathogens, potential pathogens with unknown host effects, endosymbiotic *Wolbachia* spp., and cases of unidentified diseases of natural enemies applied in biological pest control (Bjørnson & Schütte, 2003), and add recent literature of this topic. In addition, we present the results of light- and electron microscopic investigations regarding pathogens in field and mass-reared populations of natural enemies.

## Material and methods

***Populations used in diagnostics***

In the "Laboratory of diagnosis, cyto- and histopathology of arthropod diseases" of the Federal Biological Research Centre for Agriculture and Forestry, diagnostic investigations on arthropod diseases are carried out since 1953. Innumerable specimens from field populations and laboratory cultures showing signs and symptoms of diseases were diagnosed. Most of these arthropods were pest insects, and the aim of the studies was the detection of potential microorganisms that could be used for biological control. But because the market for natural enemies was growing during the last decades, diagnosis of pathogens of beneficial arthropods became increasingly important. Thus we have received many samples of beneficial arthropod populations with high mortalities and/or low fecundities.

***Light and electron microscopy***

Besides phase contrast investigations of dissected tissues and studies of stained smears, serial sections of different developmental stages of natural enemies were prepared for cyto- and histopathological studies as described by Kleespies *et al.* (2003).

## Results and discussion

***Literature review***

As many as 151 reports of pathogens, potential pathogens, endosymbiotic *Wolbachia* spp. and unidentified diseases were found for the following 24 genera of natural enemies applied in biological pest control: *Steinernema* (*Neoaplectana*), *Heterorhabditis*, *Euseius* (*Amblyseius*), *Galendromus* (*Metaseiulus*), *Neoseiulus* (*Amblyseius*), *Phytoseiulus*, *Aphidoletes*, *Chrysoperla* (*Chrysopa*), *Adalia*, *Coccinella*, *Coleomegilla*, *Harmonia*, *Hippodamia*, *Aphidius*, *Aphytis*, *Cotesia* (*Apanteles*), *Encarsia*, *Eretmocerus*, *Lysiphlebus*, *Muscidifurax*, *Nasonia*, *Opius*, *Pediobius* and *Trichogramma* (cited *in* Bjørnson & Schütte, 2003; de Barro and Hart, 2001; Zchori-Fein *et al.*, 2001; Becnel *et al.*, 2002; Mochiah *et al.*, 2002). A detailed description of most reports has earlier been published by Bjørnson & Schütte (2003). In addition to 5 reports of unidentified diseases, entities of all major pathogen groups have been reported. Nematode infections (1 report) and viral infections (5 reports) are rather scarce, whereas fungal and protozoal infections (40 reports each) are more common. Bacterial infections are most common (60 reports). This is mainly due to the enormous amount of work that has recently been done on endosymbiotic *Wolbachia* (36 reports). Host effects that may negatively influence mass production and biological control in the field, including high mortality, low fecundity, malformations, few female offspring, male-killing, reproductive incompatibility, freezing, paralysis, reduced predation capacity and behavioral changes, have been reported for 66 entities. These data clearly show that regular sanitation and screening procedures are indispensable for detection and prevention of diseases in mass-rearings of beneficials and other arthropods (for further discussion, see Bjørnson & Schütte, 2003).

***Pathogen diagnosis***

During this study, eighteen unidentified potential pathogens and 6 determined pathogen species were detected in 13 out of 15 investigated species of natural enemies (Table 1). Infections with entities of all major pathogen groups were found: nematode infections (1 shipment of 1 species) and viral infections (2 shipments of 2 species) were rather scarce, whereas protozoal infections (10 shipments of 5 species) and fungal infections (9 shipments of 7 species) were more common. Bacterial infections were most general (18 shipments of 10 species).

Table 1. Number of shipments of 15 species of natural enemies applied in biological pest control with individuals infected by either viruses, bacteria, fungi, protozoa or nematodes and those without findings (=w.f.).

| Natural enemy species | Viruses | Bacteria | Fungi | Protozoa | Nematodes | w.f. |
|---|---|---|---|---|---|---|
| *Neoseiulus cucumeris* | – | 1 | – | 4(a) | – | 8 |
| *Neoseiulus barkeri* | – | 1 | – | 3(a) | – | 5 |
| *Phytoseiulus persimilis* | – | 5 | 2 | – | – | 1 |
| *Coccinella septempunctata* | 1 | 3 | – | 1(b) | – | 6 |
| *Aphidoletes aphidimyza* | – | 1 | 1 | | – | 2 |
| *Episyrphus balteatus* | – | 1 | 1 | – | – | 1 |
| *Hydrothaea aenescens* | – | 1 | – | – | 1 | – |
| *Anthocoris nemorum* | – | – | 1(c) | – | – | – |
| *Orius laevigatus* | – | – | – | – | – | 1 |
| *Aphidius ervi* | – | 1 | 1 | – | – | 1 |
| *Cotesia glomerata* | – | – | 2 | 1(d) | – | – |
| *Encarsia formosa* | – | – | – | – | – | 1 |
| *Nasonia vitripennis* | – | 3(e) | – | – | – | – |
| *Trichogramma evanescens* | – | – | – | 1(f) | – | – |
| *Chrysoperla carnea* | 1 | 1 | 1 | – | – | 2 |

(a): *Nosema steinhausi*, (b): *Nosema coccinellae*, (c): *Aspergillus* sp., (d): *Nosema mesnili*, (e): *Arsenophonus nasoniae*, (f): *Nosema pyrausta*.

The six determined pathogen species are presented in the following list:

1. *Nosema steinhausi* was detected in *Neoseiulus* (*Amblyseius*) *cucumeris* and *N. barkeri* (Huger, 1988). Infections of unidentified microsporidia are known to reduce the fecundity and predation capacity in these predatory mite species (cited in Bjørnson & Schütte, 2003). It is unknown if these undescribed microsporidia are *N. steinhausi*.
2. *Nosema coccinellae* was diagnosed in *Coccinella septempunctata*. In earlier studies *N. coccinellae* was detected in several coccinellid species including C. *septempunctata* and *C. quinquepunctata* (cited in Bjørnson & Schütte, 2003). There are no data, however, that describe the effects of this microsporidium on these hosts.
3. *Nosema mesnili* was found in *Cotesia glomerata* (Mück *et al.*, 1981). There are earlier reports of *N. mesnili* infecting *C. glomerata* where *N. mesnili* reduced *C. glomerata* longevity (cited in Bjørnson & Schütte, 2003).
4. *Nosema pyrausta* was diagnosed in *Trichogramma evanescens* (Huger, 1984). This microsporidium is known to impair larval-pupal development and to reduce adult emergence and fecundity of this parasitoid (Huger, 1984; cited in Bjørnson & Schütte, 2003).

5. The rod-shaped bacterium, *Arsenophonus nasoniae,* was detected in *Nasonia vitripennis* (Huger *et al.*, 1985) and *N. longicornis* (cited in Bjørnson & Schütte, 2003). This bacterium is associated with the son-killing trait in both host species.
6. Moreover, one fungal species infecting *Anthocoris nemorum* was identified as *Aspergillus* sp. Studies on the virulence and host range of this fungal strain have not been conducted.

These results clearly demonstrate a potential threat of diseases in arthropod mass production. Therefore, more research is needed to increase knowledge about potential pathogens and to develop feasible and reliable diagnostic methods for known pathogens. This especially refers to mass rearing of beneficial arthropods, in order to secure optimal performance in biological control.

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# EPPO based efficacy study to control *Phyllopertha horticola* in golf courses

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**Abstract**: Four *Metarhizium anisopliae* isolates, *Heterorhabditis bacteriophora* and Dursban-2E (chlorpyrifos) were tested on lawns infested by *Phyllopertha horticola* larvae in the golf course area Igls-Rinn (Austria). The efficacy evaluation trial was based on EPPO-standards. The infestation rate was 497 larvae per $m^2$ on average with a maximum of 1574 larvae per $m^2$. Despite the use of chemical insecticides the pest population was reduced by less than 35 percent eight weeks post treatment. Both biological control agents were only moderate effective. Nema-green® caused a mortality of 19 percent after eight weeks. Similar effects could be observed for the fungal products based on *Metarhizium anisopliae* (i.e. 16 % mortality with granular formulated products; 14.4 % with spore powder formulation). Reason for the low efficacy of all control agents was a strong developed felt layer of the lawn that prevented a sufficient irrigation of the soil.

**Key words**: *Phyllopertha horticola*, *Metarhizium anisopliae,* efficacy study, biocontrol agent versus chemicals, Nematodes

## Introduction

*Phyllopertha horticola* (L.) (Coleoptera, Scarabaeidae) causes considerable damage to amenity areas (e.g. golf courses), pasture (for grazing) and orchards (Strasser, 2000). Due to its one-year life cycle the adult garden chafer causes feeding damage in orchards and stock of deciduous trees, roses and other blooming bushes. In early summer, after the eggs have been laid, the growing larvae (three larval stages) feed on roots of grasses, cultivated plants and stock of trees, which results in large damaged areas of meadows and lawns, sport facilities, recreation areas, golf courses and reforestation areas. In mountainous areas of Europe, damage to pastures can also lead to serious soil erosion. Damage is estimated to be hundreds of millions of euros each year and the pest problem is increasing. Most parts of Europe (e.g. Germany, Switzerland, Austria, Italy, France and many East European countries) are affected. The total affected area reported exceeds 20,000 ha.

In the previous EU RTD-project BIPESCO (FAIR6-CT98-4105) two *Metarhizium* strains have been identified as highly pathogenic to *P. horticola* in laboratory and preliminary field trials. *M. anisopliae* ($1 \times 10^7$ conidia $mL^{-1}$), when applied directly to larvae of *P. horticola,* caused between 67 and 93 % mortality within 6 weeks (Keller, personal communication). In 2000 the Swiss team could demonstrate in small pilot trials in Davos and Alvaneu-Bad that the drilling of granules of *Metarhizium* induced increased mortality of *Phyllopertha*.

The goal of this study was to demonstrate that biological control agents based on *Metarhizium* and *Heterorhabditis* are efficacious for the control of *P. horticola* in golf courses. The study was conducted during summer 2002 in Austria and was based on the EPPO-Standard PP1/152 (design and analysis of efficacy evaluation trials) and on the German proposal for an EPPO-guideline for testing insecticides on grubs in arable crops (I.22, October 1999).

## Material and methods

***Control agents***

Dursban®-2E (chemical insecticide based on chlorpyrifos; Dr. Stähler, Reg. Nr. 1871) and two types of biocontrol agents (BCAs) were compared (Table 1). Fungal BCAs were based on *Metarhizium anisopliae* (i.e. granular- and spore powder formulations), the nematode product was *Heterorhabditis bacteriophora* (Nema-green®, E-nema GmbH) a BCA registered and commercialised in Austria since 2001.

Table 1. *Phyllopertha horticola* control with one chemical and different biological insecticides. One application each with the recommended concentrations of the distributors.

| Treatment | Dose | Application Method | Abbreviation Code |
|---|---|---|---|
| *Metarhizium anisopliae* strain BIPESCO 5 | Equivalent spore concentration to 50 kg granular formulation per ha (Prophyta GmbH.) | Spraying | Spore powder |
| *M. anisopliae* strain BIPESCO 6 | 50 kg granular formulation per ha (Agrifutur srl.) | Drilling | Grain Type 1 (T1) |
| Commercial *M. anisopliae* | 50 kg granular formulation per ha (Schweizer) | Drilling | Type 2 |
| Commercial *M. anisopliae* | 50 kg granular formulation per ha (Andermatt Biocontrol) | Drilling | Type 3 |
| Commercial Nematodes | Heterorhabditis bacteriophora *(Nema-green® - Enema)* | Spraying | NemaG |
| Chemical Insecticide | Chlorpyrifos (Dursban 2E®; Dr. Stähler) | Spraying | Dursban |
| No treatment (Control) | | Drilling | Control |

***Experimental design***

The efficacy evaluation trial was conducted in August 2002 on a lawn heavily infested with *Phyllopertha horticola* larvae located in the golf course area Igls-Rinn (Austria). According to EPPO standard [PP 1/152] a full randomised block design was selected: two separate fields with two plots of each treatment, plot size 4 $m^2$. The barley formulations were applied with a slit seeder prototype to a depth of 2 to 4 cm in a concentration of 50 kg granules per hectare (ha). The nematodes, Dursban and the spore powder product were washed into the slit soil in a concentration and with a water content recommended by the producer. The control plots were treated only mechanically with the slit seeder.

***Evaluation of infestation levels of the garden chafer and effectiveness of insecticides***

In order to evaluate the infestation of the garden chafer and to estimate the success of the BCAs, the presence of larvae per $m^2$ and the mortality of larvae per $m^2$, respectively, were determined by means of spade sampling as follows: the control and test plots, with a dimension of 2 to 2 m, were determined with of a raster plan; three square holes per plot and sampling – 20 x 20 cm wide and up to 20 cm deep - were dug. Infestation density of the garden chafer per plot was recorded immediately before application, after 2 and 8 weeks, respectively, and 8 months post treatment.

***Isolation of* Metarhizium anisopliae *from soil***

Soil samples were taken to a depth of 10 cm by using a sampling auger, mixed, air-dried and sieved through a 2 mm sieve. Ten gram sub-samples (three replicates) were added to 40 mL

0.1 % (v/v) Tween-80, shaken at 150 rpm for 30 min and then treated in an ultrasonic bath for 30 s. Sabouraud Dextrose agar plates selective for *M. anisopliae* were inoculated with 50 µL of these soil suspensions and dilutions thereof and were incubated for 14 days at 25 °C and 60 % RH (four replicates per sub-sample). Colonies of *M. anisopliae* are given as colony forming units (cfu) per gram soil dry weight.

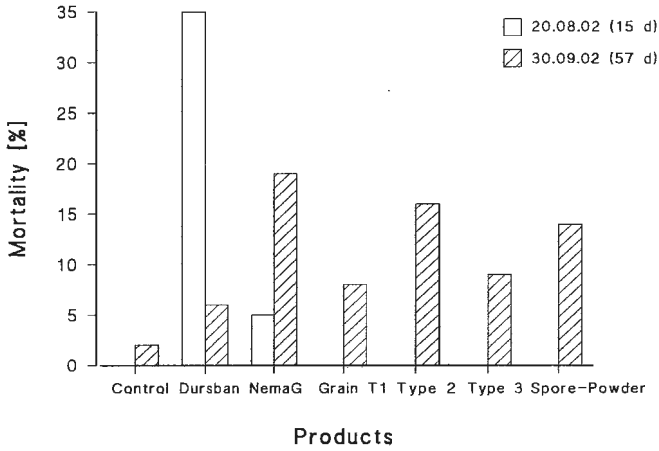


Figure 1. Assessment of *Phyllopertha horticola* mortality (%) caused by different insecticides after 2 and 8 weeks (abbreviation code of products, see Table 1).

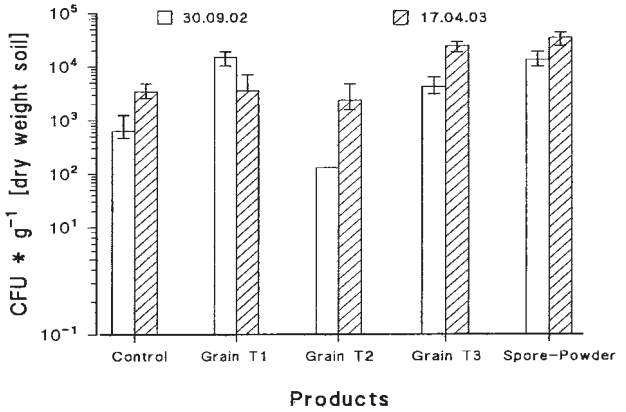


Figure 2. Abundance of formulated *Metarhizium anisopliae* BCAs (mean ± STD, n = 4) in soil samples taken from 0 to 10 cm depth after 8 weeks and 8 months post inoculation (abbreviation code of products, see Table 1).

## Results and discussion

A population density of 497 *Phyllopertha horticola* larvae per $m^2$ on average was recorded in treated and control-plots two weeks and eight weeks post treatment, respectively. This

population density led to a complete damage of the lawn caused by the feeding activity of larvae on plant roots but in a bigger dimension through collecting larva by birds, fox and badger. The control effects of all tested agents were strongly influenced by the thick felt layer of the lawn that prevented a sufficient irrigation of soil. The low moisture did not support distribution and efficacy of the chemical insecticide, the development of the applied entomopathogenic fungi and the migration of the nematodes to the larvae, respectively. After two weeks only 35 % of *P. horticola* larvae were killed with the chemical insecticide Dursban®-2E, whereas after eight weeks 19 % were killed by nematodes and less than 17 % percent by the fungus *Metarhizium anisopliae* (Fig. 1). In spring 2003, eight months post treatment, no mummified larvae could be sampled in fungal treated plots because of the rapid mineralisation of mycosed larvae. Therefore, *Metarhizum* density in soil was determined as an indirect parameter to estimate the infectivity of the BCA propagules. Based on the results of field experiments, the fungal BCAs were applied into soil to a depth of 2 to 4 cm to increase efficacy by placing the fungus in close contact with the pest. Ferron (1979) reported that a threshold concentration of $> 2 \times 10^4$ spores/g dry weight in soil is required to ensure epidemic levels in pastures. This fungal density could be achieved with one application (Fig. 2). *M. anisopliae* persistence data showed that the fungus is an indigenous entomopathogenic fungus. From untreated soil layers (0 to 10 cm depth) the fungus could be isolated in the golf course area in a concentration of $2 \times 10^3$ spores/g dry weight. The *Metarhizium* density in the treated plots remained stable or increased slightly during the first eight months after application.

The cfu numbers of *M. anisopliae* support the assumption that all the augmented propagules are persistent. There should be enough infectious propagules in soil to control the soil dwelling stage of *P. horticola*.

To conclude, under optimal climatic and soil conditions, respectively, both types of BCAs will be suitable biocontrol agents. We expect that *M. anisopliae* will reduce the garden chafer population in the same manner as *B. brongniartii* does for *Melolontha* spp. (Inglis *et al.*; 2001). After only 2 years of regular application sufficient suppression of cockchafer populations could be demonstrated. Similar promising results are referred by Enema company for *H. bacteriophora*. The Nematode product is increasingly considered for the control of *P. horticola* populations, because up to 80 percent of the pest should be killed one year post inoculation (Bart, personal communication).

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# Safety and risk assessment

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# Safety of entomopathogenic fungi

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**Abstract**: There are at least 90 genera and more than 700 species of fungi that have been identified as closely associated with invertebrates, principally insects. Insect pathologists were initially interested in protecting beneficial insects from fungal pathogens, but currently there has been a considerable interest in using fungal pathogens to control insect pests. This interest in the development of fungi as biological control agents as supplements or alternatives to synthetic chemical insecticides has been stimulated by recent widely publicised problems with these chemicals. However, fungal biocontrol agents also possess properties that lead to human and environmental unintended effects depending on the nature of the fungus and its pattern of use. We provide an overview of the potential hazards and safety concerns associated with entomopathogenic fungi with emphasis on isolates of *Beauveria bassiana* and *Metarhizium anisopliae* that are currently being developed as microbial insecticides for locust control in Spain. Special attention is given to the requirements that should be imposed on hazard identification when an indigenous fungal isolate is to be used inundatively in its native area or when it is introduced in a new environment. For that, we focus mainly on potential unintended adverse effects as competitive displacement of microorganisms and on toxigenicity and pathogenicity to non-target organisms, in hopes that this will lead to more appropriate questions and further discussions on safety relative to the use of fungal biocontrol agents.

**Key words**: Safety, microbial control, fungi, *B. bassiana*, *M. anisopliae*.

## Introduction

There are at least 90 genera and more than 700 species of fungi that have been identified as closely associated with invertebrates, principally insects (Li, 1988), being some of them important key factors in regulating their populations. Entomopathogenic fungi are unique and relevant among the insect pathogens in that they infect their host primarily through the integument, which led to consider them as the only practical means of microbial control of many important insect pests (St Leger & Screen, 2001; Quesada-Moraga, 2002). Thus, in recent years, the development of entomopathogenic fungi for the control of insect pests is under increasing challenge (Butt *et al.*, 2001). Undoubtedly, fungal entomopathogens offer some advantages compared with chemical insecticides, including safety for humans and other non-target organisms, reduction of pesticide residues in food and increased biodiversity in managed ecosystems, which it is in accord with the European Commission common agricultural policy. However, it is not possible to reduce a pest population without affecting another component of the ecosystem so that entomopathogenic fungi may pose potential adverse effects on human health and environment, even if for the moment, they are either non-existent, minimal, or have gone unnoticed (Goettel *et al.*, 2001). Cook *et al.* (1996) classified these potential adverse effects in: (1) displacement of non-target microorganisms, (2) allergenicity to humans and other animals, (3) toxigenicity and (4) pathogenicity to non-target organisms.

Given that there have been previous significant reviews on the safety of entomopathogenic fungi (Goettel & Johnson, 1992; Goettel & Jaronski, 1997; Evans, 1998; Goettel & Hajek, 2000; Goettel *et al.*, 2001), this contribution is focused on the potential safety issues associated with the use of the fungal biocontrol agents for locust control in Spain.

## Locust control in Spain: current situation

The Mediterranean or Moroccan locust *Dociostaurus maroccanus* (Thunberg), has been recorded as an important pest of pasture and crops in Spain for centuries (Vázquez-Lesmes & Santiago-Álvarez, 1993). Outbreaks also occur in other Mediterranean areas such as southern Italy, Crete, Sardinia, Morocco, Algeria and Turkey, as well as parts of Eastern Europe and the former Soviet Union. The preventive control of this locust relies on broad-spectrum synthetic insecticides, which it is likely to change because some environmental issues arising from their use have expanded the demand for biological control (Lomer *et al.*, 2001; Hernández-Crespo & Santiago-Álvarez, 1997). With this aim, entomopathogenic fungi (Hernández-Crespo & Santiago-Álvarez, 1997) are considered, together with *Bacillus thuringiensis* (Berliner) (Quesada-Moraga & Santiago-Álvarez, 2001) as the main candidates for the biological control of the Moroccan locust.

The mitosporic fungus *Beauveria bassiana* (Balsamo) Vuillemin is the dominant species in natural populations of *D. maroccanus* in the locust breeding areas in Spain, causing infection levels ranging between 1.6 and 20.5%, while *Metarhizium anisopliae* (Metschnikoff) Sorokin was found only in four *D. maroccanus* adults out of 317 collected individuals (Hernández-Crespo & Santiago-Álvarez, 1997). An indigenous isolate of *B. bassiana*, EABb 90/2-Dm is currently being developed as a mycoinsecticide for the control of *D. maroccanus* (Santiago-Álvarez *et al.*, 2002) since by laboratory assays and preliminary field trials showed better performance than any other coespecific isolates or even the *M. anisopliae* ones (Hernández-Crespo & Santiago-Álvarez, 1997; Jiménez-Medina *et al.*, 1998). Nevertheless, an alternative approach for the biological control of *D. maroccanus* is the use of non-indigenous fungal isolates. In early 2000 a collaborative European research project entitled "Protecting Biodiversity through the Development of Environmentally Sustainable Locust and Grasshopper Control" (ESLOCO), was initiated. Its aim is to reduce the environmental impact of locust and grasshopper control operations through the development of a new environmentally sustainable strategy, based on the use of a strain of *M. anisopliae* var. *acridum* (Bridge *et al.*, 1997) isolated from Africa acridoids. The insecticidal efficacy of this strain formulated in paraffine oil diluents for use in ULV sprays (Lomer *et al.*, 1997) against *D. maroccanus* has been demonstrated in several field trials conducted during a three years period (2000-2002). The mycoinsecticide has been shown to reduce significantly locust populations, although insects infected by the natural (or indigenous) *B. bassiana* strains were also detected either in control or treated plots.

## Safety concern associated with *Metarhizium anisopliae* and *Beauveria bassiana* for locust control in Spain

***Displacement of non-target microorganisms: non-indigenous vs. indigenous organisms***

Independent of the method of introduction (inundative or inoculative), an exotic fungal strain has the potential to persist in the environment and spread to other areas. Thus, it is an important concern in introducing the exotic agent into a new area, the risk that it may pose to the indigenous ones, being of great relevance to assess whether a competitive displacement of indigenous strains may occurs either in soil or locust hosts. Studies have been conducted to detect the prevalence of *M. anisopliae* var. *acridum* in soils from locusts treated areas (Maranhão, 2003). The fungus was isolated only from soil samples obtained two weeks after treatment, while the indigenous strains of *B. bassiana* were isolated during the whole 50 d post-treatment sampling period (Maranhão, 2003), suggesting that *M. anisopliae* var. *acridum* has quickly drop to non-detectable levels in the soil and indicating that it is not well adapted

to persist in the well defined environmental conditions of the locust breeding areas in southern Spain. This is also supported by the fact that *B. bassiana* is more frequently isolated than *M. anisopliae* from soils from Southern Spain (Maranhão, 2003). Besides, laboratory assays have shown that in soil co-inoculated with both fungi, baited with wax moth larvae, most of the killed larvae were *B. bassiana* infected, suggesting either that this fungus penetrated the host faster or that it had higher speed of kill (Santiago-Álvarez *et al.*, 2005). But it is also a safety concern to know whether it could interfere with the natural control of *D. maroccanus* by the indigenous *B. bassiana* isolates. With this aim, experiments has been carried out to study the *in vitro* thermal requirements of the indigenous isolates of *B. bassiana* and the *M. anisopliae* var. *acridum* strain, as well as the *in vivo* competitive infection and colonization of locust (Thomas *et al.*, 2003). *In vitro* growth of *B. bassiana* isolates and the non-indigenous *Metarhizium* strain is similar at constant temperatures whiles under fluctuating temperatures, miming the natural thermoregulating conditions, the *M. anisopliae* shows a better behaviour. Until known, *in vivo* experiments have been performed by using weekly virulent strains of *B. bassiana*, being co-infections clearly favourable to *Metharizium*, but further experiments will indicate whether a competition may exists between indigenous and non-indigenous strains for infection of the locust host.

***Toxigenicity to non-target organisms***

Entomopathogenic fungi secrete an array of relative low molecular weight secondary metabolites that may pose risk to human and animal health (Vey *et al.*, 2001). Strasser *et al.* (2000) demonstrated that the quantities of destruxins, efrapeptins, oosporein, beauvericin and beauveriolides produced by species of the important genera *Beauveria*, *Metarhizium* and *Tolypocladium in vivo* are usually much less than those secreted in nutrient rich liquid media.

Our work focused on the production of secondary metabolites among a collection of *B. bassiana* strains isolated from locusts and from the soil showed that they can be pathogenic for locust whether they secrete toxic metabolites *in vitro* or not (Quesada-Moraga & Vey, 2003). Interestingly, the toxic metabolites secreted by *B. bassiana* isolate EABb 90/2-Dm were macromolecular as they were retained by dialysis (cut-off of 6-8 kDa for globular proteins), thus it did not secrete secondary metabolites in liquid culture (Quesada-Moraga & Vey, 2003). Therefore, since the secretion of secondary metabolites represents one of the major hurdles in the registration and subsequent commercialization of entomopathogenic fungi, the strain EABb 90/2-Dm has adequate toxicological properties to be registered for *D. maroccanus* control as it poses no obvious risk to human and animal health. The secretion of toxic compounds by entomopathogenic fungi is not limited to the secondary metabolites as they may also secrete high molecular weight toxins that may be important pathogenicity determinants and that remains poorly studied compared with the small toxic molecules. In fact, the bioactive macromolecules present in the crude filtrate of strain EABb 90/2-Dm were precipitated by 90% saturation of ammonium sulphate, and the insecticidal activity was exclusively detected in high molecular mass fraction after gel filtration on Sephadex G-25 suggesting that it was proteinic (Quesada-Moraga & Vey, 2003). This protein, named Bassiacridin, has been recently purified and characterized (Quesada-Moraga & Vey, 2004). This preliminary work indicates that haemolymph extracted from EABb 90/2-Dm strain infected *L. migratoria* nymphs is toxic to the same host (data not published), indicating that the Bassiacridin is also produced *in vivo*, and suggesting its possible role in pathogenesis. Interestingly, among the assayed host, the major toxicity of the Bassiacridin has been observed against locusts (Orthoptera, Acrididae), being equally toxic to *Locusta migratoria* L. and *Schistocerca gregaria* (Forskål), and slightly more toxic to the Moroccan locust *D. maroccanus*, while it was neither toxic against the coleopteran species *Tenebrio molitor* L. nor against the lepidopteran species *Spodoptera littoralis* Boisd. and *Galleria mellonella* L..

Thus, the risk of toxicogenicity of strain EABb 90/2-Dm seems to be minimized to the acridids.

***Pathogenicity to non-target organisms***

The most common fungi produced for inundative augmentation, the anamorphic fungi *B. bassiana* and *M. anisopliae*, have shown little if any evidence of hazards to humans or wildlife (Austwick, 1980), being low the risk of the use of these mycoinsecticides to non target arthropods (Goettel *et al.*, 1990). *B. bassiana* has been isolated from over 700 species of arthropods, many of which may be considered as non-target or beneficial hosts, but most isolates are host specific and has been demonstrated to be innocuous under field conditions to many species included in its host list (Goettel & Jaronsky, 1997). Field studies conducted in pasture areas treated with *B. bassiana* to control grasshoppers demonstrated no infection among 2500 non-target field collected arthropods that were diagnosed for fungus infection (Goettel *et al*, 1996). Specifically, there are two main environmental issues arising from the use of this fungus, the impact on natural enemies of the target pest and on bees. The *B. bassiana* strain EABb 90/2-Dm is being developed as a commercial mycoinsecticide against *D. maroccanus* (Santiago-Álvarez *et al.*, 2002), but unfortunately, until now, no experiments have been conducted to evaluate its effect on the non-target fauna.

*M. anisopliae* is currently being registered against several hosts, grasshoppers and locusts, cockroaches, weevils and scarabs (Butt *et al.*, 2001). In respect to the *M. anisopliae* var. *acridum* strain, an experiment has been conducted to evaluate effects and side – effects on target locust and epigeal non-target arthropods, using simple binary (presence/absence) techniques, in the breeding area of la Serena in Spain (Valverde-García, 2003). Data of average survival time and percentage of sporulation of collected Orthoptera after treatment, showed a good insecticide action of *M. anisopliae* var. *acridum* either against the target hosts, *D. maroccanus* and *Calliptamus italicus,* or non-target Orthoptera as *D. genei*, *Decticus albifrons* and *Platystolus martinezi* (Valverde-García, 2003). In contrast, the impact of this strain on non-target insects such as Coleoptera (Cleridae, Tenebrionidae and Scarabeidae), Hymenoptera (Formicidae and Apidae), and other arthropods was not significant at any of the periods considered (1, 2 and 4 weeks) (Valverde-García, 2003). Thus, special attention has to be given to the effect of this *M. anisopliae* strain to non-target Orthoptera as it poses low risk to other non target arthropods.

## Conclusions

We provide an overview of the potential hazards and safety concerns associated with entomopathogenic fungi with emphasis on isolates of *B. bassiana* and *M. anisopliae* that are currently being developed as microbial insecticides for locust control in Spain. Special attention is given to the requirements that should be imposed on hazard identification when an indigenous fungal isolate is to be used inundatively in its native area or when it is introduced in a new environment. In respect to the possible displacement of indigenous microorganisms by non-indigenous ones, we demonstrate that the indigenous *B. bassiana* isolates are well adapted to the Mediterranean locust breeding areas whereas the non-indigenous *M. anisopliae* var. *acridum* quickly drops to non-detectable levels in the soil. We discuss also on the adequate toxicological properties of the indigenous EABb 90/2 Dm *B. bassiana* strain that could be also registered for *D. maroccanus* control in Spain. This strain secretes a protein, named Bassiacridin, that may has an important role in pathogenesis, and that it is exclusively secreted against locusts. In respect to the *M. anisopliae* var. *acridum* strain, it has not significant on non-target insects such as Coleoptera and Hymenoptera, but special attention has to be given to its effect on non-target Orthoptera.

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# Risk assessment of biological nematicides

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**Abstract**: The development of a biological control product faces many obstacles before the final goal, successful commercialization, is achieved. Although several biopesticides have already proven their ability to efficiently control pests and diseases without causing any adverse effects to the environment, there are still concerns about the fate of a microorganism after its release. However, besides the monitoring of a specific biocontrol agent in the environment, models to appropriately assess the operator or bystander exposure need to be developed or modified for microbial pesticides. The egg pathogenic fungus *Paecilomyces lilacinus* (strain 251), was chosen as a model organism to identify the parameters needed to predict its fate in the environment. To monitor the long term survival, a semi-selective medium was developed to enable monitoring of the population dynamics of *P.lilacinus* in the soil and rhizosphere. Monitoring was conducted with root knot nematodes (*Meloidogyne* spp.) as target and tomato as host plant. The population development of *P. lilacinus* was monitored depending on the application rate, formulation, temperature, method of application and the presence or absence of the target pest as well as the host plant. Initial results demonstrated that *P.lilacinus* was not able to multiply in soil or the rhizosphere of tomato plants and consequently showed a relatively low persistence.

**Key words**: *Paecilomyces lilacinus* strain 251, monitoring, persistence, biological nematicide

## Introduction

*Paecilomyces lilacinus* (Thom) Samson is a facultative egg pathogen of sedentary nematodes and the most extensively field tested biological control agent (BCA)(Kerry, 1996). It has been reported to reduce nematode populations and is considered one of the most promising and practicable BCA for the management of plant parasitic nematodes (Siddiqui *et al.*, 2000). Strain 251, deposited at the Australian Government Analytical Laboratory (AGAL; # 89/030550), is currently registered as BIOACT WG and used commercially for control of nematodes on Banana in the Philippines. This strain, isolated from a *Meloidogyne* egg mass, originated from the Philippines and was selected due to its efficacy for control of *Meloidogyne* spp. on tomato and *Globodera rostochiensis* on potato (Davide & Zorilla, 1983; Davide, 1985).

In contrast to the research on new BCAs, the potential environmental risks of biocontrol products have often been neglected. Currently, risk assessment is essential for the registration process. The biological control agents (BCAs) must be safe for humans and non-target organisms (De Jong *et al.*, 1999). The fate and behavior of the introduced organism (Vänninen *et al.*, 2000), the exposure of the worker or the applicator, the effect of the BCA on non-target organisms like mycorrhiza (Godeas *et al.*, 1999) and other microorganisms (Moënne-Loccoz *et al.*, 1998) needs to be addressed in order to register a biocontrol agent. One of the main problems is the lack of acceptable methods that would adequately address and evaluate the above mentioned risks.

Formulation plays an important role as far as it concerns the hazards that a biological agent may pose to the environment (Inynag, 2000). Different formulations may expose the

worker or the applicator to different amounts of conidia, or may affect the persistence of the introduced organism in the environment.

## Material and methods

***Persistence***

One of the major concerns of risk assessment is the fate and behavior of the introduced organism once it is applied. Hence, there is a need to monitor the population density of the biocontrol agent over time. The common method for monitoring a fungal biocontrol agent is soil dilution plating onto semi-selective media.

Although several media have been developed for the isolation and enumeration of colony forming units (cfu) of *P. lilacinus*, those media were not suitable for isolation of the particular strain 251 from soil samples. Different compounds with known antifungal activities such as Benomyl and Cyclohexemide inhibited the growth of *P. lilacinus* (data not presented). However, strain 251 was able to tolerate high concentrations of 2,6-dichloro-4-nitroaniline, the active ingredient of Dichloran. For isolations from greenhouse experiments, OHIO agar was chosen as the base medium. This agar is a common medium for the isolation and enumeration of soil-borne fungi (Johnson & Curl, 1972). For isolations from field experiments, OHIO agar was supplemented with 10 g/L NaCl and 20 mg/L 2,6-dichloro-4-nitroaniline. With this modified medium, it was possible to quantify population densities as low as 1 x $10^3$ cfu of *P. lilacinus* per gram of soil (data not presented). For pot experiments under greenhouse conditions, formulated product containing 1 x $10^{10}$ viable conidia/g was suspended in water and mixed into the substrate. The initial concentration was between LOG 6,16 and LOG 6,45/g soil dry weight. Samples were taken for up to 161 days past application and dilution plated onto OHIO agar.

***Effect of temperature***

The microbial human health risk assessment of fungal biocontrol agents is one of the major concerns during the registration of biocontrol products. The ability of a biocontrol agent to grow at temperatures of 36 °C or higher is, besides other tests, one indicator for a possible pathogenicity towards mammals. Determination of the maximum growth temperature for radial growth is currently used to evaluate the risk for fungal biocontrol agents. However, since most products contain formulated conidia or blastospores as active ingredient, these tests should be performed with the formulated products.

Conidia of *P. lilacinus* from the formulated product were plated on SDA and exposed to 36°C followed by determination of i) the germination rate after 24 and 48 hours ii) the germ tube development iii) survival rate depending on the exposure time.

## Results and discussion

***Persistence***

The data presented in Fig. 1 demonstrate that diluting field soil with sand resulted in a faster decline in the population compared to undiluted soil. Linear regression analysis resulted in significant correlation coefficients for German field soil ($r^2$ = 0,153**), soil + 25% sand ($r^2$ = 0,541***), soil + 50% sand ($r^2$ = 0,738***), soil + 75% sand ($r^2$ = 0,846***) and 100% sand ($r^2$ = 0,763***). However, field soil from Germany and Greek field soil showed only a low or now correlation ($r^2$ =0,066 for Greek soil). Results from growth chamber and greenhouse studies indicate that the persistence of *P. lilacinus* (strain 251) seems to be predictable. In none of the conducted experiments an increase in the population density was observed. This effect was independent from the presence of the target, root-knot nematodes, or the host plant,

tomato (data not presented). However, diluting field soil with sand resulted in a steeper slope in the decline, which indicates that *P. lilacinus* behaves like a typical saprophyte.

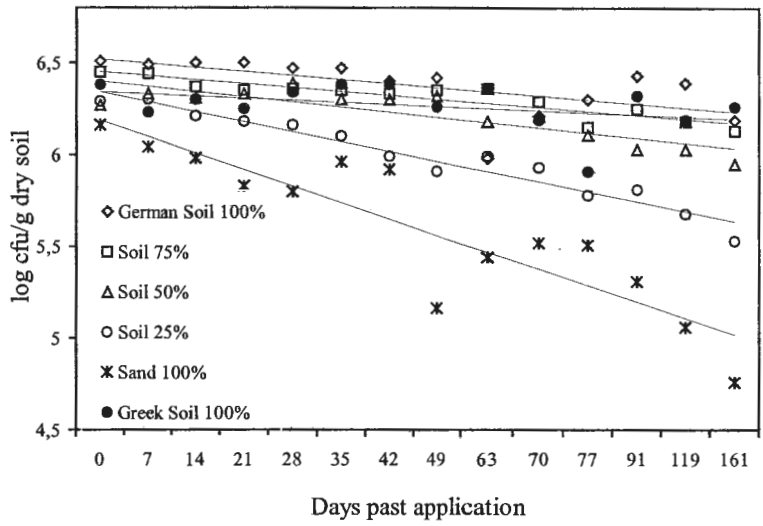


Figure 1. Effect of substrate type on the persistence of *P. lilacinus* formulated as WG under greenhouse conditions. Soil 100% = Silty loam, Germany; Soil 75% = field soil + 75% sand; Soil 50% = field soil + 75% sand; Soil 25% = field soil + 75% sand; Sand 100% = pure sand; Greek soil 100% = soil from vegetable growing area in Greece.

***Effect of temperature***

As most biocontrol fungi, *P. lilacinus* (strain 251) showed no radial growth at 36°C (data not shown). In contrast, the germination of the conidia from the formulated product was only slightly delayed compared to the control at 24°C (Kiewnick *et al.*, 2002). However, when the germ tube development was measured over a period of 168 hours, it could be demonstrated that after 72 hours of exposure and longer, the germ tubes showed deformations and stopped growing (Fig. 2). Additional staining with fluorescein diacetate and observation under UV-light revealed that those fungal structures showing deformations and lysis were no longer viable (data not presented). To verify that conidia, when exposed to 36°C, were not able to survive, the number of cfu per plate after incubation for up to 168 hours was determined. For this test 2 x $10^6$ conidia were spread on SDA plates using a spiral plater. After exposure between 96 and 168 hours a rapid decline in the cfu number was found indicating that although germination was still possible *P. lilacinus* could not survive at 36°C (Kiewnick *et al.*, 2002).

In conclusion, the persistence of *P. lilacinus* obviously depends on conditions for saprophytic growth. The presence of the host plant and target (root knot nematodes) had no effect on the persistence in the soil. Experiments are underway to evaluate the long term survival in greenhouse and field experiments. Based on these data, appropriate models to predict the fate of *P. lilacinus* in the environment need to be developed. In addition, more information is needed to assess the worker and bystander exposure, when a fungal biocontrol

product is applied. Based on these initial data, the persistence of *P. lilacinus* seems to be relatively low and predictable. This indicates that a low exposure can be expected. In addition, the evaluation of the microbial human health risk by determination of growth and survival at 36 °C leads to the conclusion that *P. lilacinus* (strain 251) is posing a low risk.

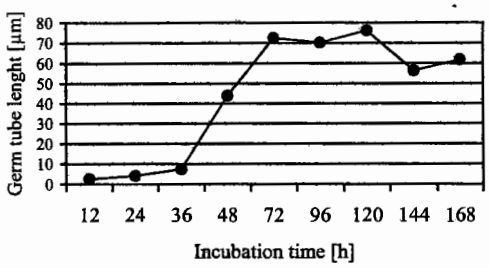


Figure 2. Effect of exposure to 36 °C for 168 hours on germ tube development of *Paecilomyces lilacinus* (strain 251) on SDA.

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# The EU BIPESCO project – latest results on safety of fungal biocontrol products

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**Abstract**: BIPESCO, acronym for Biological Pest Control, was an EU-funded project to develop entomogenous fungi for the control of subterranean insect pests like scarabs and weevils. European scientists and four industrial partners from seven different European countries participated in this multidisciplinary, multifaceted project. Particular attention focused on methods for improving production and field efficacy of fungal BCAs (Biological Control Agents). The data generated could help accelerate registration of BCAs based on promising isolates of *Beauveria* and *Metarhizium*. The consortium believe that demonstration trials in the future will help validate the efficacy and safety of these fungi for the control of chafers and weevil pests.

**Key words**: Biocontrol agent, entomogenous fungi, scarabs, weevils, soil pests, virulence, biotechnology, formulation, ecological fitness, sustainable agriculture, organic farming

## BIPESCO Team and its objectives

The aim of the BIPESCO project was to study and develop entomogenous fungi for the control of subterranean insect pests like scarabs and weevils. Leading experts from seven different European countries including four industrial partners participated in this multidisciplinary, multifaceted project. Particular attention focussed on the widespread, soil-borne, insect pathogens, *Beauveria brongniartii* and *Metarhizium anisopliae*. Strains of these fungi were developed for use in integrated pest management programmes to help replace or reduce the input of chemical pesticides in European agriculture, forestry and horticulture in accord with the reformed common agriculture policy guidelines. The specific objectives of this project were to (1) control scarabs and weevils with virulent, ecologically competent strains of insect-pathogenic fungi, (2) improve production and formulation technologies, (3) develop biochemical methods to monitor fungal virulence and nutritional (carbon) requirements, (4) use molecular techniques to characterise strains to monitor the pathogen in the field (spatial-temporal distribution, genetic stability, interactions with autochthonous, conspecific strains) and for the detection of instability factors (e.g. transposons, mycoviruses), (5) test new application systems for effective targeting of the pathogen, (6) study the impact of the pathogens on target and non-target insects, (7) conduct field trials to demonstrate/evaluate the efficacy of the fungal biological control agents (BCAs), and (8) address some of the criteria for the registration of entomogenous fungi.

## Goals achieved

The BIPESCO team kept to the schedule and achieved the goals. The BIPESCO team established appropriate test methods for conducting assays against target and non-target pests and methods for rearing these organisms.

Valuable data on morphological and physiological characteristics of the *Beauveria* spp. and *Metarhizium anisopliae* strains are now available. For example, there were qualitative and quantitative differences in enzyme and metabolite profiles of each tested *M. anisopliae* strain. Most of these enzymes were required for general metabolism (i.e. "house-keeping"), a few were shown to be important pathogenicity determinants such as the subtilisin, Pr1. This enzyme was a virulence determinant that played a major role in the infection process (i.e. cuticle degradation). Several Pr1 genes were identified and sequenced. No clear link was established between virulence and production of the secondary metabolites destruxin and oosporein by *Metarhizium* and *Beauveria*, respectively. Conidia of attenuated cultures of *M. anisopliae* differed in their adhesion properties compared with those of the original, virulent strain. Conidia could increase or decrease in adhesive properties. Spontaneous mutants of *Metarhizium* were discovered in which the Pr1 gene was silent (not expressed) or absent (Wang *et al.*, 2002). Silencing or loss of the Pr1 gene only partly explained why entomogenous fungi like *Metarhizium anisopliae* become attenuated. Molecular and biochemical methods were developed to monitor Pr1 genes and enzymes, respectively. These methods have considerable potential for quality control (i.e. identifying *Pr1-deficient* or *Pr1-silent* mutants).

Quantitative and qualitative differences in the carbon-nitrogen content of culture media influenced the growth, sporulation and virulence of *Metarhizium anisopliae.* Strains grown on carbon-rich media were usually less virulent than those grown in nitrogen-rich (but carbon poor) media. Increasing the osmolarity of the culture media usually increased/stabilised virulence but reduced conidial yield. The conidia were paler and less hydrophobic than those produced on normal media or on mycosed cadavers. Cultural conditions stabilising virulence of *Beauveria brongniartii* did not stabilise *Metarhizium anisopliae* demonstrating generic physiological differences between these fungal species. The BIOLOG™ microtitre plate system was used to elucidate the link between nutrition and virulence. Metabolism of specific carbohydrates and amino acids was species-related and altered depending on the degree of attenuation. Inter- and intraspecific variation in the carbon utilisation profiles was recorded. Some of the favoured substrates included monosaccharides, disaccharides (e.g. sucrose), sugar alcohols (e.g. xylitol) and polymers (Tween 40 and Tween 80). Extensive testing of liquid media revealed the Catroux-medium as the most suitable for mass production of *B. brongniartii.* Standard C- and N-sources could successfully be replaced by cheap compounds like molasses or brewer's yeast.

## Production of more reliable BCA products to control scarabs and weevils – second generation formulation

Environmentally benign, effective new formulations of fungal BCAs based on *Beauveria brongniartii* and *Metarhizium anisopliae* have been developed for the control of the major, economically important pests such as scarabs and weevils.In June 2000 B*eauveria brongniartii,* produced on sterilised barley kernels, was approved by the Austrian plant protectant legislation and the registered product is sold under the trade name, Melocont®-Pilzgerste. This is the first effective BCA against *Melolontha* spp. in any EU-member state.

***Available primers to monitor pathogens in the field***

Molecular methods and tools were established for characterising promising fungal strains. Protocols were developed for the molecular characterisation of fungal BCAs based on RFLPs of mtDNA, protease-gene differences and various amplified rDNA regions (18S, ITS-5.8S-ITS, 28S). These protocols were refined to optimise detection of *Beauveria* and *Metarhizium*

at the species and strain levels and are now available for further scheduled projects (Pantou *et al.*, 2003).

## Ecological studies and field trials

One of the priorities of the BIPESCO project was to evaluate the susceptibility and behaviour of a range of non-target invertebrates to *B. brongniartii* and *M. anisopliae* strains. No side effects of the application of *B. brongniartii* were estimated on earthworms and collembolans in field trials and in bioassays.

Field trials were carried out in Austria, Denmark, Italy, Germany and Switzerland under different climatic and soil conditions. In Switzerland, a commercial formulation of *B. brongniartii* was applied at a rate of 40 kg $ha^{-1}$ and fungal development in the soil monitored using selective isolation media and the *Galleria* bait method. The results were analysed in relation to presence/absence of the host and edaphic (chemical, biological) factors. The reduction of fungus density after two seasons post-application in absence of hosts was high and differed significantly from that in presence of host. A multiple regression analysis revealed a strong correlation of fungal growth with temperature, clay content and catalase activity. *Beauveria brongniartii* and *Metarhizium anisopliae* were isolated from soils treated with *B. brongniartii*. The results revealed a negative correlation between the two fungi. However, this interaction is believed to be an effect of the isolation methods and not the result of a competition in the soil.

Melocont®-Pilzgerste was tested against the common cockchafer *Melolontha melolontha* in large field trials between 1994 to 2000. It was applied in pastures in Austria, Italy and Switzerland with a slit seeder at various times of the year. Highest efficacy of the product was achieved by incorporating the inoculum into the soil at a depth of 3 to 10 cm. Inoculum density increased following successive annual applications of Melocont®-Pilzgerste. Molecular marker analysis allowed to demonstrate that the applied strain and re-isolated strains were identical. The *B. brongniartii* barley kernel product resulted in sufficient suppression of cockchafer populations after only 2 years of application. Similar results can be expected for the control of weevils with *Metarhizium anisopliae*.

## Rational risk assessment of the use of this technology and MO´s

The non-confidential information is already published and/or in press in 25 international, refereed scientific papers. Additionally, 109 BIPESCO contributions also provide information that will help end users (e.g. policy makers, registration authorities, industry) and the public in making more informed decisions regarding the use and the risks, if any, that fungal BCAs may poses to plant, human and animal health. A review has been published by members of the BIPESCO consortium on the promising entomopathogenic fungi *Beauveria, Metarhizium* and *Tolypocladium*. The review summarises data on specific secondary metabolites (destruxins, efrapeptins, oosporein, beauvericin and beauveriolides) produced by the important genera (Strasser *et al.*, 2000). Methods and strategies were suggested which could standardise the risk assessment of fungal biological control agents (Butt *et al.*, 2001).

The BIPESCO consortium strengthened its activities to be able to draft two dossiers pertaining to *Beauveria brongniartii* and *Metarhizium anisopliae,* respectively. The dossiers contain information to the fungi intended for the use as a microbial pest control agent against *Melolontha melolontha, Melolontha hippocastani, Phyllopertha horticola, Amphimallon solstitialis, Strophosoma* spp. and *Otiorhynchus* spp. The dossier format conforms to EU Commission Directive 91/414 Appendix IIB and IIIB. Comments are included to show expert authorities which of the proposed methodology can and cannot be adapted for BCAs.Since its

inception, the BIPESCO consortium has maintained a website (http://bipesco.uibk.ac.at) to ensure that the public and end users are kept informed of its progress and research activities.

## New activities

The BIPESCO consortium believed that the new methodologies developed needed refinement and validating. One initiative was an EU RTD-project (QLK1-CT-2001-01391) with the acronym RAFBCA (http://www.rafbca.com). Several members of the BIPESCO team are active members of RAFBCA which aims to assess the risks that metabolites of fungal BCAs may pose to human and the environment.

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# A risk assessment framework for biological control agents: the ERBIC approach

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**Abstract**: To ensure the continuing safety and positive public image of biological control, many countries are now requiring risk assessment for biological control agents. A methodology has been developed within the EU-financed project "ERBIC", to be used as a tool for assessing the ecological safety of exotic natural enemies. This general framework integrates information on the potential of an agent to establish, its abilities to disperse, its host range, and its direct and indirect effects on non-targets. The parameter 'host range' forms a central element in the process, because lack of host specificity might lead to unacceptable risk if the agent establishes and disperses widely, whereas, in contrast, a monophagous biological control agent is not expected to create serious risk even when it establishes and disperses well. The proposed risk assessment methodology has been applied to a number of biological control agents currently in use, including the fungi *Beauveria bassiana* and *Metarhizium anisopliae*, and the nematode *Steinernema feltiae*. Among a wide variety of agents such as parasitoids and predators, they ranked in the initial assessment in the middle range as being 'moderately safe'. These case histories indicate that the risk assessment methodology can discriminate between agents, with some species attaining low 'risk indices' and others scoring moderate or high. Risk indices should, however, not be seen as absolute values, but as indicators to which a judgement can be connected by biological control experts for granting permission to release or not.

**Key words**: natural enemies, host range, establishment, dispersal, non-target effects, regulatory policy, environmental impacts, *Steinernema feltiae*

## Introduction

Negative environmental effects of biological control have rarely been reported, although over the past 100 years more than 6000 exotic natural enemies have been imported, mass reared and released as biological control agents (e.g. Lynch *et al.*, 2001). To ensure the continuing safety and positive public image of biological control, many countries are requiring risk assessment for all biological control agents. This is new for macro-organisms in inundative use, although as classical agents these have always been strictly regulated, in particular for weed biocontrol. Microbial agents have historically first been tested and applied at a small scale, and without many restrictions. Regulatory authorities first became interested in them, when larger scale production and application became possible. The main worry in the beginning has been worker safety and the safety of consumers: is the production and application safe for the personnel involved (e.g., toxic metabolites), and for the general public if used on edible crops? As a consequence, microbial control agents have been subject to regulatory approval (e.g., EU Directive 91/414). Macroscopic organisms used in inundative control, such as nematodes, mites and insect parasitoids, usually have been exempted from these regulations.

## Reasons to regulate biological control agents

Several reasons for a more comprehensive regulatory procedure for biological control agents (BCAs) have been presented (see Blum *et al.*, 2003). These include potential human health

hazards, environmental risks, and questions of efficacy (see Hokkanen & Hajek, 2004). Consideration of these has led to differing policies in European countries. For example, the UK regulates all imports of any organisms from elsewhere, but does not regulate the use of indigenous BCAs. Norway has banned the import of all species that cannot be proven to be native to Norway, but allows other imports, and also regulates the use of native BCAs. Switzerland, Austria and Sweden also have implemented a formal registration procedure for all macro-organisms. Elsewhere, in some countries the procedures (e.g. those of Australia, New Zealand and Hawaii; see articles *in* Lockwood *et al.*, 2001) are already so strict that import and release of exotic natural enemies is extremely difficult. Other countries have no regulations at all, so any species can be imported and released. There is a general trend, however, towards more stringent regulatory requirements (e.g. Barratt *et al.*, 1999).

## Components of an ecological risk assessment

The ecological risk assessment is the most critical and difficult part of the overall risk assessment procedure in biological control. A general framework developed for such an assessment (van Lenteren *et al.*, 2003) identifies the following 7 basic steps. These consider different aspects of natural enemy biology and the environment of the system into which the natural enemy will be introduced, in order to evaluate the potential impacts on non-target species and ecological risks:

1. Defining ecological context and the selection of appropriate non-target species
2. Host specificity testing
3. Natural enemy dispersal capability
4. Potential for natural enemy establishment
5. Direct effects on non targets
6. Indirect effects on non targets
7. Risk assessment

## Risk assessment methodology

Evaluation of risks related to releases of natural enemies demands integration of many aspects of their biology, as well as information on ecological interactions. For a full risk assessment, three steps are distinguished: (1) risk identification and evaluation procedure concerning the release of a natural enemy, (2) risk management plan dealing with risk reduction and risk mitigation, and (3) risk/benefit analysis of the proposed release of the natural enemy, together with risk/benefit analyses of current and alternative pest management methods.

The risk of adverse effects caused by the release of a biological control agent is the product of the impact of likelihood (probability) and the impact of magnitude (consequence) (see Hickson *et al.*, 2000; van Lenteren *et al.*, 2003). Five groups of risks are considered related to the release of exotic biological control agents: establishment, dispersal, host specificity, direct effects, and indirect non-target effects.

| RISK = (LIKELIHOOD of adverse effect) x (MAGNITUDE of effect) |
|---|

In order to assess risks, first the likelihood and magnitude of adverse effects are established for each of the five risk groups. For details of this process, see van Lenteren *et al.*, 2003. However, without adding a numerical value to each evaluation criterion it remains a qualitative procedure, making comparison of natural enemies difficult. Therefore, the following values could be assigned to each criterion (van Lenteren *et al.*, 2003):

| Likelihood | | Magnitude | |
|---|---|---|---|
| very unlikely | = 1 | minimal | = 1 |
| unlikely | = 2 | minor | = 2 |
| possible | = 3 | moderate | = 3 |
| likely | = 4 | major | = 4 |
| very likely | = 5 | massive | = 5 |

The overall risk index for each natural enemy is then obtained by first multiplying the figures obtained for likelihood and magnitude, and then by adding the resulting figures obtained for dispersal, establishment, host specificity, direct and indirect effects. The minimum score therefore is 5 (5 times 1 x 1, see Table 1), and the maximum score 125 (5 times 5 x 5).

Table 1. Example of calculating the lowest possible risk index for a biological control agent.

| Criterion | Likelihood | Magnitude | L x M |
|---|---|---|---|
| Establishment | 1 | 1 | 1 |
| Dispersal | 1 | 1 | 1 |
| Host range | 1 | 1 | 1 |
| Direct effects | 1 | 1 | 1 |
| Indirect effects | 1 | 1 | 1 |
| | | Sum = risk index | 5 |

Table 2. Case: risk index for *Steinernema feltiae* in field crops in Finland.

| Criterion | Likelihood | Magnitude | L x M |
|---|---|---|---|
| Establishment | 3 | 5 | 15 |
| Dispersal | 1 | 1 | 1 |
| Host range | 5 | 5 | 25 |
| Direct effects | 4 | 2 | 8 |
| Indirect effects | 4 | 1 | 4 |
| | | Sum = risk index | 53 |

In a first application of this methodology, van Lenteren *et al.* (2003) analysed 31 cases of natural enemy introductions. Entomopathogens all scored intermediate values in that analysis (*Beauveria* 50, *Metarhizium* 43 *and* 45, and *Steinernema* 53), mainly because of their broad host range, but their very limited dispersal capacities strongly reduces risk. For example in the case of *S. feltiae* (see Table 2) it could be argued - and further research might show - that the actual realised host range in the agricultural fields should not score 5 x 5 but, e.g. 3 x 2, which then already would change the initial rating significantly.

It is clear that interpretation of risk indices should be done with great care, and can only be done by biological control experts knowing the biology of the natural enemy under consideration. Risk indices will also vary according to the region for which they were made. Therefore, risk indices should not be seen as absolute values, but as indicators to which a judgement can be connected when considering whether to release an agent or not.

The next step in the risk assessment process is to discuss risk management, including risk mitigation and risk reduction. For example, in some cases risks may be minimised by imposing label restrictions, such as concerning the types of crops on which the use of the organism is or is not allowed (e.g., treatment of flowering plants with a mycoinsecticide), or by requesting specific application techniques (e.g., soil incorporation only for insect pathogenic nematodes).

The final step in making a justified ecological risk analysis for a new biological control agent is to conduct a risk benefit analysis, which should include a comparative performance of pest management methods, particularly based on environmental aspects.

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