10th EUROPEAN MEETING
"INVERTEBRATE PATHOGENS IN BIOLOGICAL CONTROL: PRESENT AND FUTURE"
at / à
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editor:
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The content of the contributions is in the responsibility of the authors
Preface

This bulletin contains the proceedings of the 10th European meeting of the IOBC/WPRS Working Group "Insect Pathogens and Insect Parasitic Nematodes". Entitled "Invertebrate Pathogens in Biological Control: Present and Future", this meeting was held in Locorotondo, Bari, Italy, 10-15 June 2005, in cooperation with COST Action 842 "Biocontrol of Pest Insects and Mites, with Special Reference to Entomophthorales" and COST Action 850 "Biocontrol Symbiosis".

Nearly 200 people attended, originating from 26 countries. Most European countries were represented and there were scientists from Egypt, Kenya, Israel, Georgia, P.R. China and U.S.A also. The meeting offered plenary sessions, oral and poster contributions sessions, and workshops. Contributions were dedicated mostly to entomopathogenic fungi and insect parasitic nematodes, less to other entomopathogens. A plenary session was devoted to bioinsecticide regulation and marketing. Three workshops were organized owing to local facilities, and attracted many interested people: a workshop (in two parts) dedicated to the diagnostics of nematodes, by Patricia Stock (U.S.A), a workshop dedicated to Microsporidia, by Rudolf Wegensteiner (Austria) and Regina Kleespies (Germany), and a workshop (repeated twice) devoted to the handling of entomopathogenic fungi for identification purposes, by myself. Indeed, such true workshops met the strong demand, from people interested in entomopathogens, for being trained in the recognition and diagnosis of microbial enemies of insects they are not familiar with.

Given I was elected as convener of the Working Group in 1999, my mandate was ending and the election of a new convener took place during the meeting. It led to the election of Ralf-Udo Ehlers (Germany). I am confident of the motivation and strength of all members of the Working Group and I have no doubt about its future.

The Locorotondo meeting was undoubtedly a very great success, which opportunely and firmly marked the 20th anniversary of our Working Group. On behalf of its members and all attendants, it is my pleasure to warmly express my gratitude towards the Local Organizing Committee and especially the Local Organizer, Pr. Oreste Triggiani, Dipartimento di Biologia e Chimica Agro-Forestale e Ambientale, Università degli Studi di Bari, for the excellent organization of the meeting. Warmest thanks are due also to the staff of the hosting institution, the Basile Caramia Research Centre ITA-CRSA.

A Oreste, Eustachio, Michele, Alessandro, Antonietta, Claudia, Pierdomenico, Giacomo, Giuseppe, Serafina, Maria, ed agli tutti altri amici dell’Apulia: mille ringraziamenti!

And last, but not least, I would like to thank all the colleagues and friends who relied on me for years in the framework of the activities of the Working Group.

Bernard Papierok
Past Convener of the Working Group
"Insect Pathogens and Insect Parasitic Nematodes"
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Insect Parasitic Nematodes
Molecular approaches for diagnostics and phylogenetics of entomopathogenic nematodes: applications and implications for pest management

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Abstract: Several molecular approaches are currently used in support of biological control as fast and cost effective ways of diagnostics and in analyzing data on relationships of both the target pests and the agents involved in their control. With respect to entomopathogenic nematodes (EPN), a variety of molecular methods have been used to diagnose, delimit species and infer their evolutionary histories. Of all, DNA sequence analysis has demonstrated to yield more information about variation within and among nematode species than other methods previously used. Furthermore, analysis of sequence data has shown to be a more suitable tactic in assessing phylogenetic relationships at different taxonomic levels, as well as for delimitation of species, and in separating cryptic species. In spite of these accomplishments, there still are many milestones and challenges that need to be achieved and unravelled in the study of EPN molecular biology. For example, until now, very little progress has been made in understanding the genetics of EPN at the infraspecific level. Yet, it remains to be defined what we understand as "populations" and delimit the geographic boundaries of such populations. This level of discrimination is yet much needed not only to meet possible requirements of registration for isolates but also to provide verification tools for proprietary rights to patented nematodes. For this, it is expected that new advances in molecular biology and comparative genomics will significantly expand our gamut of molecular markers and analytical tools. A summary of the current molecular approaches for the study of EPN is herein presented and discussed.

Key words: entomopathogenic nematodes, diagnostics, phylogenetics, molecular, pest management

Introduction

Systematic studies have lead to substantial contributions to our knowledge of evolutionary history and biological diversity, but have also played an important role in several other applied biological disciplines including sustainable agriculture, biological control and pest management strategies. Indeed, agricultural development has increasingly become dependent on the interactions between systematics and biological diversity, as many of the most important organisms in agricultural systems are the least understood in the context of biological diversity and systematics (Miller & Rossman, 1995). Certainly, many important plant-pathogenic microorganisms, arthropod pests and their natural enemies are either superficially understood or the least known to humans. This limitation in taxonomic knowledge becomes a major impediment to solve crucial problems in agriculture such the consideration of proper control measures. For example, insufficient systematic knowledge of both the pest and their natural enemies may delay implementation or even preclude proper biocontrol measures.

In this presentation, focus is centered on a group of natural enemies, the so-called entomopathogenic nematodes (Steinernematidae and Heterorhabditidae), hereafter referred to as EPN. These nematodes are a ubiquitous group of lethal parasites of a wide range of insect
pests. EPN have a mutualistic association with enteric bacteria (*Xenorhabdus* for *Steinernema* and *Photorhabdus* for *Heterorhabditis*), which are released into the insect hemocoel after penetration of the host by the infective stage juveniles of these nematodes.

EPN provide an environmentally safe and economical alternative for the control of a wide range of arthropod pests (Tanada & Kaya, 1993) and interest in these nematodes has increased exponentially over the past years because of their documented efficacy. For example, EPN are effective control agents of many soil insects such as weevils, grubs, white grubs, mole crickets, and caterpillars (Gaugler & Kaya, 1993). Although these insect pests are most commonly controlled by chemical pesticides, EPN based strategies have shown to be as effective as chemical insecticides in many situations (Gaugler & Kaya, 1993). Moreover, insecticidal nematodes are widespread in nature, specific to insects, safe to non-target organisms including humans, other vertebrates, and plants, and do not pollute the environment. EPNs can also be mass-produced in large fermentation tanks, can be stored for long periods and applied by conventional methods using standard spray equipment, making them a desirable commercial alternative (Grewal, 1999).

Akin to other entomopathogens, assessment of the efficacy, persistence, safety of EPN are essential and required steps that precede their development as a new management tool for insect pest control. However, and precluding all these tests, it is critical that proper identification of the species/strains is attained. In this presentation, I provide and overview of the most recent and commonly used molecular methods and techniques for diagnostics and identification of entomopathogenic nematodes and their implication in biodiversity and pest management studies.

**EPN biodiversity and the Tree of Life**

Improved analytical methods and data have recently made systematics a far more exciting and accurate field that it had been before. However, much more research remains to be done and consideration of novel approaches and techniques (i.e. molecular tools) need to be taken into account and evaluated in combination with traditional methods (i.e. morphological diagnostic methods).

Among the important challenges that need to be considered are: 1) continued documentation of the diversity of EPN, 2) development and application of accurate diagnostic methods to help identification of these organisms, 3) estimation of their evolutionary history and, 4) improvement of methods for phylogenetic inference and testing of evolutionary hypotheses.

With the exponential recovery of new EPN species and isolates, a demand for more accurate and fast diagnostic tools (i.e. molecular techniques) has been prompted, not only to better understand the biodiversity of these organisms but also to interpret their evolutionary history (Stock & Reid, 2003). Among many others, three molecular methods: random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP) and more recently DNA sequencing, have been the most extensively applied. DNA sequence analysis is currently the approach of preference for EPN diagnostics, species delimitation and phylogenetic inference.

Many nuclear (i.e. 18S, 28S and ITS) and mitochondrial genes (i.e. COI, ND4, 12S) have been considered in this respect (Stock et al., 2001; Stock & Reid, 2003, Nadler et al., 2006). However, it is important to emphasize that certain markers that are considered good for diagnostics are not suitable for phylogenetic inference and vice versa. For example, ITS rDNA genes are good diagnostic tools, but are not reliable for inferring evolutionary relationships within steinernematids. This is because ITS sequences are quite variable in
length (> 100 bp difference between some species) and nucleotide composition, making inferences of positional homology dubious across extensive regions of sequence (Nadler et al., 2005). However, this marker is so far, the only gene considered for diagnostics and assessment of phylogenetic relationships for *Heterorhabditis* spp. The large subunit rDNA gene (28S or LSU) is a more reliable marker to assess evolutionary relationships in Steiner- nematidae, but it is too conservative to infer evolutionary trends within Heterorhabditidae (Stock et al., 2001; Nadler et al., 2006).

In addition to EPN phylogenies, two species, *Steinernema carpocapsae* (Weiser) and *Heterorhabditis bacteriophora* Poinar, were depicted as representatives of each EPN family in the first Nematoda phylogenetic molecular framework (Blaxter et al., 1998). Interpretation of 18S rDNA sequence data suggests that the Heterorhabditidae are most closely related to the Strongylida, a group of vertebrate parasites, and together sharing *Pellioiditis*, a free-living bacterivore, as the closest common ancestor. The same tree depicted the Steinernematidae as being most closely related to the Panagrolaimoidea (free-living and insect associates) and Strongyloidae (vertebrate parasites), and as a member of a larger clade that includes free-living, fungal-feeding and plant parasitic taxa (Dorris et al., 1999).

### EPN diagnostics and pest management

EPN isolates and species show significant variation in behavior, host range, infectivity, reproduction, and environmental tolerances. This biological variation has stimulated interest in more fully characterizing *Steinernema* genetic diversity because new strains and species may prove more useful than those currently used as biological control agents against agriculturally important pests (Simoes & Sosa, 1996; Brown & Gaugler, 1997; Campbell & Gaugler, 1997; de Doucet et al., 1999). In this respect, molecular approaches have also been applied to study ecological traits of these nematodes such as environmental tolerance, and to genetically manipulate these organisms to make more successful biological control agents (Fodor et al., 1990, 1994; Hashmi et al., 1995). For example, host foraging strategies have also been mapped on molecular phylogenetic trees to develop hypotheses for the evolution of these ecological traits. For example, the ambush strategy seems to have evolved once, but cruise foragers evolved 3 times, and an intermediate foraging strategy seem to be the ancestral trait (Campbell et al., 2003).

With respect to pest management, accurate identification of EPN species/isolates is of critical importance. For example, matching the right EPN species with the appropriate target insect pest is relevant for the success of biological control and IPM programs. Without knowing the correct identity of an EPN species/strain, studies which have already been done could be repeated, wasting time and financial resources. Moreover, correct diagnosis of EPN is inherent to regulatory issues (permits for importation/exportation of species/isolates). Particularly regarding the importation of species and strains that are already present in a specific region or country, or in relation to the introduction of species/strains that could out compete native species/strain therefore compromising local biodiversity. Furthermore correct diagnosis of species/strains has also implications in the commercialization of this organisms and proprietary rights for use of patents and other legal issues regarding the use and application of EPN.

### Considerations for the future

As we advance our knowledge on EPN and gain new insight on their diversity and evolutionary relationships, it is important that we expand our gene repertoire. In this respect,
multigene phylogenetic approaches may help in providing better resolution of EPN evolutionary relationships and delimitation of species. In this respect, standardization of protocols and methods needs to be considered by systematists and taxonomists dealing with the identification of new EPN species/strains.

Another critical issue that requires an immediate action is the creation of a centralized database for all species/isolates recovered and that are currently available. It is critical to record which laboratories are currently maintaining EPN and what species/isolates currently available. Also relevant is the consideration of the standardization of protocols for maintenance and/or preservation of EPN (i.e. in vivo and in vitro methods, cryopreservation techniques, etc.).

Acknowledgements

I thank Prof. Oreste Triggiani and the IOBC Organizing Committee of the 10th European Meeting of the IOBC/WPRS Working Group "Insect Pathogens and Insect Parasitic Nematodes" for the financial support provided to S.P.S. to attend this meeting.

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Control of Codling Moth, *Cydia pomonella* (Lepidoptera: Tortricidae) with nematodes (*Steinernema* spp. and *Heterorhabditis* spp.)

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**Abstract:** As entomopathogenic nematodes are known parasites of *Cydia pomonella* under natural conditions, several trials were conducted to determine the most efficient nematode species and to evaluate its efficacy in the field. In laboratory experiments the LD$_{50}$S caused by the four nematodes species *Steinernema carpocapsae*, *Steinernemafeltiae*, *Heterorhabditis bacteriophora* and *Heterorhabditis megidis* were not significantly different. However *Steinernema carpocapsae* appeared to be more effective than *Heterorhabditis megidis* in a semi field essay. Unfortunately, due to a low retrieval rate of the initially released insects, this result was based on only very few individuals. Further, in autumn 2004, trunks of apple trees in an orchard in Southern Germany were treated with *Steinernema carpocapsae*. One month after the treatment diapausing larvae of *Cydia pomonella* in the trunk bark were isolated and the mortality caused by nematodes was assessed. In the untreated control 13% were infected with nematodes, whereas a proportion of 42% larvae infected with nematodes was noticed in the treated plot had.

**Key words:** *Cydia pomonella*, *Steinernema*, *Heterorhabditis*, apple orchard, efficacy

**Introduction**

The codling moth (*Cydia pomonella*) is the most serious pest of apple production and therefore efficient means of control are important. Nowadays, *Cydia pomonella* granulovirus (CpGV) and mating disruption are used successfully for the biological control of this insect.

As suggested by several authors (Dutky, 1959; Kaya *et al.*, 1984; Unruh & Lacey, 2001), entomopathogenic nematodes could be used as a supplemental agent for reducing the overwintering populations. Reduction of the diapausing populations would not only result in a smaller population in the following spring, but would also reduce the risk of the building up of a resistance against CpGV. Since the discovery of enhanced tolerance of codling moth against CpGV in orchards in Southern Germany, such considerations are crucial to ensure that this biological means of control would remain effective also in the future.

The aim of our investigations was first to determine the efficacy of four nematode species, *Steinernema carpocapsae*, *S. feltiae*, *Heterorhabditis bacteriophora*, *H. megidis*, in laboratory conditions, and then to evaluate the efficacy of the most potential nematode in the field. In contrast to the experiments of Unruh & Lacey (2001), no cardboard trap bands were used in this field trial because it was aimed at a high praxis relevance.

**Materials and methods**

**Trial 1: Determination of LD$_{50}$ in the laboratory**

Plastic cups (3.5 dl, $Ø = 8.6$ cm) with close fitting lids were filled with 100 g moist potting soil (Ökohum Spezialerde). A hole of 0.75 mm was drilled in each lid to ensure gas exchange. Twenty-five diapausing L$_5$ of *Cydia pomonella* were placed in each cup. The larvae were given 8 days (dark, 21°C) time to burrow in the soil and to cocoon.
In a preliminary trial a dose of 2900 nematodes per cup resulted in 100% codling moth larvae mortality with each nematode species tested (Steinernema carpocapsae, S. feltiae, Heterorhabditis bacteriophora, H. megidis). Therefore, per nematode species, 2 cups were treated with 11 mL water containing 58, 290 and 1450 nematodes, respectively. 4 cups were treated with 11 mL water and served as control. After an incubation time of 7 days (darkness, 21°C) the percentage of dead larvae was assessed and probit analysed.

**Trial 2: Semi-field-trial**

On a mowed meadow, 250 codling moth larvae (L₅) per m² were evenly distributed on an area of 4 m x 4 m. The area was covered with a net to prevent birds from picking the larvae. To test the efficacy of *S. carpocapsae* and *H. megidis*, two plots of 1 m² randomly chosen out of the 16 m² of the sample area were treated with 0.125, 0.25 and 0.5 $10^6$ nematodes per m², respectively. The nematodes were applied in 2 L water per m² with a watering can 3 days after the release of the codling moth larvae. Four square metres were treated only with water as control.

Fourteen days later eclectors were set up on each plot (Fig. 1). The moths caught were counted 18, 21 and 25 days after the nematode treatment.

![Figure 1. Eclectors for catching the emerging moths of *Cydia pomonella.*](image)

**Trial 3: Field trial**

In an apple orchard (cultivar Braeburn; 8000 trees/ha) in Fischingen (Southern Germany) with an extremely high codling moth pressure, 1250 m² were treated by means of a “Myers-Nachlaufspitze” (motor spraying device) with 800 L water per ha containing 750 $10^6$ *S. carpocapsae* (= 75000 nematodes / m²). The nozzles were positioned so that 40 cm of the trunk and a soil surface of 0.75 m on each side of the tree row were sprayed.

In the same orchard 1250 m² were used as untreated control. The treatment was carried out on a rainy day in mid October. The days following were cloudy and it rained often.

Twenty days after the nematode treatment, diapausing larvae in the trunk were searched for using a knife. They were found mainly in the graft union. To guarantee for equal handling, the control parcel and the treated parcel were searched alternatingly for an hour.

The living larvae found were kept individually on moist filter paper for 15 days. All dead larvae and the larvae which died within these 15 days were dissected and the presence of nematodes was assessed.
Results

**Trial 1: Determination of LD$_{50}$ in the laboratory**

All larvae were cocooned and located in the upper 2 cm of the substrate in the cup. No significant differences between the four LD$_{50}$ could be found. However, *H. megidis* and *S. carpocapsae* showed the lowest LD$_{50}$ (Table 1).

Table 1. LD$_{50}$ caused by different nematode species on diapausing codling moth larvae in a laboratory trial.

<table>
<thead>
<tr>
<th>Variant</th>
<th>LD$_{50}$ [nematodes / cup]</th>
<th>95% threshold limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>lower</td>
</tr>
<tr>
<td><em>H. bacteriaphora</em></td>
<td>424</td>
<td>135</td>
</tr>
<tr>
<td><em>H. megidis</em></td>
<td>203</td>
<td>56</td>
</tr>
<tr>
<td><em>S. carpocapsae</em></td>
<td>239</td>
<td>69</td>
</tr>
<tr>
<td><em>S. feltiae</em></td>
<td>310</td>
<td>94</td>
</tr>
</tbody>
</table>

**Trial 2: Semi-field-trial**

As displayed in table 2, only a few of the initially distributed larvae were caught as moths in the eclectors. Theoretically around 50 codling moths per eclector should have been caught, if the survival rate had been 100%.

Table 2. Surviving moths trapped with eclectors after the nematode treatment in the semi-field-trial.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Surviving moths per 2 eclectors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. carpocapsae</em> 0.125 $10^6$/m$^2$</td>
<td>0</td>
</tr>
<tr>
<td><em>S. carpocapsae</em> 0.25 $10^6$/m$^2$</td>
<td>0</td>
</tr>
<tr>
<td><em>S. carpocapsae</em> 0.5 $10^6$/m$^2$</td>
<td>0</td>
</tr>
<tr>
<td><em>H. megidis</em> 0.125 $10^6$/m$^2$</td>
<td>3</td>
</tr>
<tr>
<td><em>H. megidis</em> 0.25 $10^6$/m$^2$</td>
<td>2</td>
</tr>
<tr>
<td><em>H. megidis</em> 0.5 $10^6$/m$^2$</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**Trial 3: Field trial**

Thirteen percent of the larvae from the control plot were infected with nematodes, whereas in the treated plot 42% were nematode infected (Fig. 2). A mortality rate of 46.4% (all dead larvae) results using the formula of Henderson & Tilton (1995). About 50% of the living larvae were parasitized by Hymenoptera and/or Diptera.
Figure 2. Infection percentages of codling moth larvae with nematodes in treated and untreated (control) tree trunks

Discussion

The laboratory and the semi-field-trials show that *S. carpocapsae* has the highest capacity to eliminate codling moth larvae. However, the differences among the LD$_{50}$s were not significant in the laboratory trial and the results of trial 2 are based on very few individuals only.

Under field conditions, application of *S. carpocapsae* ($750 \times 10^6$/ha = $0.094 \times 10^6$ per tree) reduced the diapausing larvae of *C. pomonella* in the trunk by 46.4%. Taking into account that only a portion of the whole population diapauses in this specific location, the result should be considered as unsatisfactory.

Unruh & Lacey (2001) achieved 70% mortality with a hand gun application of $2 \times 10^6$ *S. carpocapsae* per tree. They also found, that the application technique, the location of the larvae and the wetting of the trees influenced the outcome of the treatment. In contrast to the trial reported here, the trunks had been banded with cardboard traps one month before the treatment to allow the *C. pomonella* larvae to cocoon in them. Mortality was assessed in these trapped larvae.

It is likely that optimization may render a successful use of *S. carpocapsae* against the codling moth under field conditions. Further trials are necessary to achieve better results with insect parasitic nematodes against diapausing codling moths under natural field conditions.

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Effectiveness of entomopathogenic nematodes in the control of sawfly (*Hoplocampa brevis*) in pear orchards

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**Abstract:** *Hoplocampa brevis* (Hymenoptera, Tenthredinidae) has caused recently severe damages to pear fruits in Italy, particularly in organic orchards, leading to heavy economic losses. A product based on *Steinernema feltiae*, suitable for leaf treatments, was applied for the control of sawfly larvae once or twice at different doses (250,000 IJ/m² and 500,000 IJ/m²); nematode based insecticide was compared with one rotenone treatment and with a product based on rock powder and plant oils. Furthermore, in the same trial, in an area of 1 m² repeated four times, under three trees which were not treated on the leaves, two products based on *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* were applied to the soil just before the drop of sawfly mature larvae from the fruits. The results showed interesting possibility of entomopathogenic nematodes (EPNs) applications in the sawfly management of organic farms, because in the plots treated with foliar applications of *S. feltiae*, the percentage of infested fruits was the same as the one obtained with rotenone (10-25%) and moreover, in a good percentage of young pears (about 40%), EPNs were found eight days after the last treatment. The soil application of EPNs registered a good effectiveness in the control of sawfly larvae, reducing significantly the adult population in the next spring.

**Key words:** entomopathogenic nematodes, *Steinernema*, *Heterorhabditis*, *Hoplocampa brevis*, pear sawfly, biological control.

**Introduction**

The pear sawfly, *Hoplocampa brevis* (Klug) is an Hymenoptera Tenthredinidae which causes severe damages in many countries of the Mediterranean Basin such as Italy, Spain, Cyprus, Iran. Losses of production could reach up to 80% (Davoudi, 1987).

The insect completes one generation per year and attacks young pears, mostly in organic orchards. In these environments, the lack of chemical treatments against other insects has caused, in the recent years, a return of sawfly at dangerous levels.

The mature larva spends the winter in a silken cocoon, at a depth of 5-10 cm in the soil (Pollini, 1998), and pupates in spring. In Italy, the adults appear at the beginning of spring; their danger depends on some risk factors as the population size in the previous year, the coincidence between the adults flight and the pear flowering, the level of flower induction and fruit setting (Oro et al., 1994). If spring is mild, the flight of pear sawfly will last 10-12 days, until 25 days at low temperatures. The female lays their eggs singly in the flower buds, beneath the epidermis between two sepals, making an oblique incision towards the centre of the calyx with the saw-like ovipositor. The eggs hatch after a week (Arias Giralda et al., 1973; Davoudi, 1987; Huberdeau, 1995; Pollini, 1998) and the neonate larva enters the young fruit and digs a gallery beneath its epidermis, at the base of the sepals which then wither. The larva burrows towards the centre of the fruit (primary attack), finally it leaves the fruit close to the calyx and attacks another one (secondary attack), but rarely a third. It then drops to the ground.
In some years, the sawfly infestation could be very large, damaging 80% of the fruits; the populations are aggregated so the infestation is generally localized in a part of the orchard; finally, in a same pear orchard, only some varieties can be attacked (Antropoli et al., 1994).

In conventional agriculture, chemical insecticides are sprayed against neonate larvae at 50% (Davoudi, 1987) or at 70-90% (Szekely, 1977) of the petal loss, while in an integrated pest management it is suggested to monitor the adult flight by white traps; the threshold of 20 adults per trap is recommended before spraying the larvae (Antropoli et al., 1994).

In organic orchards, insecticides based on rotenone, pyrethrum and other natural substances are used; laboratory bioassays with fungi demonstrated 100% effectiveness eight days after the treatment (Jaworska, 1979), while Mermitid, Rabditid and Steinernematid nematodes were found associated to apple sawfly larvae and Heterorhabditis spp. caused 100% mortality, when distributed in water suspension in pots experiments (Jaworska, 1986). Assays in Petri dish with Heterorhabditis bacteriophora and Steinernema carpocapsae demonstrated 100% mortality in apple sawfly (Hoplocampa testudinea), while semi-field experiments showed more than 80% larval mortality and foliar applications significantly reduced secondary damage in the apple fruits (Vincent & Bélair, 1992).

Given the development of new formulations based on entomopathogenic nematodes (EPNs) suitable for foliar applications, field experiments have been carried out with the aim of checking: 1) the effectiveness of Steinernema feltiae foliar applications in the control of Hoplocampa brevis, 2) the ability of this nematode to penetrate the young pears and reach sawfly larvae in the fruits, after foliar application, 3) the effectiveness of soil treatments with H. bacteriophora and S. carpocapsae against H. brevis mature larvae, as well as the efficacy of EPNs in comparison with other insecticides, admitted in organic agriculture.

Materials and methods

The trial was carried out in the province of Bologna (Italy), in an organic pear orchard, cultivar Abate Fetel, with 4 m distance between rows and 2 m between plants, trees 4 m tall and 19 years old, grown as wall; the experimental design was a randomized block, with 6 thesis repeated 4 times and with 4 trees per plot.

Foliar treatments

All the treatments (Table 1) were applied by a sprayer doted with hand lance, pump model Comet MC 20/20, max. capacity and pressure respectively 19 litre per minute and 20 bar, engine Briggs & Stratton; the volume of water for EPNs application was 900 L per hectare, little fewer than the volume applied from the farmers, because of the scarcity of vigour in the trees. The treatments were sprayed on May; those based on S. feltiae, suitable for leaf treatments, were applied at the sunset, once or twice, at different doses; after the first application, a slight rainfall occurred, which contributed to wet the foliage in the best way.

Soil treatment

In the same pear orchard, two products based on H. bacteriophora and S. carpocapsae were applied only once onto the soil (Table 2), just before the drop of sawfly mature larvae from the fruits.

The treatment was carried out by a hand sprayer, in 20 L of water per m², in an area of 1 m² repeated four times, under three trees which were not treated on the leaves; after the application the ground was covered with a net funnel until the next spring (April 2005).
Table 1. Products and rates applied to the foliage and the young pear fruits.

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Commercial product</th>
<th>Rate</th>
<th>Application(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rock powder and plant oils</td>
<td>Unica</td>
<td>400+200 gr/hl</td>
<td>1 treatment (T) (8/5/2004)</td>
</tr>
<tr>
<td>Steinernema feltiae</td>
<td>Nemasys F</td>
<td>250,000 IJ/m²</td>
<td>1 treatment (T) (6/5/2004)</td>
</tr>
<tr>
<td>Steinernema feltiae</td>
<td>Nemasys F</td>
<td>250,000 IJ/m²</td>
<td>2 treatments (T and T+7) (6/5/2004 and 13/5/2004)</td>
</tr>
<tr>
<td>Steinernema feltiae</td>
<td>Nemasys F</td>
<td>500,000 IJ/m²</td>
<td>1 treatment (T) (6/5/2004)</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Bioroten</td>
<td>300 cc/hl</td>
<td>1 treatment (T) (8/5/2004)</td>
</tr>
<tr>
<td>Untreated (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Products and rates applied onto the soil.

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Commercial product</th>
<th>Rate</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterorhabditis bacteriophora</td>
<td>Nematop</td>
<td>500,000 IJ/m²</td>
<td>1 treatment (T) (19/05)</td>
</tr>
<tr>
<td>Steinernema carpocapsae</td>
<td>Nemasys F</td>
<td>500,000 IJ/m²</td>
<td>1 treatment (T) (19/05)</td>
</tr>
<tr>
<td>Untreated (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Assessments
Eight days after the last foliar treatment (21/5/2004), on the two central trees of each plot, all the fruits were checked and all the worm-eaten ones were removed. Later, the young pears were sectioned and the EPNs were checked inside.

In April 2005 the adults emerging from the treated ground, covered with a net funnel, were assessed by plastic white traps spread with glue, which were placed at the end of the funnel, under the net.

Statistical analysis
Data were processed by analysis of variance (ANOVA), followed by the average range test known as Least Significant Difference (LSD; p < 0.05). Data were transformed in arcsine √x.

Results and discussion
The results showed a fairly good EPNs ability to penetrate the larval hole into the pears; they were found in about 37% worm-eaten fruits in the plots treated with S. feltiae, while never in the other treatments and in the control (Fig. 1); besides, no statistical differences were found at the different EPN tested doses. The treatments were carried out under the best environmental conditions, when sawfly larvae began to migrate from the first pear (where the egg had been laid) to another one; in these conditions the EPN effectiveness to reduce the secondary damage of sawfly larvae was the same of the other organic insecticides, statistically different from the untreated control (Fig. 1).
The treatment on the soil demonstrated the effectiveness of *H. bacteriophora* and *S. carpocapsae* to control mature larvae of *H. brevis*; the adult assessment, in the next spring, showed the presence of a significant number (about 20 in all) of adults emerging from the untreated plots, while no adults were observed in the treated ones.

In conclusion, these preliminary results appear encouraging, and could serve as a contribution for further investigations in organic orchards, where sawfly represents a key insect, very difficult to control.

Figure 1. Percentage of young pears damaged by *Hoplocampa brevis* and percentage of fruits parasitized by *Steinernema feltiae*, eight days after the last treatment.

References


Nematode cryopreservation using a mechanical freezer at -140°C: a preliminary report

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Abstract: Temperatures below -130°C are known to assure long-term, and possibly indefinite preservation of various biological specimens. Recently, the Bursaphelenchus spp. I.S.Z.A. collection has been enlarged much by the acquisition of new nematode populations, referred to B. eremus (Rühm) Goodey. This species was isolated in Italy both from dying Quercus spp. and also from the bark beetle Scolytus intricatus Ratzburg as dauer larvae. So, a cryopreservation technique using a mechanical freezer at -140°C has been developed for a long-storage of all the Bursaphelenchus spp available.

Key words: cryopreservation, cryoprotectant, Bursaphelenchus spp., long-storage nematode cultures

Introduction

The necessity of repeated sub-culturing of fungal cultures, or nematode populations is not only time consuming but requires continuous efforts of specialists and it does not always assure from the risk of substrate contaminations. Additionally, the possibility of genetic changes, like loss of pathogenicity or viability due to selection pressure under laboratory conditions, is quite consistent.

Cryopreservation is a practical means of preserving organisms for a long time. Moreover, nematode cryopreservation allows us to dispose at any time of great number of living nematodes to plan experiments. However, frequently, different species show distinct requirements for cryopreservation. So, protocols have to be established empirically case by case. Nematodes with several other species of parasites and with insect embryos cannot be cryopreserved by slow cooling protocols but have an absolute requirements for vitrification. Even when slow cooling methods is suitable, the vitrification often leads to a marked improvement in the level of survival attained (James, 2004). In their treatise "Life and death at low temperatures", Luyet & Gehenio (1940) noted that to achieve vitrification, rapid cooling below the vitrification point and, rapid warming, above the devitrification point, was required. They also indicated that certain compounds such as glycerol, ethanediol and several sugars could facilitate vitrification by elevating the vitrification and devitrification points together slowing the rate of ice crystal propagation within the sample.

To date, cryopreservation procedures have been explored for the storage of animal and a few plant-parasitic nematodes (Ham et al., 1979; Triantaphyllou & McCabe, 1989; Carneiro et al., 2001). Among these are also two closely related species belonging to the pinewood nematode complex, Bursaphelenchus xylophilus (Steiner et Buhrer) Nickle and B. mucronatus Mamiya et Enda (Riga & Webster, 1991; Ogura, 2003). In these latter protocols, a glycerol solution was utilized at 15% and 25%, respectively and a successively two step cooling procedure was adopted at -70°C or -25°C, respectively, before to store the living samples in liquid nitrogen (LN) at -180°C. The percentage of motile juveniles after storage in LN was
4.8%-17.6% in the experiment carried out by Riga & Webster (1991) while Ogura (2003) observed the percentage reaching 60.3% of survival after six month of storage.

In this paper, another method is presented that improves the percentage of phytonematodes alive after a storing period in a mechanical freezer (-140°C).

Materials and Methods

Source and maintenance of nematodes
A *Bursaphelenchus eremus* population (Boscogrande, Italy, IT 20) isolated from dying oak trees in Italy was used. The nematode population was reared at 24°C on *Botrytis cinerea* plates. The cultured nematodes were isolated from the drops of water formed on top of the plates. They were rinsed in pure water and encountered before using for cryopreservation protocol.

Freezing procedures
Ethylene glycol (99% EG, BDH) solutions were prepared in distilled water at 2X final concentration (20, 50 and 70%) and an equal weight of nematode suspension and ethylene glycol solution were mixed to obtain the final concentration (10, 25 and 35%).

Nematodes were first incubated in 10% (v/v) solution for 2-12 hours at 27°C after which, a centrifugation allowed us to reduced the volume to a half and then, in cold 25 or 35% EG (v/v) for 45-60 min.

After these incubation periods, 50 µL of cold nematode suspensions were absorbed onto strips of chromatograph papers (nitrocellulose paper, Roche) and left dry on the table briefly before to transferred them in 3.5 mL cryogenic tubes and then plugged into liquid nitrogen (LN) at -196°C.

Cryovials were kept few minutes in the nitrogen bath before transferring it to a mechanical freezer at -140°C. The transfer must be done quickly to ensure that the paper strips inside the small cryogenic vials are constantly immersed in liquid nitrogen.

Thawing and viability assessment
Thawing was carried out by quickly transferring a paper strips from the freezer into a cap containing 5-6 mL of water, at room temperature. Live animals start moving within 1 hour from thawing but viability was assessed, as number of animals alive, 12 hours after thawing. The percentage of living nematodes (average of six counts of 1 mL of total suspension) was recorded from each vial after thawing.

Reproduction rate after cryoconservation
In order to check the reproduction capacity of the frozen animals, aliquot of 500 living nematodes, thawed at each interval of time (4 hours, 6 days, 1 and 2 months), were transferred to a fresh plate of *B. cinerea* and incubated, at 24°C, for a week. After that period the reproduction rate (R = Pf/Pi) was determined by the rate of the final population number (Pf) respect to the number of animals inoculated at the beginning (Pi).

Statistical analysis
Mean percentage nematode survival following each cryopreservation intervals of time were undergone to analysis of variance (ANOVA). Percentage data were arcsine-transformed prior to analysis (Fisher’s test, P = 0.05). Percentages presented in the table and followed by the same letter are not significantly different.

Results and discussion
The nematodes included in the 35% EG solution, although still alive after LN treatment, do not survived at -140°C and died upon thawing. Instead, the samples included in the lower EG
solution (25%) greatly survived. The survival percentage were assessed after a rapid thawing in water and shown in table 1. In this table, the mean number of motile nematodes, the percentage of live nematodes and the reproduction rate of the animals after treatment are reported. All data were recorded after four intervals of time: 4 hours, 6 days, 1 and 2 months at -140°C. Survival of frozen nematodes referred to a B. eremus isolate was recorded 4 h, 6 days, 1 or 2 months later and all demonstrated to survive in high percentage (a mean of 80%).

Table 1. Motility and reproduction rate of 25% cryopreserved Bursaphelenchus eremus 24 hr after thawing.

<table>
<thead>
<tr>
<th>Storage period in -140°C freezer</th>
<th>Motility of J2-adults (mean ± SE)*</th>
<th>Survival (%)</th>
<th>Reproduction rate**</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hours</td>
<td>1959 ± 438</td>
<td>84.7 a</td>
<td>13.6 b</td>
</tr>
<tr>
<td>6 days</td>
<td>1467 ± 369</td>
<td>77.0 a</td>
<td>14.5 b</td>
</tr>
<tr>
<td>1 month</td>
<td>1691 ± 138</td>
<td>85.8 a</td>
<td>9.0 b</td>
</tr>
<tr>
<td>2 months</td>
<td>2291 ± 71</td>
<td>76.6 a</td>
<td>7.2 b</td>
</tr>
</tbody>
</table>

* Average of six replicates. Percentages followed by the same letter are not significantly different (Fisher’s test) P = 0.05.

** R= Pf/Pi, average of 3 replicates.
Mean of reproduction rate for non treated animals = 2.8.

The reproduction rates of B. eremus was compared with those of animals (originating from the same culture plates) that had been kept at 12°C (Table 1). The reproduction capability of the nematodes after cryopreservation was remained as in original population. No significant differences in reproduction rate between the cryopreserved population of B. eremus and the control animals were observed.

Cryopreservation can have many benefits over the normal maintenance on B. cinerea culture plates. It obviates the labour and requirements for space and the maintenance of controlled environmental conditions, all of which are costly. The likelihood of cross infections and mutation or selection of unwanted biotypes is lessened. Furthermore, due to the easy storing and recovering, a large number of isolates can be readily maintained and individuals of each population can be safely distributed to laboratories for comparative or for other studies. Storage in liquid nitrogen has been demonstrated as a method for long-term storage of several nematode genera, including entomopathogenic ones (Riga & Webster, 1991; Ogura, 2003; Bai et al., 2004).

The new method reported here improve both the animals viability and it allows the maintaining of plant-nematodes cultures in -140°C mechanical freezer, avoiding liquid nitrogen freezer and so, minimizing the risk of potential contamination of improperly sealed vials.

Acknowledgements

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References


Effect of *Melolontha melolontha* grubs on persistence of entomopathogenic nematodes in soil

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**Abstract:** Effects of *Melolontha melolontha* grubs on persistence of three entomopathogenic nematodes, namely *Steinernema glaseri* (NC), *S. arenarium* (Ryazan) and *Heterorhabditis megidis* (01PL), were tested in two laboratory bioassays. The first test was carried out in PCV pots filled with a moist peat. The nematode infective juveniles (IJ) were applied at the rate of 10⁶ m⁻² per grub. The grubs were then withdrawn from the containers 8, 14 and 21 days after nematode application, examined for mortality and dissected to evaluate the number of nematodes present inside. All withdrawn grubs were replaced with 5 caterpillars of *Galleria mellonella* per container. After additional 72 h of incubation, the caterpillars were dissected and examined for the presence of nematodes. In general, the survival of nematodes was better in containers with no grubs. The greatest number of nematodes was observed in pots with *S. arenarium* (Ryazan) (mean 137.8 per pot), while the lowest one was in pots with *H. megidis* (01PL) (mean 37.4/pot). In the second bioassay, conducted in Petri dishes, moist sand was inoculated with 100 infective juveniles of *S. glaseri* (NC). To assess nematode persistence in presence of a grub, IJs were extracted from the sand using a decant and sieve technique. The mean number of living nematodes was significantly highest in absence of grub (61.6/dish) than in combination with the grub (53.5/dish). Persistence of tested nematodes varied according to time of their exposure, nematode species and presence or absence of the grub.

**Key words:** entomopathogenic nematodes, persistence, grubs, *Melolontha melolontha*

**Introduction**

Entomopathogenic nematodes are capable of killing many soil dwelling insects. However, these insects may also have a negative effect on the nematode population (Gaugler _et al._, 1994). Effects of the third stage larvae of the well known pest *Melolontha melolontha*, on persistence of three entomopathogenic nematodes, namely *Steinernema glaseri* (NC), *S. arenarium* (Ryazan) and the first polish isolate of *Heterorhabditis megidis* (01PL), isolated from naturally infected *Amphimallon solstitiale* white grub, were tested in two laboratory bioassays.

**Materials and methods**

The first test was carried out in PCV pots filled with a moist peat. Nematode infective juveniles (IJ) of the three nematode species were applied at the rate of 10⁶ m⁻² per grub. Experiments were carried out separately for 3 periods of time: the grubs were withdrawn from the containers 8, 14 and 21 days after the application, examined for mortality, and dissected to evaluate the number of nematodes present. All withdrawn grubs were replaced with 5 caterpillars of *Galleria mellonella* per container. After additional 72 h of incubation, the bait-insects were dissected and examined for the presence of nematodes. The sum of nematodes counted during dissection of grubs and that of caterpillars was considered as a number of active juveniles present in the soil. As a control was performed the variant without the grub, the term “start” was considered as 3 days after inoculation of IJs nematodes.
The second bioassay was conducted in Petri dishes. Moist sand was inoculated with 100 infective juveniles of *S. glaseri* (NC). To assess nematode persistence in presence and lack of a grub, IJs were extracted from the sand using a decant and sieve technique. During second bioassay we did not observe the infection of grubs because the time of their exposure to nematodes was too short.

Two variants of tests were performed: with presence or absence of the grub, each in 30 replicates.

**Results and discussion**

In the first bioassay, all nematodes survived differently according to the period of time, 8, 14 or 21 days (\(p = 0.002, \text{DF 2;12, F = 11.66}\)), presence of grubs (\(p = 0.028, \text{DF 1;12, F = 6.22}\)), and nematode species (\(p = 0.003, \text{DF 2;12, F = 9.95}\)).

In general, the survival of nematodes was better in containers with no grubs. The greatest number of nematodes was observed in peat with *S. arenarium* (Ryazan) (mean: 137.8 per pot), while the lowest one was in pots with *H. megidis* (01PL) (mean: 37.4/pot) (Fig.1, Fig.2, Tab.1).

![Figure 1](image.png)

Figure 1. Number of nematodes recovered during dissection of *Melolontha melolontha* grubs and *Galleria mellonella* larvae (\(P = 0.05\), Tukey’s procedure). Upper case – the differences between time of post-exposure, lower case – differences between nematode species.

Interestingly, the lowest number of nematodes was noted for the Polish isolate of *H. megidis*, in both variants, i.e. with and without grubs. Grubs were likely more attractive for that isolate than for *S. arenarium* (Ryazan), which could be related to the fact that this mentioned nematode species is using against the white grubs, as well as *S. glaseri* (NC). Average mortality of the grubs caused by *H. megidis* (01PL) was about 60% (unpublished data). The infective juveniles of *H. megidis* (01PL) and *S. glaseri* (NC) initiated the penetration process in insects and several among them were killed by grubs. Additionally,
there are in the literature conclusions, that the infective juveniles of Heterorhabditidae have poor natural ability to survive for long time (Molyneux, 1985).

Figure 2. Number of nematodes recovered during dissection of *Galleria mellonella* from peat without *Melolontha melolontha* grubs (P = 0.05, Tukey’s procedure). Upper case – the differences between time of post-exposure, lower case – differences between nematode species.

Table 1. Mean numbers of infective juveniles from soil in relation to nematode (*Steinernema* or *Heterorhabditis*) species and day of exposure, based on dissection of dead insects (*G. mellonella* and *M. melolontha*). Means followed by the same letter are not significantly different (P = 0.05, Tukey’s procedure).

<table>
<thead>
<tr>
<th>Nematode species</th>
<th>Days of exposure</th>
<th>Mean number of recovered IJs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8th day</td>
<td>14th day</td>
</tr>
<tr>
<td><em>S. glaseri</em> (NC)</td>
<td>114,6</td>
<td>45,5</td>
</tr>
<tr>
<td><em>S. arenarium</em> (Ryazan)</td>
<td>203,3</td>
<td>166,1</td>
</tr>
<tr>
<td><em>H. megidis</em> (01PL)</td>
<td>91,4</td>
<td>17,6</td>
</tr>
<tr>
<td>Mean</td>
<td>136,4a</td>
<td>76,4ab</td>
</tr>
</tbody>
</table>

In the second bioassay, the mean number of living *S. glaseri* (NC) nematodes was significantly highest in absence of grubs (61,6/dish) than in combination with grubs (53,5/dish). Persistence of tested nematodes varied according to time of their exposure (p ≤ 0.001, DF 4;80, F=18.54) and presence or absence of the grubs (p ≤ 0.001, DF 1;80, F=16.16).
Figure 3 shows decrease of number nematodes in combination with the grub during test. Significant differences in the number of infective juveniles were recorded between the first and the third day of the test.

The mortality of the grubs caused by *S. glaseri* (NC), *S. arenarium* (Ryazan) and *H. megidis* (01PL), which was 55, 20, and 66% respectively, was recorded only during the first test, after the first 2 weeks.

![Figure 3. Mean number of infective juveniles of *Steinernema glaseri* (NC) extracted from sand of test variant with the grubs (P = 0.05, Tukey’s procedure).](image)

**Conclusion**

The nematode survival depended on the length of exposure, presence of grubs in the soil and nematode species. It was greater in the soil with no grubs. The greatest number of nematodes was observed in the soil with *S. arenarium* (Ryazan), while the lowest one was in the soil with *H. megidis* (01PL).

**Acknowledgements**

The author thanks Maria Kozlowska for their help in conducting statistical analysis.

**References**


Effects of UV-B radiation on Steinernema apuliae (Rhabditida: Steinernematidae)

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Abstract: The effects of UV exposure on surviving entomopathogenic nematodes have been remarkably documented; however, the effects of UV exposure on Steinernema apuliae are unknown. In the bioassay conducted, a UV lamp was used that emitted medium wavelength radiation because UV-C light is known to rapidly kill entomopathogenic nematode species. The experiments were carried out on six strains of infective juveniles of S. apuliae in water. Each strain was exposed for 0 (control) and 5 and 10 minutes at different distances from the lamp with three replications. With the objective to determine the survival rates for each strain, the nematode mortality rates were analyzed after 1, 3, 5, 7, and 14 days. The survival curves were also compared to evaluate the significant difference among the strains. The ability of the irradiated infective juveniles to determine lethal infection was later tested against last-instar Galleria mellonella larvae.

Key words: entomopathogenic nematodes, Steinernema apuliae, UV exposure, survival rates

Introduction

The effects of UV-B exposure on the survival of entomopathogenic nematode species have been remarkably documented (Gaugler & Boush, 1978, 1979; Wilson & Gaugler, 2004). These noxious effects induce a decline in the reproductive capacity and a loss of pathogenicity and seem to vary among species. Steinernematid nematodes appear to be more tolerant of environmental stress than Heterorhabditidis (Gaugler et al., 1992).

No reports are available on the effects of UV radiation on Steinernema apuliae; in fact, this species was recently isolated from silt sand soil in southern Italy (Triggiani et al., 2004). For our bioassay we chose to use medium wavelength radiation (theoretical wavelength from 280 to 320 nm), since this wavelength seems to be mainly responsible for causing biologically harmful effects (Gaugler & Boush, 1979).

Materials and methods

Cultures
The bioassay was conducted with third-stage infective juveniles (IJJs) of 6 strains of S. apuliae (Table 1). The nematodes were propagated in last-instar Galleria mellonella (Lepidoptera: Galleriidae) larvae (Kaya & Stock, 1997) and passed through a nylon mesh filter (40 µm). The final suspension of nematodes was irradiated only on the day after the first emergence from the host at 21°C.

UV source
The UV lamp used (Model GL20SE, Sankyo Denki Co., Japan) emitted medium wavelength radiation (range 280-360 nm, with a peak length of about 306 nm). At a distance of 10 cm the lamp delivered 7,452 µW/cm², whereas at 30 cm it delivered 828 µW/cm². The radiation intensity was confirmed at 306 nm, based on the measure of the current obtained in a silicon
photodiode calibrated from the National Institute of Standards and Technology. Irradiation was conducted at 21°C under a vertical laminar flow hood (Bio Air s.c.r.l, Milan, Italy).

Table 1. Sites where the 6 tested *S. apuliae* strains were collected.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Locality</th>
<th>Altitude (m a.s.l.)</th>
<th>Habitat</th>
<th>Soil texture</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ItS-MS10</td>
<td>Margherita di Savoia (FG)</td>
<td>30</td>
<td>Salt pan</td>
<td>Silt</td>
<td>7.9</td>
</tr>
<tr>
<td>ItS-CS3</td>
<td>Brindisi (BR)</td>
<td>20</td>
<td>Tamarisk</td>
<td>Sand</td>
<td>8.3</td>
</tr>
<tr>
<td>ItS-TA14</td>
<td>Taranto (TA)</td>
<td>0</td>
<td>Sea coast</td>
<td>Sand</td>
<td>8.3</td>
</tr>
<tr>
<td>ItS-ZA11</td>
<td>Zapponeta (FG)</td>
<td>30</td>
<td>Uncultivated</td>
<td>Sand</td>
<td>7.8</td>
</tr>
<tr>
<td>ItS-LE13</td>
<td>Torre Pali (LE)</td>
<td>0</td>
<td>Sea coast</td>
<td>Sand</td>
<td>8.2</td>
</tr>
<tr>
<td>ItS-LD3</td>
<td>Metaponto (MT)*</td>
<td>20</td>
<td>Pinewood</td>
<td>Sand</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* Basilicata region

**Bioassay**
A water suspension of nematodes (approximately 500 IJs/6 ml) was placed in a 5.2 cm uncovered plastic Petri dish. Only 6 mL of sterilized water was required to exactly cover the bottom of the dish. Each strain of nematode suspension was exposed for 0 (control), 5, and 10 minutes at an exposure distance of 10 and 30 cm with three replications. After irradiation, the Petri dishes were kept in the dark at 21°C to determine the effects on nematode mortality.

**Nematode mortality**
The number of dead IJs was counted in each Petri dish under a stereomicroscope after 1, 3, 5, 7, and 14 days of exposure. The experiment was conducted with three replications for each strain of *S. apuliae*. The criteria used to verify the death of the IJs were adopted from Gaugler & Boush (1978). The mortality data were analyzed to determine the survival rates for each strain.

**Pathogenicity**
Pathogenicity was expressed as the capacity of *S. apuliae* to cause lethal infections in the mature larvae of *G. mellonella* (Gaugler & Boush, 1978). After each control for mortality data, the surviving IJ nematodes were collected in small wells and subsequently quickly transferred to 100 mm Petri dishes containing two No. 1 Whatman filter papers. Eight wax moth larvae were placed in each Petri dish and incubated in the dark at 23 ± 1°C. The virulence effect of the irradiated IJs on the wax moth larvae was determined after dissection under a stereomicroscope to ascertain that mortality resulted from nematode infection; larval mortality was recorded daily until the time at which death occurred in half of the *Galleria* larvae (ST50).

**Results and discussion**
This study showed that the effects of UV-B radiation on *S. apuliae* mortality, under the same conditions of exposure time (5 min), were related to the distance of the UV-B source. For all the strains analyzed, the mortality rate increased as the irradiation distance decreased from 30 to 10 cm. Twice the exposure time (10 min) caused 100% nematode mortality rate for all the distances, beginning immediately from the first day after irradiation. For this reason it was possible to determine survival rates only at the exposure time of 5 min. For each strain of *S.
*apuliae*, our data show a positive correlation between the decline in nematode population and the number of days of post-exposure control. A UV-B lamp distance of 10 cm, after 14 days of exposure, caused death in all the strains except for the strain ItS-ZA11, which showed a survival rate of about 14%. Conversely, at a distance of 30 cm, the survival rates of two strains, ItS-TA14 and ItS-ZA11, were significant higher (P<0.05) compared to those of the control groups. These strains demonstrated a higher tolerance level to UV-B exposure. Moreover, the ItS-LE13, ItS-CS3 and ItS-LD3 strains yielded a survival rate of approximately 35%, 32.4%, and 19.4%, respectively, after 14 days of exposure. Figures 1a and 1b illustrate the UV-B radiation effects on all strains at the distances of 10 and 30 cm.

Figure 1. UV-B effects on six Italian strains of *Steinernema apuliae* at the distances of 10 (a) and 30 cm (b) from the light source. The data were normalized to control value of each strain control group.
Figure 2. Median survival time (ST$_{50}$) in 4 Italian strains of *S. apuliae* exposed to UV-B radiation (control —Δ—).

This comparison was possible using survival data normalized to the control value of each strain. The data reveal the real damage caused by UV-B radiation compared to the mortality of control groups that is related possibly to various unfavourable conditions (lack of oxygen, evaporation of the water film in Petri dishes, etc.). At a distance of 10 cm from the lamp, ItS-TA14 and ItS-LE13 revealed greater resistance up to 7 days after exposure. On the contrary, at a distance of 30 cm, all strains showed overlapping behaviour within 5 days after exposure, whereas from 7 to 14 days post-exposure, 3 different behaviour types were observed: ItS-TA14 and ItS-ZA11 revealed overlapping behaviour identical to the controls; ItS-LE13, ItS-CS3 and ItS-LD3 yielded an intermediate reaction; and ItS-MS10 revealed the highest susceptibility to UV-B radiation of all the strains.

The UV-B resistance variability among *S. apuliae* strains was more significant when compared to the results for *Steinernema kushidai*, for which an exposure time of 5 min to UV-B radiation (0.9 mW/cm$^2$) caused a mortality rate of 100% in nematodes (Fujiie & Yokoyama, 1998).

Pathogenicity was expressed by means of median survival time. The virulence of irradiated IJ nematodes declined in all populations after exposure to UV-B radiation. In fact, the time at which death occurred in the 50% of *Galleria* larvae increased in all populations analyzed (Gaugler & Boush, 1978; Gaugler *et al.*, 1992; Grewal *et al.*, 2002). Greater
resistance of ItS-TA14 was reflected in the pathogenicity data: the pathogenicity caused by the treated (radiation distance of 30 cm) IJs was not reduced in comparison with the control IJs; the other 3 strains (ItS-LE13, ItS-LD3, ItS-ZA11) required more time to cause host mortality (ST50) when compared to controls (Fig. 2).

Conclusion

In conclusion, the bioassay showed that exposure to UV-B light (average wavelength of 306 nm) decreases the infectivity of *S. apuliae* and that the increase of radiation intensity causes more damage to the IJs. The same is true for all entomopathogenic nematodes (Gaugler & Boush, 1978), although the IJs of *S. apuliae* appeared rather healthy. We also observed a variation in strain persistence, which requires further evaluation. These results support the hypothesis that the resistance to UV-B radiation of different strains of *S. apuliae* may be related to the climatic conditions of the original location of the populations or can be correlated to genetic factors, as reported by Grewal *et al.* (2002). Further studies are necessary to explain the pathogenicity data on *S. apuliae*. Although laboratory data cannot be related to field conditions, this tolerance to UV-B radiation should represent an important factor from a commercial standpoint due to the potential use of *S. apuliae* as a biological control agent against insect pests.

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Efficacy and environmental impact of entomopathogenic nematodes used against nut insect pests in some chestnut woods on Etna (Italy)

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Abstract: Investigations were carried out to test the possibility of controlling the main chestnut fruit insect pests (tortricid caterpillars and curculionid larvae) on Mount Etna (Italy) by means of entomopathogenic nematodes (EPN) belonging to the genera *Steinernema* and *Heterorhabditis*. A survey of autochthonous EPN species, which should more suitable to be used locally, was conducted in many chestnut groves of Etna and various populations of nematodes, mainly belonging to *Steinernema*, were found. Laboratory tests were made to evaluate the ability of several EPN species and strains to parasitize larvae: most EPN species were shown to be effective against most insect larvae extracted from the nuts. In particular, *H. bacteriophora* showed a great efficacy both against the tortricids and curculionids. Soil insemination with different EPN species was made to test their persistence ability in the soil. The most persistent species was *H. bacteriophora*, which was found in the soil for many months after the soil insemination, that is for a time sufficient to let these nematodes find and kill the insect larvae in the soil. The environmental impact of the EPN insemination on the soil fauna was also thoroughly studied and no significant differences were found in the soil fauna composition before and after the treatment.

Key words: biological control, *Steinernema* spp., *Heterorhabditis* spp., chestnut pests, *Pammene fasciana*, Curculio elephas

Introduction

The aim of our research was to control the insect pests of nuts of some chestnut groves of Etna, by means of entomopathogenic nematodes (EPN). In Sicily the most dangerous and abundant insect pest is *Pammene fasciana* (Lepidoptera Tortricidae), but *Cydia fagiglandana* and *C. splendana* as well as some species of Coleoptera Curculionidae, among which *Curculio elephas* and *C. glandium* are also present.

Before activating a control strategy against the nut insect pests some preliminary studies were done, namely: a) a screening of the autochthonous EPN species present in the Etna chestnut soil, b) tests on the virulence of different EPN species against the main nut insect pests, c) tests on the ability of these species to persist in the soil after the insemination, and d) evaluation of a possible undesired environmental impact, especially against non-target soil insects due to the introduction of the EPN into the soil.

Two small uncultivated chestnut groves on Etna were selected as research sites. The one near the town of Fornazzo, at 800 m above sea level, and the other in locality Triciala, at 950 m above sea level. They will be indicated hereon as F and T. In each site three plots (named respectively F 1, F 2 and F 3, and T 1, T 2 and T 3) were selected for the tests.
Materials and methods

**Looking for the autochthonous EPN species present in the Etna chestnut grove soil**

A survey of EPN species was conducted punctually in each plot of the two research sites and a screening of many other chestnut groves of Etna was also started. To find out the presence of EPNs in the soil, in each sampled spot two samples of soil were collected, each of about two-three kg, taken from the upper 20-30 cm of soil. To extract the EPNs the technique of Bedding & Akhurst (1975) was followed. The nematodes found were identified till the species level both by means of morphological study and mitochondrial DNA analysis.

**Laboratory tests on the virulence of different EPN species against the main nut insect pests**

The tests were conducted on the insect larvae extracted from the collected nuts (*Pammene fasciana*, *Cydia* sp., *Curculio elephas*, *C. glandium*) and on larvae of *Galleria mellonella* as a control. The EPNs tested were the following: *Steinernema feltiae* (the autochthonous strains found on Etna at Triciala, Piano Lepre and Piano Balilla and a strain kindly provided by the bio-firm Biotecnologie B.T., Pantalla di Todi), *Steinernema kraussei* and *Heterorhabditis bacteriophora*, also provided by Biotecnologie B.T., and the commercial strain *Heterorhabditis megidis* (Nemasys ®). A selection of the larvae of the various insect species were put on a filter bed in a Petri dish where a single population of EPN per Petri dish was introduced. Every day the larvae were checked and those apparently infested were transferred to a white trap to ascertain the infestation.

**Soil insemination with different species of EPN and tests on their persistence ability**

The EPN stocks introduced were the followings: *Steinernema kraussei* (Biotecnologie B.T.), *Heterorhabditis bacteriophora* (Biotecnologie B.T.), *Heterorhabditis sp.* strain 170 (Biotecnologie B.T.), *S. feltiae* (Nemasys ®), and *H. megidis* (Nemasys ®). Most treatments were aimed only at testing the nematode persistence, while those made using *S. feltiae* and *H. megidis* were also aimed at testing their efficacy in the field and their environmental impact. At Fornazzo, the plot F 1 was treated with *S. feltiae*, F 2 with *H. megidis* and F 3 was not treated to be the control. At Triciala (where *S. feltiae* were naturally present in the site T 2), only T 3 was treated with *H. megidis*. The suspension containing the EPNs was diluted with water and distributed with a spreader to distribute about 500,000 nematodes per square meter. The persistence of nematodes was tested by sampling the treated soil monthly and looking for the EPN by the technique of Bedding & Akhurst (1975). When an EPN species was not found for three successive samplings, it was considered to be no more present or active in the soil.

**Evaluation of the environmental impact**

A survey of the soil macrofauna was made in the two experimental sites before and after the soil treatments. The sampling of the fauna was carried out by means of pitfall traps in the periods March – November 2002 and March-November 2004. The pitfall traps were constituted by plastic glasses filled with vinegar and salt, and burrowed in the soil with the aperture at soil level. The vinegar attracts the arthropods and the salt preserves the animal from decomposition for some time. At Fornazzo five traps per plot and at Triciala four traps per plot were placed. The traps were removed and substituted each month and the animals were identified mainly at the order level, excepted for the Coleoptera, of which the Staphylinidae, which are very common soil inhabitants, were sorted apart, and counted.
Results and discussion

Looking for the autochthonous EPN species present in the Etna chestnut grove soil
EPN species were found in six out of eight investigated areas. Most species belong to the genus *Steinernema* and to the species *S. feltiae*, which seems to be widespread in the Etna territory. A species of *Heterorhabditis*, not yet identified, was also found.

Laboratory tests on the virulence of different EPN species against the main nut insect pests
Table I shows the virulence degree of each species of EPN against the different species of insects, expressed as the percentage of infested larvae on the total number of larvae treated with the EPNs in laboratory. At least in the laboratory, all the EPN strains tested have a good ability of infesting the tortricid larvae and, in some cases, also the curculionid larvae. The Steinernematidae species, particularly *S. kraussei*, showed a great virulence against *Cydia* sp. Also the various strains of *S. feltiae* showed a rather high infestation ability (50-87%). The most virulent strains against the tortricids and against *C. glandium* were two autochthonous strains of Etna (Triciala and Piano Lepre). The species of *Heterorhabditis* showed a lower efficacy (no more than 60% of infested larvae) against *Cydia* sp., but a greater efficacy against *P. fasciana* (60-100% of infested larvae) and against both species of *Curculio* (100%), with the exception of *Heterorhabditis* sp. In particular, *H. bacteriophora*, seems to have a wide range of efficacy against all the insects.

<table>
<thead>
<tr>
<th></th>
<th>Cydia sp.</th>
<th>P. fasciana</th>
<th>G. mellonella</th>
<th>Curculio glandium</th>
<th>C. elephas</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Heterorhabditis</em> sp. (B.T.)</td>
<td>40%</td>
<td>100%</td>
<td>70%</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td><em>H. bacteriophora</em> (B.T.)</td>
<td>57%</td>
<td>75%</td>
<td>80%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><em>H. megidis</em> (Nemasys)</td>
<td>60%</td>
<td>60%</td>
<td>90%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><em>Steinernema kraussei</em> (B.T.)</td>
<td>100%</td>
<td>77%</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><em>S. feltiae</em> (Triciala)</td>
<td>87%</td>
<td>40%</td>
<td>79%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td><em>S. feltiae</em> (Piano Lepre)</td>
<td>50%</td>
<td>80%</td>
<td>90%</td>
<td>50%</td>
<td>0%</td>
</tr>
<tr>
<td><em>S. feltiae</em> (Piano Balilla)</td>
<td>75%</td>
<td>50%</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. feltiae</em> (B.T.)</td>
<td>60%</td>
<td>100%</td>
<td>70%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Soil insemination with different species of EPN and tests on their persistence ability
The persistence ability of different EPN species, expressed as number of days after soil treatment, is shown in table 2.

All the species introduced into the soil persisted for at least two months and, in one case, for up to about 8 months.
Also the number of estimated specimens per square meter of *H. bacteriophora* and *S. feltiae* was calculated as time passed from the insemination day, following the method of Koppenhöfer et al. (1998). The number of persisting nematodes (only 74-160 per square meter after about forty days) very rapidly decreased until very low values, that can hardly allow an efficacious action of the nematodes against the insect larvae. In our opinion, this is not a secondary aspect and it is worth of further investigation.

Table 2. Time of persistence in the soil of various strains of EPN species

<table>
<thead>
<tr>
<th>Species of EPN</th>
<th>Persistence in the soil (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Heterorhabditis bacteriophora</em></td>
<td>268</td>
</tr>
<tr>
<td><em>Heterorhabditis megidis</em></td>
<td>117</td>
</tr>
<tr>
<td><em>Heterorhabditis sp.</em></td>
<td>71</td>
</tr>
<tr>
<td><em>Steinernema feltiae</em></td>
<td>181</td>
</tr>
<tr>
<td><em>Steinernema kraussei</em></td>
<td>85</td>
</tr>
</tbody>
</table>

**Evaluation of the environmental impact**

During the year 2002, 32,493 specimens, belonging to 23 high taxa, were sorted from the traps, 32,623 specimens, belonging to 23 high taxa during the year 2004. The treatment, therefore, did not cause a decreasing of the number of individuals (which even increased) nor of the overall biodiversity. The punctual comparison of the data for each month of the two years, through the analyse of the mean and the variance of the single taxa, does not reveal any decreasing which can be attributed to the soil treatment. However, this assumption seems not to be true for Coleoptera, which greatly decrease in the plots treated with *S. feltiae* and *H. megidis* at Fornazzo; but a similar decreasing was observed also in the not treated plot at Triciala. Therefore, the variations observed could be considered as normal fluctuations of the populations, without relation to the effects of the treatment.

In conclusion, the tests on the EPN virulence showed that some species, in the laboratory, have a high impact against most nut insect parasites and that they can persist in the soil for a sufficient time to let them find the insect larvae in the soil. This potentiality, however, might not produce the desired effects in the field if the number of EPN specimens decreases too much. The laboratory tests, however, provided us with much useful information on species of EPN which have sufficient efficacy and persistence to be effectively used against all the nut insect pests. Finally, the thorough evaluation of the environmental impact of the EPN on the soil fauna diversity, which showed a virtual lack of secondary effects on the non-target species, confirms the sustainability of such a treatment, also in seminatural habitats or in protected areas.

**Acknowledgements**

We wish to thank Dr Giancarlo Rappazzo, Department of Animal Biology, University of Catania, for his help to identify the nematode species by means of mitochondrial DNA analysis.
References

Incidence of natural infection of the white grub *Polyphylla olivieri* (Coleoptera: Scarabaeidae) with entomopathogenic nematodes in Iran

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Department of Plant Protection, Tehran University, Karaj, Iran

Abstract: From June 2004 to August 2005, larvae of the white grub *Polyphylla olivieri* and soil samples were collected in fruits orchards in seven counties of the Tehran province, Iran. Whites grubs were reared in insectarium, and monitored for possible infection by EPN and subsequent isolation of the nematodes. Isolation of EPNs from soil samples were conducted using the *Galleria* bait method. Three species of EPNs were identified: *Heterorhabditis bacteriophora* (IRAN1), *Steinernema glaseri* (IRAN2) and *Steinernema* sp. (IRAN3). In bioassays against the White grub, the LD$_{50}$ of IRAN1 was 35 IJs/larva, followed by 65 IJs/larva for IRAN2. The LD$_{50}$ for *Steinernema* sp was >10000 IJs/larva and caused only 16% mortality after 25 days. Tolerance of the three Iranian EPNs (IRAN1 of *H. bacteriophora*, IRAN2 of *S. glaseri* and IRAN3 of *Steinernema* sp.) were compared. Heat tolerance study showed that the *H. bacteriophora* strain was the most tolerant nematode at 32°C, but no nematodes could survive at 36°C after a 4 – 5-h exposure. Furthermore, life cycle and natality/mortality data of the three Iranian isolates were studied in the wax moth larvae, *G. mellonella*, at a range of temperatures from 5 to 30°C.

Key words: nematodes, insects, white grubs, Iran, natural occurrence, characterization

Introduction

Entomopathogenic nematodes (EPN) have emerged as pertinent biocontrol agents for soil-inhabiting insects (Kaya & Gaugler,1993; Ehlers, 1998). Interest in these beneficial organisms has increased rapidly in recent years (Klein *et al.*, 2000). However, there is no any information about EPNs from Iran. Indeed, climatic conditions in this country are rather diverse and their fauna and flora have high diversity. The aim of this study was to look for the presence of EPNs in the white grub *Polyphylla olivieri* (Coleoptera: Scarabaeidae). This melolonthid, which is one of the key pests in Iranian fruit orchards (Fig. 1), has three larval stages and a 3-year life cycle (Rajabi, pers. commun.).

Materials and methods

From June 2004 to August 2005, more than 1180 White grub larvae (second and third stage) and 1360 soil samples were collected from 50 orchards in Karaj, Shahryar, Lavasanat, Damavand, Hashtgerd, Varamin and Chalous road counties of the Tehran province, Iran (Fig. 1). Isolation of EPNs from soils was conducted using the *Galleria* bait method, and soil characteristics were noticed. The collected White grub larvae were reared back in the laboratory to be examined individually by naked eye and under a dissecting microscope (Kaya & Stock, 1997). The infected larvae and the ones who changed in their colour (i.e. showing a brick red) were sterilized and placed onto a White trap. Dose-mortality responses of third instar larvae of *P. olivieri* submitted to infection by nematodes were assayed using 1, 10, 100, 1000 and 10000 IJs. Results were compared with the strain *Steinernema glaseri* NC
(Rutgers University, NJ, USA) against *Galleria mellonella* larvae. The study of life cycle and reproductive potential of nematodes was based on infection experiments where *Galleria* larvae (weighting 200 mg) were exposed to 100 IJs. Heat tolerance of IJs was determined according to the method by Iraki *et al.* (2000).

![Image of third instar larva, adults, and survey area](image)

**Figure 1:** Left: third instar larva of the white grub, *Polyphylla olivieri*; Middle: adults; Right: Survey Area, central province of Tehran, Iran.

**Results and discussion**

EPNs were found from soils of two types in the seven studied counties: sandy, sandy loam or loam type of soil (60-70%), and sandy-clay-loam or clay loam type of soil (30-40%). 85% of the habitats from which the nematodes were isolated were heavily disturbed (including orchards), whereas 15% were undisturbed (forest, riversides and woodland). Similar results were recorded by Hominick *et al.* (1995). Based on morphometric data, three isolates of EPNs were identified: *Heterorhabditis bacteriophora* (IRAN1), *Steinernema glaseri* (IRAN 2), and *Steinernema* sp. (IRAN3) (Table 1). The most common isolate was *H. bacteriophora* IRAN1, followed by *Steinernema* sp. IRAN3. Furthermore, the native *S. glaseri* IRAN2 was shown to be considerably more effective than *S. glaseri* NC.

**Table 1:** Incidence of entomopathogenic nematodes in natural larval populations of *Polyphylla olivieri* in seven counties in the Tehran province, Iran (data from collected larvae, and from soil samples).

<table>
<thead>
<tr>
<th>Collection sites</th>
<th>N° collected white grubs</th>
<th>N° infected white grubs by <em>Steinernema</em></th>
<th>N° infected white grubs by <em>Heterorhabditis</em></th>
<th>N° collected white grubs</th>
<th>N° soil samples + for <em>Steinernema</em></th>
<th>N° soil samples + for <em>Heterorhabditis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Karaj</td>
<td>150</td>
<td>2</td>
<td>3</td>
<td>250</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Shahryar</td>
<td>300</td>
<td>2</td>
<td>2</td>
<td>325</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Lavasanat</td>
<td>90</td>
<td>1</td>
<td>2</td>
<td>100</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Hashgerd</td>
<td>300</td>
<td>0</td>
<td>5</td>
<td>325</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Damavand</td>
<td>105</td>
<td>0</td>
<td>2</td>
<td>100</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Varamin</td>
<td>55</td>
<td>0</td>
<td>1</td>
<td>60</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Chalous Road</td>
<td>185</td>
<td>3</td>
<td>6</td>
<td>200</td>
<td>11</td>
<td>9</td>
</tr>
</tbody>
</table>

Data showed that IJs of *H. bacteriophora* and *Steinernema* sp emerged from the *G. mellonella* larvae 6 and 9 days after infection at 25°C. These data are contrary to other reports where Steinernematids emerge earlier than Heterorhabditids at the same temperature (e.g.
Wang et al., 1995). The IJs of *S. glaseri* (IRAN2) emerged from cadavers between 5 and 7 days after infection at 25°C and those of *H. bacteriophora* (IRAN1) emerged from cadavers 9 days or more after infection. *H. bacteriophora* appeared the most tolerant nematode at 32°C, but no nematodes could survive at 37°C after a 4 – 5-h exposure. Heat tolerance studies indicated that Iranian isolates of EPNs were more tolerant than European ones, as it was showed already for isolates from Middle East (Iraki et al., 2000). All isolates caused 100% mortality of *Galleria* larvae; they developed and produced progeny between 10 and 25°C. At 28°C, mortality was 100%, and no progeny was produced. The highest IJs production was at 15°C for all isolates. Host death occurred after 12 days at 7°C, 9–11 days at 10°C, 4–6 days at 15°C, 3 days at 20°C, and 2 days at 25, 28 and 30°C. Penetration of the IJs was consistently high for all isolates at 15, 20, 25 and 28°C.

Figure 2. Left: a larva of *Polyphylla olivieri* showing a Heterorhabditid infection; Right: early symptoms of infection of a larva of the same insect species by *Steinernema glaseri*.

When nematodes were produced in *Galleria* larvae at 7, 15 and 23°C, the length of the body of IJs for all isolates was in inverse proportion to the temperature.

At 26 ± 2°C, the life cycle of *H. bacteriophora* (IRAN1) revealed that the juveniles passed through 4 stages before becoming adult. The first generation of IJs produced from the mated female completed its life cycle in five days (120h) within last instar larvae of *Galleria*. In all strains, low (≤ 15°C) or high temperatures (≥ 35°C) caused the nematode to stop growing and reproducing. The nematodes developed slowly at 20°C and produced only one generation of IJs. Reproductive potential of *H. bacteriophora* (IRAN1) averaged ca. 125000 IJs/cadaver, that of *Steinernema* sp. (IRAN3) ca. 13800 IJs/cadaver. The competition studies between the two species revealed that the former one was responsible for the highest mortality rates.

Finally, a symbiotic bacterium was isolated from the haemolymph of *Galleria* infected with *H. bacteriophora* (IRAN1). The bacterial colony was absorbed with bromothymol blue on NTBA medium (Kaya & Stock, 1997). The colony was circular to irregular in shape with a clear zone around the colony. It was greenish in colour in the center, which appeared opaque and undulated.

This is the first study about natural occurrence of EPNs in Iran, and the first report of natural infection of *P. olivieri* by EPNs. Further research is needed now, first to confirm identification results using molecular studies (Stock & Hunt, 2005; Spirinidov, pers. commun.). Our results confirmed the specialization of *S. glaseri* and *H. bacteriophora* against sedentary subterranean white grubs (Sturhan, 1999). This specialization could be partly explainable by the cruiser foraging of the EPNs (Ansari, 2004; Grewal et al., 1994, 2005) and the nematode ability to overcome host defence (Wang et al., 1995). In fact, the low mortality of white grubs like *P. olivieri* may be due to their grooming behavior of mouthparts and
defence ability of their spiracles sieves (Klein & Georgis, 1992). It is interesting to note that *P. olivieri* differs from most European white grub species such as *Hoplia*, *Holotrichia* and *Phylophaga*, as regards the larval niche and overwintering depth. Research with the Iranian nematode/bacterium complex has only recently been initiated. This could lead to isolate interesting EPNs in Iran, which could be used in fundamental and practical studies.

**Acknowledgements**

We gratefully acknowledged Randy Gaugler, Ralf-Udo Ehlers and Minshad A. Ansari for their valuable suggestions. We would like also to thank Tahereh Sedighi for her moral support and Amir Hajiabadi for his technical assistance. Thanks are due to Reza Talaei for reviewing the manuscript.

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Biological characterization of *Steinernema apuliae*: first contribution

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Abstract: Eight strains belonging to *Steinernema apuliae* were collected from soils of different biotopes in Southern Italy during the last 10 years. Bioassays were performed in laboratory with the aim to assess some biological aspects of this nematode: the performance of the nematode against different species of insects, i.e. *Bactrocera oleae* (Diptera: Trypetidae), *Sarcophaga carnaria* (Diptera: Sarcophagidae), *Corythucha ciliata* and *Monosteira unicostata* (Rhynchota: Tingidae), and *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae) in comparison with the infectious activity of *S. carpocapsae* (ItS-MR7 Italian strains); the infectious behaviour depending on different temperatures using *Galleria mellonella* larvae as test insect; the desiccation tolerance upon exposure to 2 relative humidities (84% and 76% RH); the optimum storage conditions with different concentrations at different temperature levels (8°-12°-16°-20°C).

Key words: Italian entomopathogenic nematode, bioassays, biology, dessication tolerance, storage

Introduction

Surveys on the occurrence of entomopathogenic nematodes (EPNs) carried out in Southern Italy during the past 10 years pointed out the presence of numerous species and strains of EPNs in different biotopes (Tarasco & Triggiani, 1997; Triggiani & Tarasco, 2000, 2002). Among these Italian EPNs 10 strains belonging to the “Long Nematodes” (LN, *Steinernema glaseri* Group) were collected. Eight strains of these LNs belong to *Steinernema apuliae* recently described (Triggiani et al., 2004) (Table 1); 1 strain was missed (ItS-C15) and 1 strain is similar to *S. arenarium* (ItS-C31). All the *S. apuliae* strains were collected from coastal biotopes with sandy soil (except one); this species seems to prefer seaside habitats. The aim of this study was mainly to performe some different bioassays for biological characterization of *S. apuliae*.

Materials and methods

Four different bioassays were carried out in laboratory conditions:

**Test 1 - Insect mortality bioassays against different hosts**
Evaluation of infectivity potential of *S. apuliae* was carried out using different insect hosts, i.e. *Bactrocera oleae* (Diptera: Trypetidae), *Sarcophaga carnaria* (Diptera: Sarcophagidae), *Corythucha ciliata* and *Monosteira unicostata* (Rhynchota: Tingidae), *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae), in comparison with two other nematodes: *S. carpocapsae* (ItS-MR7 Italian strain) and the Long Nematode ItS-C31 (Italian strain). Different temperatures were used during the experiments: 8° – 12° – 16° – 20° – 24°C.

**Test 2 - Insect mortality bioassays at different temperatures**
Evaluation of infectious behaviour of *S. apuliae* in relation to different temperatures was carried out using *Galleria mellonella* larvae as test insect. The infectivity was determined by
larval mortality rate assays in relation to different temperature values for comparing *S. apuliae* and other 3 EPN species (Italian strains of *S. feltiae*, *S. affine*, and *H. bacteriophora*). In the experiments the percentage of larval mortality was recorded after 72 hours of exposure to IJ EPNs at 6 temperatures between 10° and 35°C, at intervals of 5°C, using IJs in aqueous suspension.

Table 1. Characteristics of the sites where Long Nematode strains were isolated in Southern Italy.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Locality</th>
<th>Altitude</th>
<th>Time</th>
<th>Habitat</th>
<th>Soil texture</th>
<th>pH</th>
<th>Org. Cont.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ItS-C15*</td>
<td>Castellaneta (TA)</td>
<td>0</td>
<td>Sep’96</td>
<td>Sea coast</td>
<td>Sand</td>
<td>7.4</td>
<td>0.4</td>
</tr>
<tr>
<td>ItS-MS10</td>
<td>Margherita di S. (BA)</td>
<td>30</td>
<td>Oct’96</td>
<td>Salt pan</td>
<td>Silt</td>
<td>7.9</td>
<td>1.15</td>
</tr>
<tr>
<td>ItS-MS3</td>
<td>Margherita di S. (BA)</td>
<td>30</td>
<td>Oct’96</td>
<td>Uncultivated</td>
<td>Sand</td>
<td>7.4</td>
<td>0.27</td>
</tr>
<tr>
<td>ItS-LD3</td>
<td>Metaponto (MT)</td>
<td>20</td>
<td>Oct’96</td>
<td>Pinewood</td>
<td>Sand</td>
<td>7.9</td>
<td>0.34</td>
</tr>
<tr>
<td>ItS-LE13</td>
<td>Torre Pali (LE)</td>
<td>0</td>
<td>Oct’97</td>
<td>Sea coast</td>
<td>Sand</td>
<td>8.2</td>
<td>0.3</td>
</tr>
<tr>
<td>ItS-ZA11</td>
<td>Zapponeta (FG)</td>
<td>30</td>
<td>Nov’97</td>
<td>Uncultivated</td>
<td>Sand</td>
<td>7.8</td>
<td>0.27</td>
</tr>
<tr>
<td>ItS-CS3</td>
<td>Brindisi (BR)</td>
<td>20</td>
<td>Apr’98</td>
<td>Tamarisk</td>
<td>Sand</td>
<td>8.3</td>
<td>0.11</td>
</tr>
<tr>
<td>ItS-TA14</td>
<td>Taranto (TA)</td>
<td>0</td>
<td>Sep’98</td>
<td>Sea coast</td>
<td>Sand</td>
<td>8.3</td>
<td>0.01</td>
</tr>
<tr>
<td>ItS-C31</td>
<td>Castellaneta (TA)</td>
<td>20</td>
<td>May 00</td>
<td>Pinewood</td>
<td>Sand</td>
<td>8.0</td>
<td>3.8</td>
</tr>
<tr>
<td>ItS-TC5</td>
<td>III Cavone (MT)</td>
<td>0</td>
<td>Mar’01</td>
<td>Sea coast</td>
<td>Sand</td>
<td>7.8</td>
<td>0.27</td>
</tr>
</tbody>
</table>

**Test 3 - Dessiccation tolerance bioassays**

Evaluation of desiccation tolerance of *S. apuliae* was carried out at 2 relative humidities (84% and 76% RH). The survival of *S. apuliae* upon direct exposure of IJs to 2 relative humidities (84% and 76% RH at 20°C) on membrane filters was compared with 4 other species collected in Italy: *Steinernema feltiae* (ItS-MA12), *S. affine* (ItS-AR1), *S. carpocapsae* (ItS-MR7) and *Heterorhabditis bacteriophora* (ItH-CE1). Approximately 200 IJs in 1 mL of water suspension were placed on a 2.5-cm cellulose filter (20 µm pore size); after the water had been removed with a vacuum pump. Then each filter was immediately transferred to a desiccation chamber. The desiccators were incubated at 20°C for 5 days and the survival percentage was recorded 2 times per day. Rehydrated IJs were used to check their infectivity releasing them 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.

**Test 4 - Storage bioassays**

Evaluation of the optimum storage conditions was carried out using different suspensions and concentrations at different temperatures. The comparison was made among *S. apuliae* (strains: ItS-MS10, ItS-LD3, ItS-LE13), *S. carpocapsae* (ItS-MR7) and the Italian Long Nematode ItS-C31. The storage temperatures were 8° – 12° – 16° – 20°C, and 20,000 – 50,000 – 100,000 – 1,000,000 IJs were placed in flasks containing 30 and 50 mL of tap water. Three
replicates were performed per flask. Control of mortality and infectivity was carried out each month for 1.5 years.

Results and discussion

Test 1 - Insect mortality bioassays against different hosts
Different results were obtained with Diptera: *S. apuliae* infected high percentages of *B. oleae* last instar larvae, above all between 12 and 24 °C (60-80% of mortality), while the percentage of *S. carnaria* larvae infected was quite low (25%). Between the Tingidae *C. ciliata* and *M. unicostata*, the best overwintering adults mortality was achieved for *C. ciliata* at 24°C (75%) while *S. apuliae* infected only 22% of *M. unicostata*; at lower temperature less than 20% of mortality was obtained. The performance against *T. pityocampa* overwintering larvae was quite good, above all between 16°C (more than 80%) and 24°C (almost 100%).

Test 2 - Insect mortality bioassays at different temperatures
The infectious activity of *S. apuliae* after 72 hours was almost zero at 10°C, started at 15°C with 7% of larval mortality, continued at 20°C with 60% and reached 80% at 25°C and 86% at 30°C, its maximum; at 35°C the percentage of larval mortality decreased at 44%. The optimum range for *S. apuliae* infectivity is between 25° and 30°C.

Test 3 - Dessiccation tolerance bioassays
*S. carpocapsae* showed the best percentages of IJs survival after 5 days at both the RH values (84% and 76%), while *S. apuliae* IJs died after 8 hrs with a behaviour similar to *S. feltiae* and *Heterorhabditis bacteriophora*. If we compare our findings with the data reported by Kung *et al.* (1991) regarding the survival ability of *S. glaseri*, *S. apuliae*, which belongs to the “Steinernema glaseri Group”, is more susceptible to low RH.

Test 4 - Storage bioassays
The lowest percentage of *S. apuliae* IJs mortality (5-10%) was recorded at the storage temperatures of 12° and 16 °C along all the storage period after 4, 8, 12 and 16 months, and similar results were obtained with the Italian long nematode ItS-C31. The percentage of IJs mortality was quite higher at 4° (20-40%), 8° (20-30%) and 20 °C (15-20%). Thus the best survival condition for *S. apuliae* was with a low concentration of IJs: 20,000 in 30 or 50 mL of tap water.

References


Molecular characterization of Italian EPN strains by RFLP analysis of the ITS region of the ribosomal DNA repeat unit

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Abstract: The ITS region of 51 Italian strains of entomopathogenic nematodes (EPNs) belonging to 4 species isolated in the last 10 years in Southern Italy was amplified by PCR and the resulting products were digested with 9 different enzymes; the fragments generated were then separated by agarose electrophoresis. The strains were previously identified upon morphological examination using morphometric data. For many of the strains RFLP analysis confirmed morphological identification (Steinernema feltiae, S. affinae, Heterorhabditis bacteriophora), whereas for some RFLP analysis evinced different data from the morphometric examinations. In particular, the RFLP profiles of 3 strains from Sardinia showed differences compared with the profiles of the known species and were quite similar to a strain called MY2 (S. litorale), which until now has only been found in Japan.

Key words: DNA analysis, indigenous entomopathogenic nematodes, Southern Italy

Introduction

The identification of nematodes by standard morphological methods is rarely straightforward, why many researchers have turned to molecular techniques, based on the Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP).

The ribosomal DNA (rDNA) repeat unit is an ideal choice for identification purposes, because it is present as a multi-copy tandem repeat in the genome of most organisms. The repeat unit contains highly conserved regions and potentially highly variable regions. The most useful region is the Internal Transcribed Spacer (ITS), which separate the genes. RFLP profiles of the ITS region exist for several species of Heterorhabditis and Steinernema. The aim of this study was to identify 51 strains of entomopathogenic nematodes isolated in Southern Italy by using the PCR-RFLP molecular technique. The strains were previously identified by morphological examinations using morphometric characters. EPNs were collected during the last 10 years from more than 700 different biotopes (68 locations). Less than 10% of the total soil samples was positive for EPNs.

The isolated nematodes were morphologically identified as: Steinernema feltiae (3.4% of the total), S. affinae (1.3%), S. carpocapsae (1 strain), S. apuliae (1%), Heterorhabditis bacteriophora (2.5%) and Steinernema spp. (2 strains).

Materials and methods

Nematode isolation and storage

The 51 isolates of EPNs were collected using the Galleria baiting technique (Bedding & Akhurst, 1975) during a soil survey in different biotopes of Southern Italian Regions: Apulia,
Basilicata, Sardinia, Campania, Molise and Calabria. (Tarasco & Triggiani, 1997; Triggiani & Tarasco, 2000).

Each soil sample was nearly amount of 100 g and taken from 0-12 cm depth. The soil samples were treated with the last-instar wax worm *Galleria mellonella* L. (Lepidoptera, Galleriidae) larvae at a temperature of 22°C. To obtain fresh infective juveniles (IJs), 10 wax worms on a 9 cm diameter Petri dish with one 90 mm filter paper were treated with ca. 2,000 IJs in 1.5 mL of tap water. Two weeks after treatment, wax worms were put on modified White traps (White, 1927) for the recovery of new generations of IJs. Then, the recovered IJs kept in lab-standard Ringer solution (9.0 g NaCl, 0.42 g KCl, 0.37 g CaCl₂*2 H₂O, 0.2 g NaHCO₃ and 1 L aqua dest) at 8°C until using.

The Italian EPNs previously identified upon morphological examination using morphometric data of infective juveniles, males and females:
- 25 *S. feltiae* strains: ItS-MA12, ItS-MO1, ItS-MF1, ItS-GR1, ItS-SAR2, ItS-SAR4, ItS-SAR6, ItS-G16, ItS-CE2, ItS-SA1, ItS-MSA3, ItS-MSA4, ItS-TE1, ItS-LE1, ItS-Q1, ItS-TG4, ItS-CL2, ItS-MU1, ItS-MV1, ItS-CS6, ItS-CZ19, ItS-CZ23, ItS-BQ1, ItS-CO1, ItS-OT3
- 7 *S. affine* strains: ItS-AR1, ItS-ST12, ItS-FO1, ItS-FO2, ItS-QU3, ItS-CZ7, ItS-CZ10

**DNA Extraction, PCR condition and RFLP analysis**

The ITS region of the 51 Italian strains of EPNs belonging to 4 species was amplified by PCR and the resulting products were digested with 9 different enzymes; the fragments generated were then separated by agarose electrophoresis.

Nematodes were propagated in last instar larvae of *Galleria mellonella*. DNA of each strain was isolated from approximately 2,000 infective juveniles by the DNasey Tissue Kit (Qiagen, Germany). The PCR amplification was carried out in a reaction volume of 100 µL for each strain, containing 75.6 µL of H₂O, 2 µL of dNTPs (2mM), 10 µL of 10x PCR-Buffer, 1 µL of Primer Forward (200 µM), 1 µL of Primer Reverse (200µM), 0.4 µL of Taq polymerase (5U/µL) and 10 µL of purified DNA. In this step, Vrain A and Vrain B were used as the primers, as described by Vrain *et al.* (1992). The amplification was carried out using a Perkin Elmer Cetus DNA thermal cycler-480. The samples were placed in the thermal cycler that was preheated to 94°C and kept at 94°C for 3 min followed by 40 cycles of 94°C for 30 sec., 42°C or 44°C for *Heterorhabditis* strains, 53°C or 55°C for *Steinernema* strains for 1 min and 72°C for 1.5 min. At the end of the 40 cycles, the samples were incubated at 4°C until they were digested with the restriction enzymes Alu I, Hae III, Hind III (Eurogentec), Dde I, Hha I, Hinf I, Hpa II, Rsal I and Sau 3Al (Pharma). All digestions were carried out using 200 µL of PCR product at 37°C for 3 hours. The resulting fragments were separated on a 2 % (w/v) agarose gel (Sigma) in 0.5x TBE at 6V/cm for 1.5 hours. The fragments were visualised by ethidium bromide staining and photographed.

**Results and discussion**

The RFLP patterns were compared with results published by Stock & Hunt (2005) or identified with the help of Alex Reid (Scottish Agricultural Science Agency, East Craigs, Edinburg, UK). For many of the strains the RFLP analysis confirmed the morphological identification (21 *Steinernema feltiae*, 5 *S. affiniae* and 18 *Heterorhabditis bacteriophora*), while for some of them the RFLP analysis gave different data. In particular the RFLP profiles of 4 *S. feltiae* (ItS-SAR2, ItS-SAR4, ItS-SAR6, ItS-SA1, 2 *S. affiniae* (ItS-FO1, ItS-CZ10) and
H. bacteriophora (ItH-C6) RFLP analysis evinced different data from the morphometric examinations. In particular the strains from Sardinia Region (ItS-SAR2, ItS-SAR4 and ItS-SAR6) showed similarities with the profiles of the new species, Steinernema litorale, recently described from Japan (Yoshida, 2004). Cross hybridisation tests among the Sardinian strains with S. feltiae and S. litorale using the "drop hemolymph" technique are in progress.

References

Efficacy of *Steinernema carpocapsae* against the cockroach *Periplaneta australasiae*

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**Abstract:** Cage tests showed that the nematode *Steinernema carpocapsae* is able to infect and to kill the cockroach *Periplaneta australasiae*. Mortality of 85% was reached using different traps. To lure the cockroaches into the traps different baits and foods were offered. Coffee and banana were as good as commercial products. Besides banana was the preferred food. When nematodes were applied to cockroaches for two minutes 80% of exposed insects died. The mortality increased even to 95% after 30 minutes. Moreover the number of nematodes on the cockroach’s body was counted. Cockroaches in contact with nematodes for ten minutes had average 431 *S. carpocapsae* on their bodies. If the cockroaches were isolated after the confinement for 24 hours the number of nematodes amount to 147. We suppose that the nematodes enter the host through the spiracles: 83% of the cockroaches died if the nematodes were placed at the side of thorax and abdomen, compared with lower mortality when applying the nematodes on other places (65% dorsal, anus 38%, ventral 33%, mouth 25%).

**Key words:** *Periplaneta australasiae*, *Steinernema carpocapsae*, biological control, trap station, botanical gardens

**Introduction**

According to our enquiry in Germany populations of *Periplaneta australasiae* increased rapidly in botanical and zoological gardens in the last years. The main reason could be the increased use of biological control methods against plant pests, give the use of insecticides in the past have had also an effect of cockroaches. The caused damage of imported cockroaches to plants is generally not important but some species are often causing plant destruction, such as *Pycnoscelus surinamensis* for example. Furthermore, cockroaches are common potential vectors of infections. Therefore alternative control strategies are necessary.

We have concentrated our studies on the cockroach *P. australasiae* and the entomopathogenic nematode *Steinernema carpocapsae*. The cockroach is 24 to 33 mm long and dark reddish-brown coloured. The key characteristics are the yellow stripes on the outer edge of the front wings (Gurney & Fisk, 1991; Weidner, 1993; Bohn, 2000). The duration of developmental time requires six to 12 month. A single female can produce average 650 eggs in the life span. We chose the nematode *S. carpocapsae* because of his behaviour – the invective juveniles nictate on the substrate surface and wait for their hosts to pass by (Ishibashi & Kondo, 1990).

The purpose of our investigations is to develop a biological nematode-based bait station. A bait station must lure the cockroaches, the nematodes have to infect the cockroaches and the cockroaches have to leave the bait station. Laboratory investigations in Petri dishes with *S. carpocapsae* against cockroaches showed a mortality of 50% to 97% (Zukowski, 1984; Corpus & Sikorowski, 1992; Mathur *et al.*, 1996).
**Materials and methods**

The nematodes were used as gel (nematodes, sand, polyacrylat and water). According to studies by Corpus & Sikorowski (1992) and Koehler et al. (1992), the formulation per 1 cm² contained approximately 5000 nematodes.

**Different lures**

20 nymphs and 10 adult cockroaches were placed in a container (60 x 50 cm) with refugia and water. From the literature various fodders can be used for rearing cockroaches (Beier, 1967). In four experiments with each five replicates, different food was offered: a) pieces of bread, rusk and rolled oats, b) pieces of apples, bananas, tangerines and fruity sweet, c) fish food, vitamin powder and bananas, and d) rusk, dog food, coffee and bananas. Indeed coffee was used as an attractant to catch cockroaches.

Further banana, coffee and two commercial products (a pheromone tablet and a bait tablet) were tested in a main tropical greenhouse. Four trap jars with one bait and an unbaited jar (control) were distributed for 20 times. The upper inside surface was coated with margarine to prevent the cockroaches from escaping. The confined cockroaches were counted.

**Form of the bait station**

Also the form of a trap is very important because the cockroaches have to get in contact with the nematodes. But the question is: where do the nematodes have to be attached? Four stations in cages with 20 nymph and 10 adult cockroaches were compared (Table 1).

<table>
<thead>
<tr>
<th>Station</th>
<th>Size and form of the station</th>
<th>Nematodes</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. dish</td>
<td>Petri-dish, 11 cm² bottom, top gel</td>
<td>bottom, top</td>
<td>gel</td>
</tr>
<tr>
<td>2. labyrinth 1</td>
<td>17.5 x 9.6 cm, three courses with each 3 cm broad</td>
<td>bottom, top</td>
<td>gel</td>
</tr>
<tr>
<td>3. labyrinth 2</td>
<td>size such as labyrinth 2, cotton wool on the sides of the courses</td>
<td>side, top</td>
<td>water</td>
</tr>
<tr>
<td>4. tube</td>
<td>felt, 15 cm, 1.5 cm in the diameter</td>
<td>all over</td>
<td>gel</td>
</tr>
</tbody>
</table>

The nematodes were placed on the bottom and at the top (dish and labyrinth 1), respectively on the side and at the top (Labyrinth 2) or all over (tube). Five replicates with each 30 cockroaches were used for each treatment. Dry dog food was offered as food.

**Duration of infection**

The duration of infection was investigated. In each case, 40 cockroaches were added to nematodes for 1, 2, 5, 10, 15, 30 to 60 minutes. After it they were isolated.

**Number of nematodes on the cockroach’s body**

To count the number of nematodes on the cockroach’s body, 40 cockroaches were confined in contact with nematodes for 10 min. Then 20 of them were killed and rinsed with water immediately while the other 20 cockroaches were killed and rinsed only after 24 hours (to allow them to possibly clean themselves).

**Possibilities to enter the host**

According to Ishibashi & Kondo (1990), the nematodes enter the host through natural openings (mouth, anus and spiracles). To find out where *S. carpocapsae* have to enter exactly
*Periplaneta australasiae*, nematodes were applied onto different places on the cockroach’s body: mouth, anus, spiracles, dorsal, ventral (per each cockroach approximately 1000 nematodes were applied). In each case 40 cockroaches were used.

**Treatments in greenhouses**
The efficacy of *S. carpocapsae* was also explored in greenhouses. In the first experiment nematodes were applied weekly in bait stations (10 dishes) and with a watering can (50 millions). The experiment took place in separate small greenhouses (100 m² each) for eight weeks. The second experiment with bait stations took place in a tropical greenhouse for four month (10 dishes distributed on 100 m²).

Before the tests started the initial cockroach population size was estimated using trap jars two times. The average value was set equivalent to 100%. The number of caught cockroaches was converted in percentage.

**Results and discussion**

**Different lures**
Banana was clearly preferred. The highest average food consumption amounted to 0.9 g per day. Cockroaches like sweets, too. But they don’t like tangerines, coffee and vitamin powder as food.

To lure cockroaches, banana and coffee were as good as commercial products. Most cockroaches were caught in the jars with coffee and the commercial bait tablet. In jars with bait tablet the mean value amounted to 20 cockroaches (the high number of 147 nymphs was found once in such a jar). Only 13 cockroaches on average were caught with the pheromon bait, and 14 cockroaches were confined in the control.

**Form of the bait station**
An average of 26 cockroaches, which made 85%, died when the nematodes were attached at the top and on the side (labyrinth 2) (Fig. 1). Less than 15 insects, which made less than 50%, died by using the other stations. The station had most efficacy, where the nematodes infect the cockroaches from the side and from above.

![Figure 1. Number of nematode-infected cockroaches according to the form of the bait station.](image)

**Duration of infection**
Contact with nematodes for two minutes is sufficient to cause 80% death of the cockroaches. The mortality increased even to 95% after 30 min.
**Number of nematodes on the cockroach’s body**
Cockroaches confined with nematodes had an average of 431 *S. carpocapsae* on their bodies. If the cockroaches were isolated after the confinement for 24 hours the number of nematodes was 147.

**Possibilities to enter the host**
When the nematodes were added to the spiracles, 83% of the cockroaches were infected (Fig. 2). When applied dorsally 65% died whereas less than 40% died in the other trials. It is therefore assumed that the nematodes enter the host preferably through the spiracles.

![Figure 2. Percentages of infection in *Periplaneta australasiae* cockroaches according to the site *S. carpocapsae* nematodes were applied on the cockroach’s body.](image)

**Treatments in greenhouses**
The tests in greenhouses confirm the efficacy of *S. carpocapsae*. In the first experiment (small greenhouses), most cockroaches were found in the untreated greenhouse (48% of live cockroaches) while 13% (bait stations) and 29% (water application) in average were caught in the treated greenhouses. The most effective application was therefore the one using bait stations.

In the second experiment (tropical greenhouse), the population of the cockroaches decreased to 14% in the bait trial. The population still remained on the same level for 8 weeks after the stations were removed. In the unbaited control the population increased to nearly the before-test level.

**References**


Strategies to control woodlice with entomopathogenic nematodes

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Abstract: The efficacy of entomopathogenic nematodes in different formulations against woodlice was investigated. Individual Porcellio scaber and Armadillidium spp. woodlice treated with nematodes were more susceptible to Steinernema carpocapsae than to S. feltiae. Experiments with different nematode doses, nematode carriers and bait substances revealed that after 14 days incubation time, 86 to 100% mortality was reached when S.carpocapsae was formulated into quartz sand with compost as bait. Tests with cucumber plants indicated that older plants are protected from woodlice attack by the nematode bait formulation.

Key words: entomopathogenic nematodes, Steinernema carpocapsae, Porcellio scaber, Armadillidium spp., biological control, cucumber, greenhouse

Introduction

While contributing to the decomposition of organic litter in terrestrial ecosystems, isopods may cause severe damage in cellar store rooms on vegetables and fruits, and in organic greenhouse cultivations and botanical gardens by feeding on living plants. Initial work had shown that the commonly used drench application of nematodes with 500,000 nematodes/m² was ineffective in controlling woodlice. Apparently, the nematode dose is not sufficient in such a broadcast application. Moreover, the woodlice predominantly live on the soil surface while the nematodes stay in the soil; hence contact is less probable with a drench application. The aim of the present research was to develop a bait formulation to control woodlice with entomopathogenic nematodes.

Material and methods

Woodlice
The woodlouse species Porcellio scaber, and Armadillidium spp. (i.e. A. vulgare and A. nasatum) were collected every two weeks in the field or in the Botanical Garden, Kiel. They were kept in plastic boxes with potting compost at a temperature of 20°C until the experiments started. The two Armadillidium species were not distinguished further in these initial examinations.

Assessing damage by woodlice
The experiments were carried out in plastic containers (34 cm x 19 cm) which contained potting compost. Porcellio scaber was added at densities of 10, 20 and 30 woodlice per container directly after sowing 6 Cucumis sativus plants and 7 days after sowing 6 plants. Plant damage was recorded one week later.

Susceptibility of woodlice to entomopathogenic nematodes
12 P. scaber and 12 Armadillidium spp. in 1 mL potting soil were treated individually with 800, 1600 and 3200 nematodes of the species Steinernema carpocapsae and S. feltiae. After 14 days incubation at 25°C, woodlice mortality was evaluated.
**Behaviour observations and luring substances**

Petri dishes (Ø 15 cm) were subdivided into 4 equal sectors. In each sector a round, non-transparent plastic lid was placed with the lateral opening to the center. Bait substances were placed under these lids. Choice tests were carried out either between two different substances or between a luring substance and a control (no bait). One woodlouse was placed into the center of the Petri dish. The behaviour was observed over a period of 10 min and the residence time inside a lid with the respective bait was recorded. The experiments were carried out with the species *P. scaber* and *Armadillidium* spp. Luring substances tested were 0.005% ammonia solution, compost, the soil bacterium *Pseudomonas chlororaphis*, potatoes and 2-3 week old woodlouse-faeces (Schliebe, 1989; Zimmer et al., 1996; Hassall & Rushton, 1985).

**Development of a bait formulation: experiments under greenhouse conditions**

The nematode species *S. carpocapsae* was formulated at concentrations of 53,000, 86,000 and 110,000 nematodes per woodlouse (corresponding to 0.8, 1.3 and 1.6 $10^6$ *S. carpocapsae*/g formulated product) into the carrier quartz sand and diatomaceous earth (Celite), and were streaked onto the luring substances potato and compost. The tests were carried out with the species *P. scaber* and *Armadillidium* spp. in plastic containers (34 cm x 19 cm) at 20-25°C and 80-100% RH. The containers were filled with moist potting compost and 30 woodlice were added. Mortality was assessed 7 and 14 days after treatment.

In a further experiment the damage to cucumber plants by *P. scaber* was examined. Three treatments were assessed. In the first treatment (control) only compost and 30 *P. scaber* were added directly after sowing of 6 *C. sativus* plants. The second treatment contained the optimized nematode formulation (110,000 nematodes/woodlouse). Thirty woodlice were added directly after sowing 6 *C. sativus* plants. The third test variant contained 7 days old cucumber plants and 30 *P. scaber* together with the optimized formulated nematodes. Plant damage and woodlouse mortality was assessed after 7 and 14 days.

**Results**

*Porcellio scaber* caused greater damage to seedlings than to 7 days old cucumber plants. Within a week, three of 6 seedlings were destroyed by 10 and 20 woodlice and 4 of 6 by 30 woodlice. From the 7 days old plants only 1 of 6 plants were destroyed one week after 20 or 30 woodlice were added. Due to the low number of plants treated, none of the difference proved to be statistically significant (Fisher’s exact Test; $p > 0.05$).

*Armadillidium* spp. proved to be less susceptible than *P. scaber* to nematode infection. The nematode *S. carpocapsae* proved to be more virulent to *P. scaber* than *S. feltiae*. No decrease in woodlice mortality with decreasing nematode doses was observed (Table 1).

It turned out that compost and woodlouse faeces are preferred significantly to other baits (Fig. 1). *Porcellio scaber* spent most time at these lure substances. Compost was also attracting *Armadillidium* spp. (Fig. 1).

When testing different baits and carriers for the nematodes bait formulation, best results were obtained in the combination of the quartz sand with compost (Table 2). Within the range tested no decrease in mortality of *P. scaber* with decreasing nematode concentration was seen (Fig. 2, left graphic). Nearly all woodlice died after 14 days incubation time. The less susceptible species *Armadillidium* spp. was also controlled successfully (Fig. 2, right graphic) with this formulation. The lower susceptibility of this species is only indicated by the lower mortality after 7 days.

Damage to cucumber seedlings by *P. scaber* added directly after sowing could be reduced from 6/6 plants to 4/6 plants if the nematode bait formulation was applied simultaneously. In constrast to the experiment without nematode treatment (see above), none of the 7 days old...
seedlings died from woodlice damage if the nematode formulation was applied. Also in the presence of cucumber plants all woodlice were killed 14 days after applying with 0.11 \(10^6\) nematodes/woodlouse in a bait formulation.

Table 1: Number of dead *Armadillidium* sp. and *Porcellio scaber* 14 days after individual treatment with the nematodes *Steinernema carpocapsae* and *Steinernema feltiae*. Number of woodlice per treatment: 12. No mortality was recorded in the control (untreated).

<table>
<thead>
<tr>
<th>Nb nematodes / woodlouse</th>
<th><em>Porcellio scaber</em></th>
<th><em>Armadillidium</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. carpocapsae</em></td>
<td><em>S. feltiae</em></td>
</tr>
<tr>
<td>800</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>1600</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>3200</td>
<td>11</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 1: Average residence time ± standard error (in sec) of *Porcellio scaber* on the baits: 0.005% ammonia on potato, woodlouse faeces, potato, compost and *Pseudomonas chlororaphis* on agar and of *Armadillidium* spp. on the bait substance compost (n.s.: \( p > 0.05\); **: \( p \leq 0.01\); t-Test).

Table 2: Mortality (%) of *Porcellio scaber* 14 days after treatment with *Steinernema carpocapsae* (110000 per woodlouse). n.s. and *: comparison between treatments and control. Large letters: comparison between the baits. Roman numerals: comparison between the carriers. Different symbols indicate a significant difference between the treatments (Fisher’s exact Test, \( p<0.05\)).

<table>
<thead>
<tr>
<th>Nematode carrier</th>
<th>Bait substance</th>
<th>Celite</th>
<th>Quartz sand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>6% n.s. A I</td>
<td>80% *A II</td>
<td></td>
</tr>
<tr>
<td>Compost</td>
<td>23% * B I</td>
<td>100% *B II</td>
<td></td>
</tr>
<tr>
<td>Untreated control (potato + Celite)</td>
<td>6%</td>
<td></td>
<td></td>
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</tbody>
</table>
Discussion

This study showed that the woodlice *P. scaber* and *Armadillidium* spp. can be killed successfully with entomopathogenic nematodes in simulated greenhouse conditions. Only by formulating the infective juveniles into quartz sand a sufficient mortality could be reached. Compared to other applications of entomopathogenic nematodes, the nematode dosage used was very high and a decrease in woodlouse mortality was not noted within the range tested. Further studies are needed to establish the most economic nematode dosage without compromising efficacy. The experiments with the cucumber plants had shown that older plants can be protected completely from woodlouse attack by the nematode product while the damage of younger plants can only be reduced from 100 to 70%. It is therefore recommended to apply the nematode product at least 7 days before sowing of plants. Further research is being continued to find out the optimum treatment time as well as the optimum nematode doses and the area covered by one bait lump. The new product will be tested in biological horticulture under field conditions. In house, the bait will have to be enclosed inside a bait station.

![Mortality of Porcellio scaber (left graphic) and Armadillidium spp. (right graphic) 7 and 14 days after treatment with three different doses of Steinernema carpocapsae (Nb nematodes/woodlouse) formulated into the carrier quartz sand and then streaked onto compost. Small letters indicate significant differences between the treatments. An asterisk indicates a significant increase in mortality from 7 to 14 days incubation time (Fisher’s exact Test).](image)

In average the woodlouse spent about 300 sec. into the attractive baits. Judging from the woodlouse mortality this contact time seems to be sufficient to infect the woodlouse with nematodes. The mixture of several leaf litter colonizing microorganisms and their metabolites seems to be more attractive to the woodlouse than those from one soil living bacterium species alone. The attractiveness of the woodlouse faeces can be an advantage for the new nematode product because the woodlouse stay in the bait and the excrement would accumulate there.

Acknowledgements

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Entomopathogenic nematodes and forest insects in Italy

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Abstract: Entomopathogenic nematodes (EPNs) in Italy are used above all in greenhouses and nurseries. Usually not indigenous but commercial EPNs are used from northern European producers. Application of EPNs in forest biotopes is uncommon and only experimental trials have been conducted on few forest pests. It is important to consider that during the past 10 years, the occurrence of EPNs (Rhabditidae: Steinernematidae and Heterorhabditidae) was investigated in pine and oak woods in Southern Italy. Six percent of pine woods and 15.5% of oak woods were positive for the presence of nematodes. Steinernema feltiae was the most common (42%) species in both pine and oak woods. S. feltiae, S. apuliae and Heterorhabditis bacteriophora were collected in the pine woods and S. feltiae and S. affine in the oak woods. Many of the following indigenous EPNs isolated from forest soils were used in laboratory and field trials to control some dangerous insect pests of forest and urban trees in the Apulia region: Thaumetopoea pityocampa (Lepidoptera: Thaumetopoeidae), Corythucha ciliata (Rynchota: Tingidae), Galerucella luteola (Coleoptera: Chrysomelidae) and Tomicus piniperda (Coleoptera: Scolytidae). Indigenous EPNs were also used against Balaninus (= Curculio) elephas (Coleoptera: Curculionidae) in Sicily and the Lazio region, whereas indigenous and commercial strains of EPNs were used to control Cephalcia arvensis (Hymenoptera: Pamphilidae), Zeuzera pyrina (Lepidoptera: Cossidae), Cossus cossus (Lepidoptera: Cossidae), Parenthrene tabaniformis (Lepidoptera: Sesiidae) and Cryptorhynchus lapathi (Coleoptera: Curculionidae) in the northern regions of Italy.

Key words: Steinernematidae, Heterorhabditidae, woodlands, urban trees

Introduction

Entomopathogenic nematodes (EPN) are used in Italy above all in greenhouses and nurseries, also against xylophagous insects. No registration is required at the moment and the market is improving. There are no producer companies of EPNs in Italy, only imported EPNs are used and the impact of these exogenous EPNs is still unknown. There are few data on the EPNs in the Italian forest ecosystems, some of them regard the occurrence of EPNs in forest soils and some other the application of EPNs against forest pests.

Natural occurrence of entomopathogenic nematodes in Italian forests

During the past 10 years, the occurrence of EPNs (Rhabditidae: Steinernematidae and Heterorhabditidae) was investigated in pine and oak woods in Southern Italy. Six percent of pine woods and 15.5% of oak woods were positive for the presence of nematodes (Tarasco & Triggiani, 2000, 2005). Steinernema feltiae was the most common (42%) species in both pine and oak woods. S. feltiae, S. apuliae and Heterorhabditis bacteriophora were collected in the pine woods, and S. feltiae and S. affine in the oak woods. Many of these indigenous EPNs isolated from forest soils were used in laboratory and field trials (Triggiani & Tarasco, 2002, 2004; Tarasco & Triggiani, 2005) to control some dangerous insect pests of forest and urban trees in the Apulia region: Thaumetopoea pityocampa (Lepidoptera: Thaumetopoeidae),
Corythucha ciliata (Rhynchota: Tingidae), Galerucella luteola (Coleoptera: Chrysomelidae) and Tomicus piniperda (Coleoptera: Scolytidae). Indigenous EPNs were also used against Balanus spp. (Coleoptera: Curculionidae) and tortricids in Sicily and Lazio region, whereas indigenous and commercial strains of EPNs were used to control Cephalcia arvensis (Hymenoptera: Pamphiliidae), Zeuzera pyrina (Lepidoptera: Cossidae), Cossus cossus (Lepidoptera: Cossidae), Parenthrene tabaniformis (Lepidoptera: Sesiidae) and Cryptorhynchus lapathi (Coleoptera: Curculionidae) in the northern regions of Italy.

Field experiments with entomopathogenic nematodes to control forest insects in Italy

The first experiments in Italy using EPNs in forest biotopes were conducted against Tomicus piniperda (Triggiani, 1983); field tests showed that pairs of T. piniperda in process of excavating their nuptial chamber in trunks of pine trees could be infected with H. bacteriophora through the injection of the nematode into the entrance of the tunnel leading to the chamber. After this, further experiments against xilophagous insects were carried out using commercial EPNs against Zeuzera pyrina (Lepidoptera: Cossidae) (Deseò et al., 1984), Parenthrene tabaniformis (Lepidoptera: Sesiidae) and Cryptorhynchus lapathi (Coleoptera: Curculionidae) (Cavalcaselle & Deseò, 1984) and Cossus cossus (Lepidoptera: Cossidae) (Rovesti, 1989), in the northern regions of Italy. The results of these trials were quite good and proposed the EPNs as effective promising biocontrol agents to use in forest biotopes.

More recently an important field test was conducted in the Asiago Forest, Venetian Prealps to evaluate the efficacy of 4 EPN strains (Heterorhabditis sp. HL 81, S. kraussei SK, S. feltiae IS 389 and S. carpocapsae IS 230) against the spruce web-spinning sawfly Cephalcia arvensis (pest of Picea excelsa, fir-tree) (Battisti & Masutti, 1994; Battisti, 1994). The 2 most effective strains were S. feltiae IS 389 and S. kraussei SK, which appeared well adapted to low temperature. However, there was a negative effect also: the nematode S. feltiae was shown to parasitize also two ichneumonid parasitoids (Xenoschesis fulvipes and Ctenopelma lucifer).

Field trials were then conducted in chestnut and oak woods in Lazio, Apulia and Sicily with different EPN species (Heterorhabditis bacteriophora, H. megidis, Steinernema kraussei and S. feltiae) against Pammene fasciata (Lepidoptera: Tortricidae), Curculio elephas, C. glandium and C. propinquus (Coleoptera: Curculionidae) (Vinciguerra & Clausi, 2005; Paparatti et al., 2003; Triggiani & Tarasco, unpublished data) to evaluate EPN infectivity and persistence. Most of the used EPNs showed a great efficacy both against the tortricids and Balaninus, and their persistence in the soil was also evaluated.

Another important experiment was performed in Southern Italian pinewood reforestations with Steinernema feltiae in polyacrylamide gel to control overwintering larvae of Thaumetopoea pityocampa (Triggiani & Tarasco, 2002). S. feltiae killed more than 50% of caterpillars after 28 days with a real feasibility to reduce the overwintering larval populations of T. pityocampa by injecting EPN in their nests; the nematode gel suspension do not percolate and adhere to the bristles of larvae and the excrements in the nests and nematodes reach the adult stage and complete their life-cycle in the larvae inside the nest. Moreover there were no effects on the parasitoid Phryxe caudata (Diptera: Tachinidae).

Finally H. bacteriophora, S. carpocapsae and S. feltiae were used against Galerucella luteola (Coleoptera: Chrysomelidae) (a pest of elm-tree) (Triggiani & Tarasco, unpublished data) and Corythucha ciliata (Rhynchota: Tingidae) (a pest of plane-tree) (Triggiani & Tarasco, 2004). The potential of these 3 Italian EPNs as biological control agents for control of C. ciliata, was evaluated through both laboratory and field experiments. In the laboratory
Steinernema feltiae, S. carpocapsae and Heterorhabditis bacteriophora were compared in Petri dishes with filter paper, against overwintering adults of C. ciliata. All nematodes produced high levels of mortality: S. carpocapsae and H. bacteriophora produced significantly greater adult mortality than S. feltiae. The efficiency of these EPNs was then evaluated in field trials, spraying EPN suspensions on tree trunks, where the adults of C. ciliata usually spend the winter under the bark. The mortality percentages in field conditions were quite low if compared with laboratory bioassays. Among the nematodes H. bacteriophora produced significantly greater adult mortality than S. carpocapsae and S. feltiae. Even though more studies and experiments are needed to improve the EPN potential in field conditions, these preliminary results showed that EPNs, and H. bacteriophora in particular, are good candidates for further evaluation as biological control agents against C. ciliata overwintering adults.

References


Chestnut pest control with entomopathogenic nematodes – prospects and constraints

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Abstract: Laboratory and field experiments were carried out to evaluate the biological control potential of entomopathogenic nematodes against the chestnut weevil *Curculio elephas*. Ten nematode strains were screened for virulence against *C. elephas* in the laboratory and four commercial nematode products were applied under semi-natural conditions in the field. In the laboratory, strains of *Steinernema bicornutum*, *S. feltiae* and *Heterorhabditis megidis* caused highest pest mortality. Soil applications of commercial nematode products into PVC tubes (depth: 40 cm) interred in a chestnut orchard showed no significant control effects. Reasons for lacking effects and possibilities to improve biological control of this pest are discussed.

Key words: biological control, field experiments, *Curculio elephas*, *Steinernema bicornutum*, *S. carpocapsae*, *S. feltiae*, *S. glaseri*, *S. kraussei*, *Heterorhabditis bacteriophora*, *H. megidis*

Introduction

The chestnut weevil, *Curculio elephas* Gyll. (Coleoptera: Curculionidae), is one of the most serious insect pests of chestnuts throughout Mediterranean countries. In Southern Switzerland, *C. elephas* is very abundant and generally damages 10-20% of fruits, but in some years and locations 50% or more of harvested fruits can be infested (Bovey et al., 1975). From beginning of October mature larvae leave fruits through exit holes, enter the soil and form earth cells at 10-70 cm soil depths for diapause. Larvae hibernate one to four times prior to pupation and adult emergence (Soula & Menu, 2005). Thus, chestnut weevils spend most of their life time as larvae in the soil, where they can be targeted with entomopathogenic nematodes and/or fungi (Paparatti & Speranza, 1999).

In Southern Switzerland, the majority of chestnut trees are grown in traditional orchards and the use of pesticides is strongly restricted. No insecticides are registered for the control of *C. elephas*. In recent years attempts were made to improve fruit quality and productivity of chestnut forests as well as to push new planting of modern orchards to satisfy increasing consumer demands for local chestnut products. In this context, biological control strategies received increasing attention and we hypothesised that the use of entomopathogenic could offer a new, environmentally sound approach for chestnut weevil control in this environment.

In the present study laboratory bioassays were carried through to screen both commercial nematode products and Swiss laboratory strains on virulence against *C. elephas*. In a second step, weevil larvae were exposed to commercial nematode products in soil columns in a chestnut orchard. Nematode induced mortality was assessed and different application times and techniques were evaluated.
Material and methods

Nematodes and insects
Entomopathogenic nematodes used in the present study were either laboratory strains of Agroscope FAW or commercial nematode products provided by the companies. Laboratory strains originated from different locations in Switzerland; they were isolated from soil samples, identified on the species level and reared on Galleria mellonella for several generations. The species and strain names considered in laboratory and field experiments are listed in Table 1. Strain SF Tor (Steinernema feltiae) had been isolated from a cultivated chestnut orchard in Southern Switzerland in 2004.

For the evaluation of commercial nematode products Carponem (S. carpocapsae), Traunem (S. feltiae) and Black vine weevil nematodes (BVWN; H. megidis), all supplied by Andermatt Biocontrol AG (Grossdietwil, Switzerland), as well as Exhibitline (S. kraussei; cold active strain from Syngenta Bioline Ltd., Essex, UK), were selected. Weevil larvae derived from natural populations and were collected by putting infested chestnuts on metal grids over catch boxes. Collected larvae were used in the experiments within 36 h after emergence from fruits.

Table 1. Nematodes used in laboratory and field assays.

<table>
<thead>
<tr>
<th>Laboratory strains</th>
<th>Commercial products</th>
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<tbody>
<tr>
<td>Species</td>
<td>Strain</td>
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<tr>
<td>Heterorhabditis bacteriophora</td>
<td>HB 1</td>
</tr>
<tr>
<td>Steinernema bicornutum</td>
<td>SB 45</td>
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<tr>
<td>S. carpocapsae</td>
<td>SC 14</td>
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<tr>
<td>S. feltiae</td>
<td>SF 19</td>
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<tr>
<td>S. “glaseri” type</td>
<td>SF Tor</td>
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<td>SG 26</td>
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Laboratory experiments
Screening assays were done with 250 IJs/host applied in 0.5 mL of water into 12-well plates in sandy soil at 15°C, 75% RH and permanent darkness. 48 individual larvae each were exposed to nematodes in the treatments and the untreated control. Mean mortality was assessed after 7 days.

Field experiments
Altogether four commercial nematode products were applied into PVC tubes (Ø 10 cm, depth 40 cm) that had been previously interred in a chestnut orchard in Cadenazzo (Southern Switzerland). Nematode concentration applied was 2-4 x 10⁶ IJs/m². In 2003 only Carponem was applied, whereas in 2004 Traunem, BVW nematodes and Exhibitline were considered. Nematode applications were done in 200 mL of water generally one day before the first weevils were put into the soil columns. PVC tubes were inoculated with up to 53 pest larvae per tube during 10 days respectively and if not stated otherwise, the evaluation of nematode induced mortality was done within 10 days to 3 weeks after final inoculation. In 2003, nematode applications were carried out either in autumn, in spring, or in a combined treatment (autumn + spring). As a consequence, exposure time of weevil larvae in the tubes was significantly longer in the spring and the combined treatments, compared to the autumn treatments.
In 2004, only autumn applications were done. In addition to the standard treatment (2 x 10^6 IJs/m^2 on top of the soil columns), we also included one treatment with two fold higher (4 x 10^6 IJ/m^2) nematode concentration, as well as a third treatment where the nematodes were applied simultaneously on top and bottom of the soil columns with 2 x 10^6 IJs/m^2 each. These top+bottom treatments were done by taking out PVC tubes from the soil and applying nematodes into the upside down tubes, followed by a standard treatment on top of the columns after repositioning the tubes in the soil. Top + bottom applications were assumed to improve vertical distribution of nematodes in the tubes and to increase mortality at increased soil depths. Larval mortality was assessed by rinsing the soil over a sieve to collect and count both intact and dead weevil larvae at increasing soil depths.

**Results and discussion**

The laboratory screening assay revealed that mature pest larvae were susceptible to all tested nematode species and strains and that all of the nematode strains were able to propagate in *C. elephas*. The effect of nematode treatments on pest mortality was highly significant (Anova: F_{10,33} = 10.59, p<0.0001). The Swiss laboratory strain SB 45 caused highest mortality (56 ± 17.2%), followed by SF Tor (46 ± 16%) and commercial *H. megidis* (BVW nematodes; 40 ± 12.5%). On the other hand, cold active *S. kraussei* (Exhibitline; 6 ± 4.2%) were significantly less virulent (LSD post hoc tests, p<0.05) and did not differ from the untreated control (Fig. 1). The other strains achieved pest mortalities that ranged between 13 and 33%.

![Figure 1](image)

**Figure 1.** Mortality of chestnut weevil larvae after treatment with laboratory and commercial strains of entomopathogenic nematodes in multi-well plates at 15°C, 75% RH and permanent darkness. SB = *S. bicornutum*, SC = *S. carpocapsae*, SF = *S. feltiae*, SG = *S. “glaseri” type*, HB = *H. bacteriophora*, BVWN (*H. megidis*), Traunem (*S. feltiae*), Carponem (*S. carpocapsae*), Exhibitline (*S. kraussei*).

In the soil column experiments in 2003, pest mortality was significantly higher in spring and combined (autumn + spring) applications than in the autumn applications (F_{5,30} = 13.39, p<0.0001). However, no significant differences were found between Carponem treatments and the control neither in autumn and spring applications nor in the combined applications (Fig. 2). This was probably due to the relatively high natural mortality found in all control treatments and may have blurred any effect of nematode application in the treatments.
In the 2004 experiments weevil mortality was slightly higher in the treatments than in the untreated control (Fig. 3). However, none of the commercial nematode products caused significantly increased pest mortality ($F_{3,56} = 0.39$, $p>0.05$). The effect of nematode treatment was similar for all application types (standard, two fold, top+bottom) and for all nematode products ($F_{9,50} = 0.62$, $p>0.05$). Again, natural mortality was relatively high in all the untreated controls and may have covered again the potential effects of nematode treatments.

In all soil column experiments the total amount of recovered larvae increased with increasing soil depths and was highest at 31-40 cm both in the treatments and the control. These findings correspond with observations made by Bovey et al. (1975), showing that preferred soil depths of diapausing larvae were between 20 and 60 cm. Our data further revealed that larval mortality generally decreased at increasing soil depths, meaning that more larvae died in the upper soil levels.

From these initial experiments it is difficult to draw clear conclusions concerning the potential of entomopathogenic nematodes for chestnut weevil control. Although laboratory experiments showed promising results, these findings could not be confirmed under semi-
natural conditions. We assume that biotic and abiotic factors may have limited biological control in our experiments. One reason for unsatisfactory results may lay in reduced nematode activity at lower temperatures. When weevil larvae enter the soil in late autumn, soil temperatures are rapidly decreasing from around 15°C to far below 5°C in the upper soil levels. In addition, the steady entry of pest larvae into the soil over several weeks makes it difficult to maintain optimal conditions for nematodes. Pest larvae may therefore partly escape nematodes, first because nematodes may need more time for successful infection under these conditions, and secondly because pest larvae reach soil depths for entering diapause that may be adverse to nematodes. In future works both strain selection, application time and application technique have to be improved and combinations with other biocontrol strategies may be considered as well. For a long-term reduction of pest populations it is further strongly recommended to clean chestnut orchards by removing all fallen nuts as soon as possible and thus reducing the potential for future infestation.

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References

Control of the hazelnut borer, *Curculio nucum*, with entomopathogenic nematodes

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**Abstract**: One of the most important insect pests for hazelnuts is the weevil, *Curculio nucum*. In laboratory trials using the sandy loam from the hazelnut orchard, three nematodes species, *Steinernema carpocapsae*, *S. feltiae*, and *H. bacteriophora* were applied to larvae collected in August 2002 in Cancon, France. The first assessment revealed 76%, 33% and 23% mortality 7 days after treatment with 100 infective juveniles/larvae of the three species, respectively. In a second experiment the *S. feltiae* that had propagated in the infected weevils were used and caused 88% mortality at a rate of 100 infective juveniles/larvae. A further trial was made in June 2004 with larvae from the same location using *H. indica* at rates of 5, 10 and 50 infective juveniles per cm². Larval mortality was >60% for all the doses tested. In field trials with artificially infected soil in buried buckets in hazelnut orchards, the mortality was 41, 65 and 75% in after applying *S. feltiae*, *H. indica* and *H. bacteriophora*, respectively, at a rate of 2.2 million/m², whereas 14% died in the untreated control. When repeating the experiment in 2004 with a dose of 0.5 million/m² larval mortality was not significantly different from the untreated control. Most larvae were found at a depth of 20 to 30 cm in the soil columns but some larvae were found down to 40 cm. Only 5% of the larvae were at 0 to 10 cm depth. All the nematodes applied did establish on the trial site as shown by baiting the soil with wax-moth larvae one year after treatment. However, most bait insect were infected in plots treated with *H. bacteriophora* which indicates the superior persistence of this species compared to *S. feltiae* and *H. indica*. The emergence of beetles two years after nematode treatment was significantly lower in the plots treated with *H. bacteriophora* or *S. feltiae*, while no difference to the untreated control was found in plots treated with *H. indica*. It is concluded that *H. bacteriophora* is the most promising candidate for controlling the hazelnut weevil pest to its good persistence and efficacy.

**Key words**: *Steinernema feltiae*, *Heterorhabditis indica*, *Heterorhabditis bacteriophora*, *Curculio nucum*, biological control, hazelnuts

**Introduction**

The hazelnut borer, *Curculio nucum*, is a serious pest in hazelnut plantations. Without control, the beetle cause losses of about 20% in the first years and after many years without treatment up to 80%. Even in well managed orchards with 3 to 4 chemical treatments per year against the adults with endosulfan and/or carbaryl pest damage is about 5 to 10% (Akça and Tuncer, 2005). The adult weevil lays single eggs into the fruits in early spring. The larvae feed inside the fruits for about 4 weeks and fall down with the fruits in July/August. Within a few days, the larvae leave the nuts and bury themselves into the soil. A part of the larvae pupate next summer and emerge in spring a year later, some pupate one or two years later. The beetles overwinter in diapausis inside the soil before laying eggs in early spring. In the Cancon region in France, the new adults start laying eggs into the young nuts in late spring around 10th of June. The larvae feed and grow inside the nuts and the infested nuts fall down from end of
July to the beginning of August. They leave the nuts and burrow themselves into the soil for overwintering. They pupate next summer and emerge in spring of the next year, some of the larvae may reside in the soil for 3 to 4 years before emerging as adults. As an alternative control strategy to the chemical treatment, the use of entomopathogenic nematodes was evaluated.

**Material and methods**

**Laboratory screening**

Full grown hazelnut borer larvae were collected from infested nuts collected in an orchard near Cancon (France). In a first laboratory screening, the susceptibility of the larvae to *Steinernema feltiae*, *S. carpocapsae* and *Heterorhabditis bacteriophora* was compared. Single larvae were put in cell wells of 1.5 cm diameter filled with 1 mL of moist sand (10%) and 100 nematodes added per larva. After 8 days incubation at 22°C, mortality was assessed. The weevil larvae infected with *S. feltiae* were put on white traps to harvest the emerging nematodes. These were evaluated for their ability to kill further weevils at a lower dosage (50 n/larvae).

Since the nematode *H. indica* was used in the field trials, it was later also evaluated in a different laboratory test. Twelve plastic boxes (10 x 10 cm) were each filled with 200 mL of the original soil and 10 full grown *C. nucum* larvae. Each three boxes were treated with a dose of 500, 1000 and 5000 nematodes (equivalent to 0.05, 0.1 and 0.5 million nematodes / m²). The mortality was assessed 7 days after incubation at 25°C. Means ± Standard errors are given in the text.

**Field trials**

Field trials were conducted in an orchard near Beaugas (France) with 12 year old hazelnut trees variety CORABEL. Trees were planted in 3 meter spacing and rows were 6 m apart. The soil was a calcareous clay. A micro sprinkler system with a water flow of 20 L/hour/tree was installed. Eighty plastic buckets (38 cm diameter, 50 cm depth) were buried into the soil. In 2003, 48 of these container were spiked with 53 weevil larvae each. These containers were treated on 1st August 2003 at a dose of 2.2 million per m² (12 containers per treatment and 12 untreated containers). Treatments were arranged completely randomised. 2 months after treatment, 3 containers per treatment were destructively sampled and the number of living larvae as well as the position of the larvae was examined. Another group of containers were treated again in 2004, on 18th August with 0.5 million per m². In total , there were 5 buckets per treatment and per assessment year (5 x 4 x 4 = 80 container). They were destructively sampled 2 months after treatment and the number of living larvae and the position of the larvae was recorded. Nematodes used were, *Heterorhabditis indica* (LN2), *H. bacteriophora* (e-nema) and *Steinernema feltiae* (e-nema).

Nematode establishment in the soil at the trial site was tested after soil samples were sent to Germany. They were baited with *G. mellonella* larvae. Presence of *H. bacteriophora* or *H. indica* was visible by the red coloration of infected larvae and was verified by measuring the luminescence of the dead larvae in a luminometer (Lumat; Berthold GmbH). Larvae infected with *S. feltiae* were distinguished from larvae died for other reasons by their lighter colour and the integrity of the dead insect. Carcasses infected with fungi differ in that they become hard and dry and those infected with other bacterial saprophytes disintegrate rapidly. Soil samples from control plots were used to verify that there were no native populations of one of the three nematode species used.

Beetle emergence was established from 12 containers per treatment which had been spiked with larvae and been treated with nematodes in 2003 (except in 6 of the containers.
treated with *H. indica*, where the treatment took place in 2004). To keep the beetles from leaving the containers, the lids were kept closed during the period of emergence.

**Results and discussion**

In the first laboratory screening with 100 nematodes per larva mortality was highest for *S. carpocapsae* (76%) followed by *S. feltiae* (33%) and *H. bacteriophora* (23%). Interestingly, the *S. feltiae* emerging from infected hazelnut weevil larvae appeared to be more virulent (88% mortality). The second laboratory trial was performed to test the infectivity of *H. indica* which was not included in the first trial. There was a significant mortality after treating weevil larvae at doses of 0.05, 0.1 and 0.5 million nematodes per m². However, there was no significant increase in larval mortality with increasing dosage (6 ± 1; 4.3 ± 1.3; and 6.5 ± 2.3 dead larvae from 10, respectively).

Despite the high virulence of *S. carpocapsae* in the laboratory assays, it was not included in the field trials since this species is known to reside on the soil surface while the hazelnut weevil larvae move down a few centimeters.

In field trials the mortality was 41, 65 and 75% after applying *S. feltiae*, *H. indica* and *H. bacteriophora*, respectively, at a rate of 2.2 million/m², whereas 14% died in the untreated control (Fig. 1). The reduction in the number of living weevil larvae was statistically significant only for *H. bacteriophora* (ANOVA). When repeating the experiment in 2004 with a dose of 0.5 million/m² larval mortality was lower and not significantly different from the untreated control for none of the nematodes used (Fig. 1).

Most weevil larvae were found at a depth of 20 to 30 cm in the soil columns but some larvae were found down to 40 cm. Only 5% of the larvae were at 0 to 10 cm depth. There was no significant effect (ANOVA) of the different treatment on the vertical distribution of the larvae but larvae were found slightly deeper in 2003 (mean = 24 ± 1 cm) than in 2004 (mean = 20 ± 1 cm).

![Figure 1: Number of Curculio nucum larvae found in container 2 months after treatment with 2,2 million nematodes/m² of three different species (Heterorhabditis indica, H. bacteriophora and Steinernema feltiae) in 2003 (■) and 2 months after treatment with 0,5 million/m² in 2004 (♦).](image-url)
All the nematodes applied did establish on the trial site in 2003 as shown by baiting the
soil with wax-moth larvae. In 2004, however, the persistence of *H. bacteriophora* was clearly
superior to the persistence of the other two nematode species (Fig. 2). The tropical species *H.
indica* is likely to have suffered from low temperatures. It does not survive well at
temperatures below 10°C (Strauch *et al.*, 2000).

![Graph showing the number of infected bait insects from soil samples from the field trial site two
months after nematode treatment in 2003 (■) and 2004 (♦). Means ± Standard error.]

Beetle emergence in 2005 was still low. This might be due to heavy rainfall which may
have killed part of the larvae. Also, not all beetles do emerge in the second year after settling
in the soil. There is a clear difference in emergence between the treated and non-treated plots.
No beetles did emerge from the 12 plots treated with *H. bacteriophora*, 2 beetles from one of
the 12 plots treated with *S. feltiae* and 3 beetles from 2 of the plots 12 plots treated with *H.
indica*. The total number of beetles emerging from the 12 untreated control plots was 8. These
encouraging results warrant further investigation in the control of the hazelnut weevil with
entomopathogenic nematodes. To optimise the efficacy of nematodes against the hazelnut
weevil, the timing of nematode application should be modified to hit the larvae while they are
in the top soil layer. In a comparable environment, the citrus orchards in Florida infested with
the weevil *Diaprepes abbreviatus*, nematode application via the sprinkler application system
gives excellent control of the pest (Bullock *et al.*, 1999). The nematode application via the
application system should also be tried to control the hazelnut weevil.

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Storage and formulation of the entomopathogenic nematodes *Heterorhabditis indica* and
The effect of linearly polarized light on pathogenicity and reproduction in entomopathogenic nematodes

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Abstract: The aim of the study was to find out the effect of linearly polarized visible radiation on pathogenicity and reproduction in entomopathogenic nematodes. Exposure to light positively affected the pathogenicity of invasive larvae for test insect, Tenebrio molitor. The irradiated nematodes killed insects in greater numbers and more rapidly in comparison with the not illuminated ones (control). This positive effect of linearly polarized light on the pathogenicity of S. carpocapsae was not followed by better reproduction of the microorganism from dead insect bodies.

Key words: Steinernema carpocapsae, polarized light, pathogenicity, Tenebrio molitor

Introduction

Polarized visible radiation has been applied in medicine for treatment of severe burns, healing wounds, ulcerations and eczemas (Fiedorowicz, 2004). The exposure of human skin to polarized light improves an immunological response in peripheral blood (Samoilova et al., 1998). However, a mechanism of the effect polarized light has on biological systems is still not known. Kubasova et al. (1984, 1988) reported that polarized light would change some properties of a cell membrane and finally would produce functional changes in irradiated cells. The aim of this study was to estimate preliminarily the influence of polarized light on entomopathogenic nematodes, and especially on such biological functions of the microorganisms as pathogenicity for test insects and reproduction from the insect bodies.

Materials and methods

A strain of the entomopathogenic nematode Steinernema carpocapsae from the collection of the Department of Agricultural Environment Protection was used in the experiment. Nematodes were cultured in Tenebrio molitor L. (Coleoptera: Tenebrionidae) larvae according to the commonly accepted procedure. Samples containing a known number of invasive larvae were suspended in 250 mL of water in a glass flask. Each sample was exposed to light for 1, 2, 3 and 4 hours from the distance of 30 cm. For this purpose an illuminator type 57-400 (Optel Opole, Poland) with a 150W xenon lamp (XBO 150, Oriel, England) was used. A polarizing filter (Polaroid, USA) and glass filter cutting out wavelengths below 500nm were fixed on between the illuminator and the sample. The light source was emitting radiation with continuous intensity in the visible range. Its energy flux at the place of the sample was 8 mW · cm⁻², as checked with a YSI radiometer (Yellow Springs, USA). The method has been developed by Fiedorowicz (2004). The sample was steadily mixed at 500 rpm in the flask using an Electromagnetic Stirmer mixer type ES21H (Wlgo, Poland). A control sample
consisted of nematodes placed in a glass flask, not exposed to irradiation and mixed with the same appliance, as the illuminated one. After 1, 2, 3 and 4 hours two thousands of *S. carpocapsae* invasive larvae were taken in three replications from illuminated and control objects, put into Petri dishes and placed on the filters. The test insects were introduced into the dishes containing the nematodes; for this purpose *T. molitor* larvae were used. Then the mortality of test insects was recorded starting three hours after the insects had been put into the dishes. Insect bodies were carried to islands of watch glasses laid in the Petri dishes to allow nematodes to reproduce. After a 21-day period reproduction estimates were determined and, to make the results comparable, converted into relative values per 1 g DM of a test insect. Obtained data were subjected to statistical analysis with the use of a single factor analysis of variance and the means were differentiated by the Duncan test at a significance level *p*=0.05.

Table 1. Effect of duration of exposition to polarized light on pathogenicity of *S. carpocapsae* nematodes (expressed in % mortality of test insect, *Tenebrio molitor*).

<table>
<thead>
<tr>
<th>Time of exposure EN to light (hours)</th>
<th>Test insect mortality (%) after:</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>6 h</td>
</tr>
<tr>
<td>irradiated + mixer</td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>16.9 b* 19.8 bc 29.0 bc 30.0 cd 36.4 cd 64.4 cd 84.8 de 93.5 bc 99.4 a</td>
</tr>
<tr>
<td>2 h</td>
<td>23.3 b 33.1 c 38.2 c 38.2 de 45.0 d 71.9 de 88.9 de 100 d 100 a</td>
</tr>
<tr>
<td>3 h</td>
<td>16.2 b 18.1 bc 26.5 bc 29.7 cd 38.0 cd 65.1 cd 77.6 cde 96.7 cd 100 a</td>
</tr>
<tr>
<td>4 h</td>
<td>28.1 b 28.1 bc 40.0 c 55.0 e 67.4 e 88.3 e 92.3 e 100 d 100 a</td>
</tr>
</tbody>
</table>

mixer (control)

<table>
<thead>
<tr>
<th>Time of exposure EN to light (hours)</th>
<th>Test insect mortality (%) after:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>3.3 a 9.6 ab 13.0 ab 20.0 bc 21.4 abc 46.6 bc 70.1 cd 93.5 bc 98.9 a</td>
</tr>
<tr>
<td>2 h</td>
<td>1.1 a 1.7 a 4.3 a 9.6 ab 13.2 ab 29.7 ab 46.6 ab 83.8 ab 95.5 a</td>
</tr>
<tr>
<td>3 h</td>
<td>2.2 a 10.4 abc 19.8 bc 26.2 cd 27.7 bcd 53.4 bcd 63.4 bc 89.8 bc 98.3 a</td>
</tr>
<tr>
<td>4 h</td>
<td>0 a 0.6 a 3.3 a 3.3 a 8.2 a 18.3 a 31.6 a 70.8 a 94.8 a</td>
</tr>
</tbody>
</table>

*means marked with the same letters in columns do not differ significantly at level *p*=0.05

Results and discussion

The experimental results of the influence of various periods of exposure of linearly polarized light on the pathogenicity of *S. carpocapsae* for *Tenebrio molitor* are given in Table 1. Illumination of the nematodes positively affected their pathogenicity for the test insects in comparison with the control (not exposed) ones. The nematodes from the irradiated object killed the insects more quickly and in greater numbers when set beside the nematodes from the control object and staying in the mixer. At twelfth hour of mortality observation of the test insects it was noticed that the nematodes illuminated for the longest time (4 hours) killed the greatest number of test insects. Similar levels of pathogenicity were indicated in nematodes
from both objects not earlier than at forty-fourth hour of test insect exposure to the nematodes. Mean values of test insect mortality in the irradiated and control object are shown in Figure 1. A positive effect of linearly polarized light on pathogenicity of *S. carpocapsae* was not followed by a better reproduction from dead insect bodies. In contrast with the illuminated nematodes, significantly higher numbers of invasive larvae were obtained from bodies of the insects killed by the control nematodes. Reproduction data from the treated and control nematodes from dead insect bodies are presented in Figure 2.

Our investigations demonstrated a distinct stimulation of *S. carpocapsae* entomopathogenic nematodes caused by linearly polarized light effect. Studies on the effect of entomopathogenic nematodes exposure to radiation yielded various results. While testing low ionizing radiation doses on entomopathogenic nematodes, it was showed previously that a dose of 0.01kGy (the lowest of the used ones) twice increased the infectiveness of infective larvae toward test insects (Poleszuk, Pezowicz, Kamionek & Jarmul, personal communication). The authors have stated that ionizing radiation causes morphological changes in the tested nematodes. On the other hand, while researching the effect of ultraviolet radiation (302 nm) on *S. carpocapsae* and *Heterorhabditis bacteriophora* Gaugler et al. (1992) observed an intensive decline in pathogenicity towards test insects during irradiation of nematodes lasting for over 4 minutes. In the later studies no stimulated pathogenicity of entomopathogenic nematodes under the influence of applied radiation was observed.

![Figure 1](image.png)

**Figure 1.** Mean values of % mortality caused, in test insect, *Tenebrio molitor*, by the nematodes *S. carpocapsae* exposed to polarized light for 4 hours or not (control).
*means marked with the same letters do not differ significantly at level p=0.05

Figure 2. Influence of duration of exposition to polarized light on reproduction of *S. carpocapsae* nematoda from bodies of test insect, *Tenebrio molitor*.

References


Laboratory trials of microbiological control of *Agelastica alni* L. with *Beauveria bassiana* (Bals.) Vuill.

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Abstract: The authors tested the entomopathogenicity of *Beauveria bassiana* isolates, obtained from forest soils, against the chrysomelid *Agelastica alni*, a pest of alder trees. In 2004, conidial suspensions of three isolates of the hyphomycete (Bba T01, BbaT06 and BbaT08) were adjusted in the laboratory to a concentration of nearly \(3 \times 10^6\) cfu/mL and sprayed on the leaves of apical shoots of *Alnus cordata* (Italian alder). Controls were sprayed only with water. Treated and control shoots were placed on damp filter paper inside Petri dishes of 15 cm Ø with five *A. alni* various instar larvae. Each experiment was repeated four times. Dishes were kept under laboratory conditions and controlled daily. All *B. bassiana* isolates were entomopathogenic against *A. alni*, but showed differences in the survival time of the chrysomelid.

Key words: *Agelastica alni*, *Beauveria bassiana*, microbiological control

Introduction

There is considerable interest in the exploitation of entomopathogenic fungi from soil for use as an alternative to chemical insecticides in the control of forest pests.

The authors have collected soil samples in several broad-leaf and conifer plantations in Tuscany (Italy) from which various fungal isolates were obtained. *Beauveria bassiana* (Bals.) Vuill. was the most representative fungal species, with strains having different levels of entomopathogenicity. Their pathogenicity has been tested in the laboratory against larvae of the wax moth *Galleria mellonella* L. and adults of the cerambycid *Monochamus galloprovincialis* (Olivier), an important pest in forestry as vector of the pine nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (Francardi et al., 2003; Rumine et al., 2004; Shimazu et al., 1992, 1995). The *B. bassiana* isolates are part of the Collection of Entomopathogenic Microorganisms of the Istituto Sperimentale per la Zoologia Agraria (ISZA - Firenze).

Adults and larvae of *Agelastica alni* L. (Coleoptera: Chrysomelidae) can cause severe defoliation of alder trees, which often predisposes the trees to decline. Recently, there have been severe attacks by *A. alni* in several plantations of *Alnus* spp. in a large wooded area in Tuscany (Valdarno - Arezzo) where no chemical treatments are allowed. Therefore, laboratory trials were conducted to test the pathogenicity of three *B. bassiana* isolates against *A. alni* larvae with the aim of identifying the most effective strains for the control of this forest pest.

Materials and methods

The entomopathogenicity of three *B. bassiana* isolates (BbaT01, BbaT06 and BbaT08), obtained from forest soils in Tuscany (Italy), was tested in 2004 in laboratory trials against *A. alni* larvae. Conidial suspensions of the isolates were prepared in 250 mL flasks containing 100 mL of PDB (Potato Dextrose Broth). Inoculum was added to the culture medium and the flasks were placed in a stirred (100 rpm) thermostat at 25°C for 7-15 days.
The fungal concentration was evaluated with a Thoma haemocytometer and then adjusted to values of nearly $3 \times 10^6$ cfu/mL.

The suspensions were sprayed on the leaves of apical shoots (10-12 cm length) of *Alnus cordata* (Italian alder) while controls were sprayed only with water. Treated and control shoots were placed on damp filter paper inside Petri dishes of 15 cm Ø. Five various instar larvae of *A. alni* were then put in each dish. Each experiment was repeated four times.

Dishes were kept under laboratory conditions at 23-25°C. Relative humidity inside the dishes ranged from 80 to 90%. The dishes were monitored daily during the trial period.

Data were processed with life tables, survival function analysis and pairwise comparison of survival experience using the Wilcoxon (Gehan) statistical software (SPSS ver.10.1.3).

**Results and discussion**

All *B. bassiana* isolates were entomopathogenic against *A. alni*, but showed differences in the survival time of the chrysomelid (Fig. 1).

![Figure 1](https://via.placeholder.com/150)

Figure 1. Survival function analysis of *Agelastica alni* larvae in microbiological control trials using three strains of *Beauveria bassiana*.

The BbaT08 isolate caused the mortality of all larvae within 3 days, BbaT06 within 7 days and BbaT01 within 11 days. The mortality of *A. alni* larvae in the BbaT01, BbaT06 and BbaT08 trials was significantly different from that observed in the control trials ($P=0.0000$).
in which there was only one dead larva. Moreover, the mean mortality induced by BbaT08 was significantly higher than that induced by BbaT06 and BbaT01 (P=0.0000), while there was no significant difference between the last two isolates (P=0.0764).

These data indicate that BbaT08 is a highly effective strain against larvae of A. alni. However, detailed toxicological studies (in progress) are necessary to clarify these results in view of possible applications in Integrated Pest Management programs.

References


Entomopathogenic fungi isolated from various substrates in Italian pine woods

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Abstract: From 2001 to 2004, investigations were carried out in several maritime (Pinus pinaster Aiton) and nigra pine (Pinus nigra Aiton) stands of Tuscany (Italy) to isolate entomopathogenic fungi from various substrates (soil, bark and dead infected insects). Fungi were isolated in laboratory using different methods. The results showed a widespread presence of entomopathogenic fungi; in particular, Beauveria bassiana was obtained from all the substrates while Paecilomyces spp. were detected in both bark and insect samples. The entomogenous fungi Lecanicillium evansii and Nectria inventa were also recorded.

Key words: entomopathogenic fungi, soil, bark, mycosed insects, Beauveria bassiana

Introduction

Several species of entomopathogenic fungi can be isolated from various substrates, such as soil, parts of plants and insects (Poinar & Thomas, 1982). In this way, it is possible to evaluate the presence and spread of these microorganisms in the environment, offering the possibility to increase the pre-existing inoculum by various management strategies, e.g. dissemination or massive inoculation. Because of their broad spectrum of activity and slow action, entomopathogenic fungi cannot supplant chemical pesticides (especially in intensive agricultural systems) or stop rapidly expanding insect pest populations. However, they could be a valuable resource in Integrated Pest Management programs to optimize the control of target pests.

Beauveria bassiana has been used on several occasions as a biological insecticide to achieve greater control of forest or agricultural pests (Inglis et al., 2001). To evaluate the presence and spread of entomopathogenic fungi in forest environments, investigations have been carrying out to isolate and characterize the fungal species present in various substrates in conifer stands, in particular the entomopathogenic and/or entomogenous fungi. The aim of the present paper is to report the data obtained from analyses of various substrates collected in pine forests of central Italy.

Materials and methods

Investigations were conducted from 2001 to 2004 in several maritime (Pinus pinaster) and nigra pine (Pinus nigra) stands of Tuscany (Italy) with the aim of isolating entomopathogenic fungi from soil, bark and dead infected insects. The fungi were isolated by different laboratory methods according to the type of sample.

For soil samples, larvae of Galleria mellonella were added to the substrate (about 0.5 Kg) in plastic boxes and kept wet at room temperature (20-25°C) for 2-3 weeks. Larvae
showing the typical symptoms of fungal infection were used to isolate fungi: mycelium and spores were placed directly on growth medium (PDA - Potato Dextrose Agar) in Petri dishes, and the isolates were then purified by repeated transplanting.

For bark samples, fungi were isolated from scales of about 5 x 10 cm taken from trunks of *Pinus pinaster* with a chisel. The outer surface of the scales was washed with a flush of sterile water (40 mL) containing 0.01% of a wetting agent (Tween 80). One mL of washing per sample was drawn with a sterile pipette and spread over a Petri dish filled with selective medium (Doberski & Tribe, 1980).

Naturally infected insect samples (larvae or adults) collected in pine stands were put in a moist chamber to promote the external development of the fungus. Mycelium was then taken from the sample, directly plated on PDA and later purified by repeated transplanting.

All inoculated plates were incubated at 25°C and examined daily. Finally, all the fungal isolates obtained were identified.

**Results and discussion**

Isolation results are presented in Table 1. Several isolates of *Beauveria bassiana* were identified from soil samples collected near the base of pine trees, while *B. bassiana*, *Paecilomyces farinosus* and *P. lilacinus* were obtained from bark samples.

*B. bassiana*, and species of *Paecilomyces*, i.e. *P. farinosus*, *P. javanicus* and *P. lilacinus*, were isolated from dead infected insects (larvae and adults) collected in the same pine forests, i.e. *Arhopalus syriacus*, *Monochamus galloprovincialis* and *Thaumetopoea pityocampa*. Only *B. bassiana* was obtained from all the substrates, while *Paecilomyces* spp. were detected from both bark and insect samples.

<table>
<thead>
<tr>
<th>Entomopathogenic fungus</th>
<th>Substrate</th>
<th>Environment/Host</th>
<th>Province</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>Soil</td>
<td><em>Pinus pinaster</em> forest</td>
<td>Firenze</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td><em>P. pinaster</em> forest</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td><em>P. nigra</em> forest</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td><em>P. pinaster</em> forest</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td><em>P. pinaster</em> forest</td>
<td>Pistoia</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td><em>P. pinaster / Quercus cerris</em> mixed forest</td>
<td>Pisa</td>
</tr>
<tr>
<td>&quot;</td>
<td>Bark</td>
<td><em>P. pinaster</em></td>
<td>Firenze</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td><em>P. pinaster</em></td>
<td>Arezzo</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td><em>P. pinaster</em></td>
<td>Pistoia</td>
</tr>
<tr>
<td>&quot;</td>
<td>Insect</td>
<td><em>Monochamus galloprovincialis</em></td>
<td>Firenze</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td><em>Arhopalus syriacus</em></td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>Insect</td>
<td><em>Thaumetopoea pityocampa</em></td>
<td>&quot;</td>
</tr>
</tbody>
</table>

| *Paecilomyces farinosus* | Bark      | *P. pinaster* forest                         | Arezzo   |
| "                       | Insect    | *Monochamus galloprovincialis*               | Firenze  |

| *Paecilomyces javanicus* | Insect    | *Monochamus galloprovincialis*               | "        |
| *Paecilomyces lilacinus* | Bark      | *P. pinaster*                                | "        |
| "                       | Insect    | *Monochamus galloprovincialis*               | "        |
In addition to the above-mentioned species, the two other entomogenous fungal species *Lecanicillium evansii* and *Nectria inventa* were also recorded.

The results showed a widespread presence of entomopathogenic fungi in the substrates, especially *B. bassiana*. Because of its broad diffusion and entomopathogenicity, this Hyphomycete could be used as an inoculum against populations of phytophagous and xylophagous insect pests in pine stands. Moreover, the three species of *Paecilomyces*, isolated from *Pinus pinaster* bark and from the cerambycid *Monochamus galloprovincialis* living on the same tree, could contribute to microbiological control in pine forests.

**References**


Does *Beauveria* spp. produce toxins after application when the product is present at the crop?

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**Abstract:** The question of possible production of toxins by *Beauveria* after applications seems to be utmost important especially for regulation authorities in Europe. Members of the RAFBCA consortium were confronted with these issues because little is published: (i) about the concentration range of metabolites produced by fungal BCAs; ii) about whether relevant metabolites enter the food chain, therefore posing a risk to human and animal health as well as the environment; (iii) about relevant studies about workers tested concerning exposure risk to toxins with focus on exposure to fungal products and to toxicologically relevant compounds in the products. As a matter of fact, there is sufficient information from literature which demonstrates that *Beauveria* does not produce relevant metabolites (toxins) during or after application. No risks to workers and bystanders are expected. There is no indication of environmental risk, nor do relevant metabolites enter the food chain. *Beauveria* spp. is therefore a safe biological control agent which should be registered in Europe without any restrictions.

**Key words:** fungal BCA, mycotoxin, exposure control, safe crop protection

**Introduction**

*Beauveria* spp. are ubiquitous soil fungi and have been used as microbial pest control agents for many years with no protective clothing of workers at very high degrees of exposure to conidia both in the production process and in application. Thus there is a long history of human exposure to the fungi.

Both *Beauveria bassiana* and *B. brongniartii* are able to secrete an array of low molecular weight primary and secondary metabolites. The metabolites secreted in submerged cultures include oxalic acid, bassianin, beauvericin, oosporein and tenellin (Roberts, 1981; Khachatourians, 1991; Strasser et al., 2000b; Vey et al., 2001).

Toxicologically relevant excreted *Beauveria* metabolites are primarily produced in the liquid fermentation step (i.e. submerged culture) and in infected pest larvae, where the secondary metabolites exhibit antibacterial activity and moderate insecticidal properties, respectively (Khachatourians, 1996; Strasser et al., 2000a). This excludes beauvericin which will be accumulated in low concentration in the mycelium. None were detected in plants (Abendstein et al., 2000). Investigations on environmental enrichment and the significance of secondary metabolites released by *Beauveria* have been realised by the EU funded project RAFBCA (QLK1-CT2001-01391). Major goal of the project was to detect and quantify relevant fungal metabolites of *Beauveria* spp. in the crop or produce, to identify possible exposure routes and to assess the risk they pose to human and animal health. *B. brongniartii* has been used as model organism, a closely related species to *B. bassiana*, known to secrete the same kinds of secondary metabolites (Strasser et al., 2000b).
Material and methods

Risk assessment approach
Risk assessment studies were based on EPPO draft PP1/New 1: “Efficacy evaluation of insecticides, white grubs” and EPPO Standards P1/135: “Phytotoxicity assessment”. Further details on reagents, buffers and adsorption experiments are described in Seger et al. (2005a, b).

Quantification of oosporein in biological matrixes
To quantify oosporein in biological matrixes (e.g. potato, maize, sugar beet, barley) the biomass was suspended in the extraction solvent (BR5.5-MeOH) and ultrasonicated (3 x 5 min). The solution was purified by centrifugation (Hermle Z383 (Hermle Labortechnik, Wehingen, Germany) centrifuge at 2500 rpm, 20 min, room temperature) over a Mr 10 000 cutoff membrane (Vivaspin2, CTA membrane, Sartorius, Göttingen, Germany) and used for HPLC analysis without further treatment. The recovery rate was determined with spiking experiments using BR5.5-MeOH extraction solvent, fortified with equals of oosporein/L (Seger et al., 2005a).

HPLC-DAD conditions
HPLC analyses for oosporein were performed using a HP 1090 liquid chromatograph (Agilent, Waldbronn, Germany) equipped with a diode array detector (DAD), an automatic injector, an autosampler, and a column oven. Separations were performed on a Phenomenex Synergi Hydro-RP 80A column (150 x 2 mm) with a particle size of 4 µm (Phenomenex, Torrence, CA). The binary elution gradient consisted of water (solvent A) and acetonitrile (solvent B), both containing 0.1 % (v/v) acetic acid and 0.9 % (v/v) formic acid. The course of the gradient was 5-60 % B in 6 min, followed by 60-98% B in 2 min and was kept constant for a further 5 min at a flow rate of 0.3 mL/min. The column was kept at 23 C (thermostated). Between analyses, the column was reequilibrated for 7 min. The injection volume was 2 µL, and chromatograms were recorded at 287 nm (sample bandwidth 4 nm, reference wavelength 450 nm, reference bandwidth 80 nm; according to Seger et al., 2005a).

Results and discussion

Residues in or on treated products, feed and fodder
Secondary metabolites were primarily produced by Beauveria spp. during submerged fermentation (e.g. oosporein 270 mg/L), and the levels produced on a solid substrate were less than 7 mg/kg. Oosporein was detectable in infected pest larvae (0.23 mg per larva max.; in Strasser et al., 2000a). Although oosporein is accumulated by the fungus in low quantities (about 1 mM), the metabolite is not mobile in biological matrices (Seger et al., 2005b). For example, at this concentration range, the adsorption to soil is nearly quantitative and irreversible. Spiking experiments with sandy soil confirmed that 100 percent adsorption was observed for 300 mg oosporein kg⁻¹ soil and 98 percent adsorption was observed for 3 g oosporein kg⁻¹ soil, respectively. Since Beauveria spp. cannot infest plant parts (e.g. potato tubers, blade of grass), the likelihood of oosporein to enter edible roots or tubers can be considered extremely low (Abendstein et al., 2000). Even if a plant matrix is infested deliberately with oosporein, as in the case of the formulated product Melocont®-Pilzgerste (i.e. barley kernels are sterilized, crushed and incubated with a spore suspension), the oosporein concentrations found are about forty fold lower (about 7 ppm) than in the culture broth. Thus an environmental risk emanating from the biocontrol agent can be excluded.

During the RAFBCA project we isolated spiked oosporein from different plant matrices with high recovery rates. We are studying the role of Beauveria metabolites in pathogenesis, antagonism and survival. These studies suggest that oosporein plays a minor role in the
infection process. The toxicity of purified oosporein was tested against seedlings of five different plant species. All the fungi were not sensitive to oosporein at all the concentrations tested (0.1-1 mM). It was shown that oosporein is negatively charged and readily binds to positively charged materials especially metal ions. Oosporein degrades quickly under moderate alkaline conditions (half life time is 12 days at 23 °C, pH 8), under moderate acidic conditions it is more stable (half life time is 74 days at 23 °C, pH 6). Elevation of the temperature reduces the stability of oosporein remarkably (half life time is 0.3 days at 53°C, pH 8).

Conclusion

As a result of the RAFBCA project none of the secreted *Beauveria* metabolites must be defined as a “relevant” metabolite (i.e. metabolite of toxicological and/or ecotoxicological or environmental concern; see also amended EU directive 91/414/EEC, Annex II, Section 4. Analytical Methods, p 43). Looking at our model organism *B. brongniartii* the fungal BCA can be characterised as follows:

(i) *Beauveria* spp. is not a plant pathogen.
(ii) *Beauveria* production strains do not grow on plant material.
(iii) Data on metabolite production by commercial isolates of the genus *Beauveria* (e.g. Melocont®-Pilgerste, Beauveria-Schweizer, Engerlingspilz-Andermatt, Boverol®, Melocont®-WG) is hard to come by. Only oosporein was characterised as a major secondary metabolite in submerged culture, in the final product and in mycosed pest organisms (Strasser *et al*., 2000b; Seger *et al*., 2005a).
(iv) There is no evidence of metabolites transferred to plants (RAFBCA studies, unpublished observations).
(v) As can be derived from the chemical and physical characterization of oosporein (Seger *et al*., 2005b), the metabolite degrades quickly under moderate alkaline conditions. Oosporein is not volatile and, therefore, cannot be inhaled/taken up by workers as MVOCs. An adsorption into soil and charged biological matrices is nearly irreversible; however, oosporein can be washed off from the cuticula of crops and fruiting vegetables with tap water.
(vi) Exposure risks of toxins for workers and users is not relevant because formulated products are free of toxicologically “relevant” *Beauveria* metabolites. *Beauveria* metabolites have no relevant antibiotic activity, no cytotoxic or apoptotic effects (Abendstein & Strasser, 2000 and unpublished results).
(vii) Hypothetically speaking, even if the fungus would show saprophytic growth on plant materials, the production of metabolites still would not be relevant. Referring to the EU Directive 91/414/EEC, Annex IIB, item 2.8; no metabolites which are produced by *B. brongniartii* show unacceptable effects on human health and/or the environment during or after application.

In conclusion, there is sufficient information available from literature which demonstrates that *Beauveria* does not produce relevant metabolites (toxins) during or after application (Strasser *et al*., 2000b; Seger *et al*., 2005a). No risks to human health are expected (Strasser & Kirchmair, 2006). The authors have summarized the literature on the safety of biological control agents with specific reference to human infection, allergies, and intoxication. They have provided an overview of the European standards for testing the safety and they gave an updated review on the biological/toxicological knowledge and analysed if potential hazards will influence future biological control. There is no indication of environmental risk, nor do relevant metabolites enter the food chain. *Beauveria* spp. is therefore an effective safe biological control agent.
Acknowledgements

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Insect Pathogens and Insect Parasitic Nematodes

Intraguild interactions involving the entomopathogenic fungus
*Pandora neoaphidis*

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Abstract: Cage arenas were used to study the interactions between three natural enemies of the pea aphid, *Acyrthosiphon pisum*; the entomopathogenic fungus *Pandora neoaphidis*, the coccinellid predator *Coccinella septempunctata* and the hymenopteran parasitoid, *Aphidius ervi*. *A. ervi* and *C. septempunctata* significantly reduced populations of *A. pisum* when introduced as individual species whereas *P. neoaphidis* had no effect on aphid population size. Foraging by both *C. septempunctata* and *A. ervi* increased the abundance of *P. neoaphidis* which may be sufficient to initiate an epizootic. The benefits of increased transmission by *C. septempunctata* therefore outweighed the fitness costs to the fungus of intraguild predation. Although *P. neoaphidis* was associated with a decrease in the reproductive success of *A. ervi* in the cage arenas (where abiotic conditions were optimal for the fungus), preliminary experiments done under sub-optimal conditions indicated that *A. ervi* did not incur a fitness cost from foraging in patches containing *P. neoaphidis*.

Key words: *Pandora neoaphidis*, *Coccinella septempunctata*, *Aphidius ervi*, intraguild interactions

Introduction

Intraguild interactions occur between species from different higher taxonomic groups that share a similar resource (Hochberg & Lawton, 1990; Polis & Holt, 1992). Such interactions occur between many taxa, including parasitoids and fungus and, coccinellids and fungus (Fuentes-Contreras et al., 1998; Furlong & Pell, 1996; Roy & Pell, 2000). Potentially, intraguild interactions between species introduced as biological control agents may positively or negatively influence their effectiveness.

The outcomes of direct and indirect intraguild interactions between arthropods and entomopathogenic fungi will directly affect the population size of both the natural enemies and their prey. Laboratory studies have indicated that foraging arthropods have positive effects on entomopathogenic fungi through enhanced transmission and negative effects through competition for hosts/ predation of infected hosts (Fuentes-Contreras et al., 1998; Furlong & Pell, 1996; Roy & Pell, 2000; Powell et al., 1986). However, the majority of these studies were done at a small spatial scale e.g. in Petri dishes or on single plants. In the experiments described here we used cage arenas to assess the interactions between the aphid-specific entomopathogenic fungus *Pandora neoaphidis* (Remaudière & Hennebert) Humber, the generalist predator *Coccinella septempunctata* (L.) and the aphid parasitoid *Aphidius ervi* (Haliday) at the population scale and under abiotic conditions optimal for the fungus.

Materials and methods

Four replicate Perspex cages (0.5m² x 1m high), each containing a Perspex frame designed to support nine 85mm diameter plant-pots, were prepared. The frame allowed movement of
insects between plants without contact being made with the matting on the base of the cage (which was kept wet at all times to create a relative humidity greater than 90%). Nine 15-day-old dwarf bean plants, *Vicia faba* L. (cultivar The Sutton) planted in individual pots were placed in the frame and the soil at the base of each plant covered with filter paper. Three hours prior to the start of the experiment the eight peripheral plants were infested with either 30 eight-day-old (4th instar) pea aphids, *Acyrthosiphon pisum* (Harris), (experiment A) or ten *A. pisum* (experiments B and C). Immediately prior to the start of the experiment the cages were supplemented with the natural enemy species (see Table 1 for species and quantity of natural enemies added). In cages containing *P. neoaphidis*, six water agar discs each supporting five *P. neoaphidis*-sporulating cadavers (isolate X4 from the Rothamsted Research collection) were placed in random positions on the leaves of the central plant. Mixed sex cohorts of laboratory reared *C. septempunctata* (≤12 weeks old) that had been starved for 24h prior to the start of the experiment were used whereas *A. ervi* (≤5 days old) were transferred directly from an insectary culture. *C. septempunctata* and *A. ervi* were released directly onto the central plant. The cages were maintained at abiotic conditions optimal for the fungus (18°C, 16L:8D).

Table 1. Natural enemies of aphids added to treatment cages in experiments A-C (*Pn = P. neoaphidis*, *Cs = C. septempunctata* and *Ae = A. ervi*). Number of individuals added shown in brackets.

<table>
<thead>
<tr>
<th></th>
<th>Cage 1</th>
<th>Cage 2</th>
<th>Cage 3</th>
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<tr>
<td>Exp. A</td>
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<td>Exp. B</td>
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<td>Exp. C</td>
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The experiments were assessed as follows; experiment A: the number of aphids and *P. neoaphidis*-sporulating cadavers on each peripheral plant recorded after 8 days, experiment B: the number of sporulating cadavers and *A. ervi* mummies recorded on each peripheral plant after 16 days and, experiment C: the number of aphids on four peripheral plants recorded after 8 days and the number of sporulating cadavers and *A. ervi* mummies on the remaining four plants after 16 days. Each experiment was replicated on four occasions with each treatment having been done in each cage once. Data were transformed using a log(n+1) transformation and ANOVA used to assess differences in the population sizes of the aphids and their natural enemies.

**Results and discussion**

*Pandora neoaphidis* did not significantly affect the aphid population (*p*>0.05) whereas foraging by both *C. septempunctata* and *A. ervi* reduced the aphid population by approximately 50% (Figs 1 & 3). Multiple natural enemies (*P. neoaphidis* plus *C. septempunctata* and/or *A. ervi*) reduced the aphid population to a level below that using the individual natural enemy species alone and, in many cases, the aphids were excluded from the bean plants entirely. Multiple natural enemies may interact synergistically, additively or antagonistically, and this interaction will be reflected in the size of the pest population (Roy & Pell, 2000). Although it cannot be assessed statistically, the total aphid mortality when using multiple
natural enemies was approximately the sum of using each natural enemy species alone. Therefore, it appears that there was an additive interaction between *P. neoaphidis*, *C. septempunctata* and *A. ervi*.

Figures 1-4. Summary of results for experiments A (Fig.1), B (Fig.2) and C (Fig.3 & 4). *Cs = C. septempunctata, Ae = A. ervi and Pn = P. neoaphidis. Aphids = □, P. neoaphidis-sporulating cadavers = ▲ and A. ervi mummies = ▼.

These results support previous findings which show that the presence of foraging arthropods increases the abundance of entomopathogenic fungi (Fuentes-Contreras *et al.*, 1998; Furlong & Pell, 1996; Roy & Pell, 2000). Although the presence of foraging *C. septempunctata* and *A. ervi* significantly increased the abundance of *P. neoaphidis*-sporulating cadavers (p<0.001) (Figs 1 & 2), there were no significant interactions between *P. neoaphidis* and either *C. septempunctata* or *A. ervi* that further reduced the aphid population (p>0.05). Therefore the increased transmission of *P. neoaphidis* in the presence of either *C. septempunctata* or *A. ervi* cannot be regarded as being a synergistic interaction. The increased number of sporulating cadavers recovered in the presence of *C. septempunctata* and *A. ervi* may have been as a result of increased transmission or vectoring of conidia. Unlike *C. septempunctata*, which is known to vector *P. neoaphidis* between aphid colonies, there is no evidence of parasitoids acting as vectors of fungal conidia (Roy & Pell, 2000). It is therefore likely that the increased number of sporulating cadavers recovered in the presence of *A. ervi* is a result of increased transmission. However, the presence of *P. neoaphidis* significantly reduced the number of *A. ervi* mummies recovered (p<0.05) (Fig. 2). This decrease in *A. ervi* mummies is
likely to be a result of *P. neoaphidis* out-competing *A. ervi* for aphid hosts and supports the findings of Powell *et al.* (1986) that an antagonistic interaction occurs between the parasitoid *Aphidius rhopalosiphi* (De Stefani Perez) and *P. neoaphidis*, where dominance is determined by the relative timing of attacks.

Although the abundance of *P. neoaphidis* was found to increase in the presence of either *A. ervi* or *C. septempunctata*, this was not found in the presence of both *A. ervi* and *C. septempunctata* after 8 and 16 days (p>0.05) (Fig. 4). This result may be due to multiple natural enemies reducing the aphid population to a threshold below which transmission could occur or, due to negative intraguild interactions occurring between *P. neoaphidis*, *A. ervi* and *C. septempunctata*. The results show that fewer sporulating cadavers and *A. ervi* mummies were recovered from the treatment containing *C. septempunctata* plus *P. neoaphidis* plus *A. ervi* compared to the number recovered from treatments containing *P. neoaphidis* and *A. ervi* only (Fig. 4). Although the decrease in the population size of *A. ervi* could be as a result of competition with *P. neoaphidis* and vice-versa, it is more likely that the decreased *A. ervi* and *P. neoaphidis* populations are as a result of asymmetric intraguild predation by *C. septempunctata*.

However, these experiments were done under abiotic conditions that were optimal for the fungus and under natural abiotic conditions, where both the temperature and humidity fluctuate with diurnal cycles, the competitive outcome may be different. In preliminary experiments using a polytunnel arena at Rothamsted (Day ≈ 34°C, 20% R.H.; Night ≈ 8°C, 90% RH) *P. neoaphidis* did not have a negative effect on the reproductive potential of *A. ervi*. Further studies under natural abiotic conditions and larger spatial scales are required.

**Acknowledgements**

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**References**


Molecular tools to study natural occurrence, ecology and phylogeny of Entomophthorales

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Abstract: Molecular tools based on Polymerase Chain Reaction (PCR) technology provide opportunities to study the ecology and natural occurrence of entomophthoralean fungi in greater detail. Here, we give a short review on studies in which PCR technology has been implemented to investigate host range, geographic origin, life history traits and taxonomy of Entomophthorales. Preliminary results from some of author’s current projects are also present.

Key words: DNA, PCR, RAPD, sequencing, specific primers

Introduction

Over the last decades many molecular methods have been developed and in the current review we aim to compile the studies in which PCR technologies have been implemented to investigate natural occurrence, ecology, phylogeny and taxonomy of Entomophthorales. Essentially two PCR-based approaches have been used: 1) multiple locus analysis (fingerprinting), which mostly relies on random primed PCR and 2) single locus analysis, which relies on PCR amplification of one specific target region. In the following section we briefly introduce the two different approaches and then go through the different studies where they have been applied.

Multiple locus analysis

The most common fingerprinting method used in connection with Entomophthorales is Randomly Amplified Polymorphic DNA (RAPD) (Table 1). RAPD is based on the use of short single primers of around 10 bp. The PCR reactions are run under low stringency conditions, e.g. low annealing temperatures and as a result random primed PCR produce amplicons of different length from regions randomly distributed across the genome (Fig. 1A).

Other fingerprinting methods such as Enterobacterial Repetitive Intergenic Consensus (ERIC), Interspersed Simple Sequence Repeats (ISSR), Universal Primed PCR (UP-PCR) and Amplified Fragment Length Polymorphism (AFLP) have also been used to study Entomophthorales. The first three methods resemble RAPD in many ways, but are generally more reproducible due to the use of longer primers. AFLP relays on a different principle. For AFLP the DNA initially is digested with restriction endonucleases and subsequently restriction fragments are selectively amplified by PCR.

The mentioned fingerprinting methods have several advantages a) no prior knowledge of target DNA sequence is required b) multiple loci are analysed simultaneously and c) the methods are rather cheap and quick. However, it has also several disadvantages a) availability of pure template DNA from a single specimen requires in vitro culturing and b) PCR products of equal size are not necessary homologous (size homoplasy).
**Single locus analysis**

The specific primed PCR approach is based on the use of two primers of approximately 20 bp lengths, which are specifically designed to hybridize to the target region. The same primer pair can be used to amplify and compare homologous DNA sequences of different species depending on the level of conservation of the primer annealing site among target species. For such comparisons direct sequencing of the PCR products or Restriction Fragments Length Polymorphism (RFLP) subsequent to the PCR have been used (Table 2).

Species specific primers can be designed using variable primer annealing sites for diagnostic purposes and allows detection of single species in DNA mixtures such as bulk soil DNA extracts or infected insects. A nested PCR approach in which a second PCR with primers hybridizing to the amplicon internally of the first primer set can be applied to further increase specificity (Fig. 1B). The advantages of specific primed PCR are a) isolation and cultivation of the Entomophthorales is not required, b) the target region is known and c) it can be used to study both closely and distantly related organisms. But often it requires some prior information in order to select the target region and the primers.

![Figure 1: A) RAPD patterns of *E. muscae* in vitro cultures isolated from nine different fly specimens. The *M. domestica* and the *D. radicum* were collected at different locations in DK, whereas the *S. stercoraria* originates from the same fly population. Each host fly species harboured a single specific *E. muscae* genotype evidenced by different fingerprints between hosts and identical within hosts. B) Nested PCR amplification of ITS from bulk soil DNA extracts using the *P. neoaphidis* specific primer ITS1_E432 (Thymon et al., 2004) together with ITS4 (White et al., 1990) followed by a nested amplification with primer FARISArev (Sequerra et al., 1997) together with the entomophthoralean specific primer Nu-5.8S-5’ (Jensen & Eilenberg, 2001).](image)

**Exploitation of molecular methods to study Entomophthorales**

In the mid 1990’ties the first studies on Entomophthorales were published where researchers took advantages of molecular methods. Since then numerous studies addressing aspects like natural occurrence, ecology, phylogeny and taxonomy of Entomophthorales applying molecular methods have tremendously increased our knowledge on this group of fungi.

The fingerprinting approaches are particularly useful to determine relationships below the level of species and are often capable to differentiating between isolates. Within Entomophthorales studies have been conducted on five different genera; *Entomophthora*, *Entomophaga*, *Pandora*, *Neozygites* and *Zoophthora* (Table 1).

RAPD and UP-PCR were performed on a collection of *Entomophthora muscae* isolates originating from different host fly species (Jensen et al., 2001) in order to evaluate the intraspecific variation. Investigations revealed that each host fly species harbour a single
specific *E. muscae* genotype. RAPD fingerprinting also allowed demonstration of high host specificity within members of the *Entomophaga grylli* species complex. Analysis of isolates belonging to three pathotypes resulted in pathotype specific fingerprints. (Bidochka *et al.* 1995). Conversely, no such genotype-host correlations were detected in corresponding studies of *Pandora neoaphidis* with either RAPD, ISSR or ERIC (Nielsen *et al.*, 2001; Rohel *et al.* 1997; Tymon & Pell, 2005). *Neozygites parvispora* similarly to *P. neoaphidis* displayed high intraspecific variation, and several RAPD profiles were detected among isolates obtained from different *Thrips tabaci* collected simultaneously in a single field. This suggests that several *N. parvispora* genotypes are involved in an epizooty (Grundschober, 2000).

Table 1: Fingerprinting by multi locus PCR analysis in Entomophthorales.

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<tr>
<th>Method</th>
<th>Taxa</th>
<th>Objectives</th>
<th>References</th>
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<tr>
<td>RAPD, UP-PCR</td>
<td><em>Entomophthora</em></td>
<td>Intraspecific variation and host specificity</td>
<td>Jensen <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>RAPD, AFLP</td>
<td><em>Entomophaga</em></td>
<td>Pathotypes, intraspecific variation and origin</td>
<td>Bidochka <em>et al.</em>, 1995</td>
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<td>Nielsen &amp; Hajek, 2005</td>
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<tr>
<td>RAPD, ISSR, ERIC</td>
<td><em>Pandora</em></td>
<td>Genetic variation</td>
<td>Nielsen <em>et al.</em>, 2001</td>
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<td>Rohel <em>et al.</em>, 1997</td>
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<td>Tymon &amp; Pell, 2005</td>
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<td>RAPD</td>
<td><em>Neozygites</em></td>
<td>Genetic variation on a local scale</td>
<td>Grundschuber, 2000</td>
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<td>RAPD</td>
<td><em>Zoophthora</em></td>
<td>Monitor released strains and trace isolate origin</td>
<td>Hajek <em>et al.</em>, 1996</td>
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<td>Hodge <em>et al.</em>, 1995</td>
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RAPD and the more robust fingerprinting method AFLP have been used to unravel the origin of the alfalfa weevil pathogen *Zoophthora phytonomi* and the gypsy moth pathogen *Entomophaga maimaiga*, respectively (Hajek *et al.*, 1996; Nielsen & Hajek, 2005). The host species were, in both cases, introduced to North America. In these studies North American isolates of the pathogens were compared to isolates from the proposed ancestral source populations Results suggested that both North American pathogen populations were introduced from their suspected source regions, *Z. phytonomi* from Eurasia due to its affinity with Israeli isolates, whereas for *E. maimaiga* evidence for a Japanese origin was found.

In order to evaluate the establishment of Serbian isolates of *Z. radicans* for control of the potato leafhopper in New York, RAPD analysis were performed on the released isolates and a few isolates recovered from leafhoppers collected in the test plot. Similarities of the RAPD profiles confirmed the successful release and establishment of the Serbian isolates in the test plot (Hodge *et al.*, 1995).

The nuclear ribosomal gene cluster, in particular the small subunit (SSU rDNA), the large subunit (LSU rDNA) and the internal transcribed sequence (ITS) have been the primary targets in specific PCR approaches of the Entomophthorales (Table 2). Parts of the rRNA gene cluster are highly conserved (e.g. the SSU rDNA) and useful for evolutionary divergence studies; where as other regions are more variable (e.g. ITS) and suitable to study closely related taxa.

Phylogenetic analysis of Entomophthorales using the SSU rDNA have been performed to examine the relationship within the order and to elucidate its evolutionary position in the fungal history (Jensen *et al.*, 1998; Nagahama *et al.*, 1995). Analyses support the monophyly of Entomophthorales and confirm the exclusion of *Basidiobolus*, and did further more support the taxonomic values of nuclear characteristics for the family Entomophthoraceae.
Since the mentioned 1998 publication dealing with phylogeny of Entomophthorales, the number of entomophthoralean SSU rDNA sequences in Genbank has increased. This encourages us to perform new phylogenetic analyses. In figure 3 we present a part of a neighbour joining tree in which 44 Entomophthorales, 27 other fungi including representatives of all the five fungal phyla and a single protozoan were included. Due to the wide range of species only 867 alignable characters were included and distance estimation was based on Juke and Cantor's equation. Several analyses were performed on subsamples of this sequence collection, e.g. an analysis only including the Entomophthorales or the Erynioideae family. In each of these analyses more alignable characters were included. All the analyses supported the two new subfamilies; Entomophthoroideae and Erynioideae, recently suggested by Keller & Petrini (2005). However, analyses did not support the current taxonomy within Erynioideae and the subgenus Conidiobolus seems to be polyphyletic.

The high sequence divergence of Neozygites compared to all the other fungi analysed indicate an accelerated evolutionary rate and due to the long branch attraction phenomenon, (groupings of unrelated clusters with long branches). The true position of Neozygites within the fungi or the eukaryotes still needs to be clarified, possibly by the aid of other gene sequences. The SSU rDNA sequences, however, contain information which allows for species delineation within Neozygites and was used to verify the new species *N. tanajoae* (Delalibera *et al.*, 2004).

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<th>Method</th>
<th>Taxa</th>
<th>Objectives</th>
<th>References</th>
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<tr>
<td>Sequencing of SSU</td>
<td>Entomophthorales</td>
<td>Phylogeny</td>
<td>Jensen <em>et al.</em>, 1998</td>
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<td>Nagahama <em>et al.</em>, 1995</td>
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<tr>
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<td><em>Entomophthora planchoniana</em></td>
<td>Identification</td>
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<td>Sequencing of SSU</td>
<td><em>Neozygites tanajoae</em></td>
<td>Species delineation</td>
<td>Delalibera <em>et al.</em>, 2004</td>
</tr>
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<td><em>Pandora neoaphidis</em> and close relatives</td>
<td>Genetic variation</td>
<td>Tymon <em>et al.</em>, 2005</td>
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<td>RFLP of ITS II and LSU</td>
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<td>Life history and taxonomy</td>
<td>Jensen &amp; Eilenberg, 2001</td>
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<td>Thomsen &amp; Jensen, 2002</td>
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<tr>
<td>RFLP of ITS II and LSU</td>
<td><em>Eryniopsis</em></td>
<td>Taxonomy</td>
<td>Hajek <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>RFLP of ITS</td>
<td><em>Pandora</em></td>
<td>Taxonomy</td>
<td>Francis <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Diagnostic primers in ITS</td>
<td><em>Pandora neoaphidis</em></td>
<td>Epidemiology and survival in the soil</td>
<td>Tymon <em>et al.</em>, 2004</td>
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<td>Fournier (<em>unpubl.</em>)</td>
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It is impossible to determine most species of Entomophthorales based on resting spore morphology and since only a few species produce conidia and resting spores in the same cadaver, it thus can be difficult to link resting spores to their conidial counterpart. PCR products from resting spores can, however, be compared to PCR products of known isolates. A nested PCR approached was used by Thomsen & Jensen (2002), to tackle problems with limited amount or impure DNA from resting spores. In an attempt to elucidate the life histories of several *Entomophthora* species, resting spores from different fly species were determined by their RFLP profiles. Results demonstrated that members of the *E. muscae* complex are able to complete their life cycle in one host and that each host-pathogen system is independent.

Recently species specific primers have been developed for *P. neoaphidis* and *P. kondoiensis* and used to screen field collected aphids for fungal infection (Tymon *et al.*, 2004).
We have used the *P. neoaphidis* specific primers and a nested PCR approach (see Fig 1 for further details) to detect *P. neoaphidis* in soil. In this experiment four top soil samples were obtained cross wise around a nettle plant infested with *P. neoaphidis* infected aphids in fall and spring. *P. neoaphidis* was detected in all eight soil samples, while in deep soil samples obtained below the nettle plant *P. neoaphidis* was not detected. It is the first time the presence of Entomophthorales in soil has been detected by a cultivation independent manner.

**Figure 3.** Part of a neighbour joining tree constructed using Entomophthoralean and other fungal SSU rDNA sequences deposited in Genbank (see text for details).

**Conclusion**

Molecular methods have helped to improve our knowledge about the natural occurrence of Entomophthorales in many aspect and they will remain an important tool also in the future.
New methods like cultivation independent detection of species by specific PCR will offer great opportunities to efficiently study Entomophthorales that are difficult to isolate, to cultivate or to track in nature.

Acknowledgements

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Development of a new cultivation independent tool for monitoring the *Beauveria brongniartii* biocontrol agent in the field

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**Abstract:** Monitoring of applied fungal biocontrol agents traditionally depends on re-isolation and cultivation of the organism prior to identification. We have developed a cultivation independent PCR strategy for *Beauveria brongniartii*, the fungus used in biological control of *Melolontha melolontha*. For this the microsatellite tool used in the cultivation dependent approach has been adapted for direct application on bulk soil DNA samples.

Five soil samples obtained from a plot treated with the *B. brongniartii* BCA and five soil samples obtained from an untreated control plot have been investigated with the traditional cultivation dependent as well as with the new cultivation independent strategy. Both approaches confirmed the successful establishment of the applied BCA strain in the treated plot. The cultivation independent approach was shown to be reliable and it correlated with results obtained with the cultivation dependent approach. Yet, due to the smaller soil sample size used in the cultivation independent strategy the sensitivity is reduced as compared to the cultivation dependent strategy. The cultivation independent strategy provides a very efficient new approach, which allows to circumvent the time consuming cultivation step of the traditional monitoring strategy. The same approach can be used to analyse bacterial and fungal community structures, which eventually will allow to study potential effects of BCA application on other soil microbial populations in the same sample.

**Key words:** microsatellite, genotype, monitoring, bulk soil DNA, biocontrol

**Introduction**

Efficient monitoring tools for studying fate and possible ecological effects of organisms released into natural or agricultural ecosystems are crucial for proper risk assessment. Traditionally these tools depend on cultivation of the organism and subsequent morphological, biochemical or genetic identification (Enkerli *et al.*, 2004). Our goal is to develop and establish new molecular genetic tools that are based on a cultivation independent strategy.

The fungus *Beauveria brongniartii* (Sacc.) Petch (mitosporic fungi) is a highly specific pathogen of the May beetle (*Melolontha melolontha*). It is successfully applied as a biological control agent (BCA) to control the soil dwelling larvae of *M. melolontha*, a serious pest in grasslands and orchards in Switzerland. A *B. brongniartii* based product for biocontrol of *M. melolontha* has been registered in Switzerland and is commercially available since 1991. The highly specific and well characterized *B. brongniartii* BCA provides an ideal model system to develop and establish new monitoring tools.

We have developed microsatellite markers that allow for strain-specific identification of *B. brongniartii* isolates (Enkerli *et al.*, 2001) and we have successfully used these markers to monitor both, persistence of applied *B. brongniartii* BCA strains as well as genetic diversity of indigenous *B. brongniartii* populations in a cultivation dependent assay (Enkerli *et al.*, 2004). Here we report on our efforts to circumvent the time consuming cultivation step. We have adapted the microsatellite tool to a cultivation independent PCR strategy for identification of applied strains in bulk soil DNA extracts.
Materials and methods

In fall 2004 five soil samples were collected across each of two adjacent 20 x 20 m grassland plots in Lungeren, Central Switzerland. One of the plots was treated with Beauveria brongniartii fungus colonized barley kernels (40 kg/ha, strain BIPESCO 2) in spring 2002 and the other plot served as untreated control. *B. brongniartii* density in soil samples was assessed by applying the selective medium (SM) method (Kessler et al., 2003) and genotypes of selected subcultured single colony isolates determined by use of six microsatellite markers (Enkerli et al., 2004).

Bulk soil DNA was isolated from 600 mg fresh soil of each of the ten samples according to Bürgmann et al. (2001). DNA extracts were purified using Extract-II DNA purification columns (Machery & Nagel, GmbH&Co KG, Düren, Germany). DNA content was quantified fluorometrically (Sandaa et al., 1998) and concentrations expressed as µg DNA g⁻¹ soil [dry wt].

For cultivation independent identification of *B. brongniartii* in the soil samples, the six microsatellite loci were amplified directly from bulk soil DNA. Identification of a particular genotype was only accepted if alleles for all six loci were detected. The number of PCR amplification cycles needed to reach a certain product concentration depends on target DNA (*B. brongniartii*) concentration and locus specific amplification efficiency. Therefore, the number of cycles applied for individual reactions (10 samples, 6 microsatellite loci each) was adjusted according to target DNA concentrations and amplification efficiencies, they ranged from 28 to 40. PCR contained 50 ng bulk soil DNA, 0.2 µM each primer, in a volume of 25 µL of 1x iQ SYBER Green Supermix (BioRad Laboratories, Hercules, CA, USA). Amplification cycles consisted of an initial denaturation at 95°C for 3 min followed by a reaction specific number of cycles of 94°C for 40 s, 58°C for 40 s, 72°C for 30 s and a final extension of 5 min at 72°C. PCR products were purified (Montage PCR Reaction Cleanup Kit, Millipore, Bedford, MA, USA) and analyzed for microsatellite allele sizes using an ABI3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and the software genotyper V3.7 (Applied Biosystems).

Results

Five soil samples from a *B. brongniartii* BCA strain (BIPESCO 2) treated plot and five soil samples from an adjacent control plot were obtained and *B. brongniartii* densities determined for each sample (Table 1). Densities varied between 0 and 724’000 CFU/g soil. In the five samples obtained from the plot treated with the BCA strain, *B. brongniartii* was detected in all samples and densities ranged from 573 to 229’266 CFU/g soil. In the control samples *B. brongniartii* was detected in three samples in which densities ranged from 261 to 724’000. Successful introduction of the BCA strain was verified by using the cultivation dependent protocol, i.e. re-isolation, cultivation, DNA extraction and subsequent determination of the genotype of individual isolates based on analysis of six microsatellite loci. Twenty-four isolates were collected from SM plates of six soil samples (Table 1) and their genotypes were determined. Three genotypes BIPESCO 2, A, and B were identified among the 24 isolates. Genotypes BIPESCO 2 and A have 4 and genotype B has 5 unique alleles. Isolates originating from the same soil sample always displayed the same genotype. Soil samples (1, 4, 5) of the treated plot contained either isolates with genotype BIPESCO 2 or A, whereas soil samples (3, 4, 5) of the control plot contained isolates with genotype B.

The ten soil samples analyzed above according to the cultivation dependent strategy were used to establish the new cultivation independent PCR strategy. Bulk soil DNA was extracted
from each soil sample and the microsatellite tool used for direct detection of the genotypes in soil DNA samples (Table 1). In the five soil samples obtained from the treated plot the BCA strain was detected in samples 1, 2, 3 and 5 whereas in sample 4 genotype A was identified. In the control samples genotype B was detected in sample 5.

Table 1. Genotypes detected with the cultivation dependent and -independent techniques

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>Cultivation dependent</th>
<th>Cultivation independent</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CFU / g soil</td>
<td>No. of collected isolates</td>
</tr>
<tr>
<td>BCA applied</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>573</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>14'383</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>118'463</td>
<td>0</td>
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<tr>
<td>4</td>
<td>120'904</td>
<td>8</td>
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<tr>
<td>5</td>
<td>229'266</td>
<td>4</td>
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<tr>
<td>Control</td>
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<td>1</td>
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<tr>
<td>3</td>
<td>261</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>816</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>724'441</td>
<td>8</td>
</tr>
</tbody>
</table>

n.a., not analyzed
n.d., not detectable

Discussion

Cultivation dependent as well as cultivation independent analyses of the ten soil samples demonstrated the successful establishment of the BCA strain in the treated plot. In four of the five soil samples of the treated plot *B. brongniartii* concentrations (10^3-10^4 CFU/g soil) considered to result in good white grub control were detected (Keller *et al*., 2002). In addition, genotype analysis demonstrated the importance to not only rely on fungal density analyses but also to include verification of genotypes in order to not assign fungal density to a wrong strain (BCA or indigenous strain). For example, the *B. brongniartii* density detected in soil sample 4 of the treated plot originated from an indigenous isolate with genotype A and not from the applied BCA strain or the *B. brongniartii* densities in control samples 3, 4, and 5 originate from an indigenous isolate with genotype B. Moreover, results clearly demonstrated the inhomogeneous distribution of the BCA strain even after homogenous application of the BCA product across the plot by use of a seed tilling machine.

The main goal of the present study was to establish a cultivation independent monitoring strategy for *B. brongniartii* and to compare it to the conventional cultivation dependent strategy. Results showed that for all samples in which *B. brongniartii* was identified both strategies revealed the same genotypes (treated 1, 4, 5; control 5). The fact that in control
samples 3 and 4 genotype B was not detected with the cultivation independent strategy indicated the higher detection limit of this approach. In the cultivation independent strategy about 200 times less soil was subjected to analysis as compared to the cultivation based strategy, which may explain the observed difference in sensitivity. Also, at the current stage the cultivation independent strategy is limited to a qualitative approach. Additional efforts will have to be undertaken in order to assess feasibility of a quantitative approach. Furthermore, analyses of the various soil samples have revealed that the cultivation independent approach allows for detection of applied as well as indigenous isolates. Therefore, the cultivation independent approach is also suitable for assessing genetic diversity of B. brongniartii in soil.

The cultivation independent strategy has substantial advantages over the cultivation based strategy: 1) soil samples can be much smaller, 2) time necessary to perform an analysis is greatly reduced (1 week versus to 2-3 month), 3) analysis of bulk soil DNA allows to detect applied BCA strains as well as B. brongniartii diversity in the same assay, and 4) the same bulk soil DNA sample can be used for B. brongniartii detection as well as for analysis of fungal and bacterial soil community structures (Widmer et al., 2001). Such analyses eventually will allow to investigate potential effects of B. brongniartii BCA applications on other soil microbial populations.

Acknowledgments

We thank Ch. Schweizer for setting up the field trial and assistance in collection of soil samples and isolation of B. brongniartii. This work has been supported by the Swiss Agency for the Environment, Forests and Landscape (SAEFL).

References


Fungal BCAs in the European Union: *Beauveria brongniartii* (Sacc.)

Petch as the model organism to address key questions

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Abstract: Significant progress has been made in the development of fungal biocontrol agents (BCAs) for the control of insect pests. Nevertheless, no mycoinsecticide has yet been approved under European Union registration procedures. Critical issues in the registration of fungal BCAs are (i) the efficacy of the product, (ii) potential effects on non-target organisms and biodiversity, and (iii) the toxicity as well as risks posed by compounds synthesized by the microorganisms. *B. brongniartii* was used as a model organism to assess these issues and whether the ERBIC Risk Index would be adequate for the evaluation of fungal BCAs. It was demonstrated by accomplishing slight adaptations, the Index can be effectively used for the assessment fungal BCAs. The application of the ERBIC Risk Index to *B. brongniartii* resulted in a very low Risk Index of 15 on a scale of 125.

Key words: *Beauveria brongniartii*, efficacy, non-target organisms, metabolites, risk assessment, registration

Introduction

Biological control, or biocontrol, is defined as “the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be” (Eilenberg et al., 2001).

Concerns about negative side effects of chemical pesticides on human health and the environment, and the resulting bans of substances such as methyl bromide, organochlorine or other compounds with unacceptable side effects have increased the demand for environmentally friendly alternatives.

Fungi are considered to have high potential regarding the development of biocontrol agents (BCAs). There is a large number of different fungi available that can potentially be used for the control of a wide variety of pests including weeds, nematodes, fungal pests, and insects.

From an ecological perspective the advantages of fungal BCAs include their capability for a longterm pest regulation, the relatively narrow host range of many fungal strains and their potential to encourage organic farming through the availability of alternatives at a time where the political trend goes toward more environmentally friendly pest control strategies (Goettel et al., 2001). From a commercial perspective fungal BCAs have some disadvantages that may have been a factor preventing stronger presence of these products on the market. Currently, biocontrol products account for only 2 % of the global pesticide market, with fungi contributing a far lower fraction than bacterial products based on *Bacillus thuringiensis* Berliner (Bidochka, 2001).

While environmentally desirable, the narrow host ranges of many fungal BCAs both increase the development and registration costs and decrease the revenues generated from the sale of a single product. False expectations in fungal BCAs caused by an inadequate...
understanding of their ecology often result in the dismissal of biological products as inefficient, especially in comparison to chemical pesticides (Bidochka, 2001).

Despite these limitations there is a strong potential for a growing market for fungal BCAs, especially now when the European Union is determined to phase out noxious chemical substances, creating a demand for alternatives. Key issues to be dealt with in the risk- and efficacy assessment of fungal BCAs are addressed in this work using *B. brongniartii* as a model organism taking into account the legal background of the European Union (Council Directive 91/414/EEC).

**Material and methods**

**Efficacy study**

An efficacy study of Melocont®-Pilzgerste for the control of *Melolontha melolontha* L. was conducted in potato fields located in Tyrol, Austria. Treatments comprised the application of three different doses of Melocont® as well as sterile barley kernels: (i) 25 kg Melocont® ha−1 (half of recommended amount), (ii) 100 kg Melocont® ha−1 (double of recommended amount), (iii) 33 kg sterile barley kernels ha−1 (control; equals 50 kg Melocont®) and (iv) 50 kg Melocont® ha−1 (recommended amount). Melocont®-Pilzgerste was applied with a sowing machine and worked into the soil to a depth of 20 cm. After the application potatoes were planted. Quantification of *B. brongniartii* in soil was done following Strasser et al. (1996), larval density of *M. melolontha* was determined by digging 50 cm × 50 cm sample holes to a depth of at least 50 cm and manually screening the removed soil for larvae. Harvest was assessed by recovering potato tubers from each whole dug and classifying them as “healthy tubers” and “tubers damaged by white grub feeding” (for more details see Laengle et al., 2005).

**Assessment of biodiversity - Fungi**

Soil samples were mixed, air-dried, and sieved (2 mm). Ten gram sub-samples were added to 40 mL sterile 0.1% (w/v) Tween 80 solution. Potato-Dextrose-Agar plates supplemented with Streptomycin (100 mg L−1), Tetracycline (50 mg L−1) and Dichloran (2 mg L−1 as 0.2% w/v ethanol solution) were inoculated with 50 µL of soil suspensions and dilutions, thereof. Plates were incubated at 25°C and 60% RH. After one week the colonies were counted and assigned to the taxa *Acremonium* spp., *Mortierella* spp., *Alternaria* spp., *Paecilomyces* spp., *Aspergillus* spp., *Penicillium* spp., *Beauveria* spp., *Trichoderma* spp., *Cladosporium* spp., *Ulocladium* spp., *Gliocladium* spp., *Metarhizium* spp., Zygomycetes, and others using light microscopy.

**Assessment of biodiversity - Bacteria**

Pooled soil samples from each treatment were frozen at −20°C until processing and were allowed to equilibrate at 4°C for 24 h before the extraction procedure was started. Bacterial cell extraction from soil was performed following Insam & Goberna (2004). Extracts were diluted 1:100 in sterile NaCl (0.9% w/v). Ecolog® microtitre plates were inoculated with 130 µL per well and incubated at 20°C for one week. Three replicate plates per sample, equivalent to nine replicates for each of five samples, were inoculated. Plate readings at a wavelength of 592 nm were conducted every 12 h starting at the inoculation time.

**Toxicity assessment of oosporein – Seed germination and emerging**

Pure oosporein (purity > 93%) was dissolved in NaHCO3 buffer (0.1% w/v, pH 7) and diluted (2 mM, 1 mM, 500 µM, 250 µM, 125 µM, 62.5 µM).

According to the ISTA Rules for Seed Testing (International Seed Testing Association, 1996) seeds were placed in a pleated, accordion-like paper strip, with 50 pleats. Two seeds were placed in each pleat. The paper strips were then placed in sealable polypropylene
containers, watered with 40 mL of testing solution and incubated at 20°C. The number of germinated seeds was counted after 3 days. Depending on the species the final assessment of seedlings was done after 7-12 days of incubation. All seedlings were classified according to ISTA. The most sensitive species was furthermore assessed by measuring root elongation and seedling biomass.

Soil from field plots was dried and placed in sealable polypropylene boxes (350 g per box). According to the ISTA Rules 50 seeds were placed in each box, covered with soil, watered with 80 mL of testing solution and incubated at 20 °C for 8 days. At the end of the incubation period the number of seedlings emerging from the soil were counted.

Data processing
Agricultural Research Manager 6 and Excel 97-2000 were used for data management and processing. Statistical analyses and graphing were performed with Statistica 6.0, SPSS 11.5, Sigmastat 5.0, SigmaPlot 8.0 as well as GraphPad Prism 4.0. Multivariate datasets (fungal diversity data, community level physiological profiles) were subjected to Principal Component Analysis and Discriminant Function Analysis to detect differences on the community level and to identify which variables (taxa, carbons sources) contribute to a potential separation of cases (Quinn & Keough, 2002). All univariate data were tested for normality (Lillifors correction, SigmaStat 3.0). If normality tests were passed for all datasets of a group, means with standard deviations were calculated and statistical comparisons were conducted with parametric methods (t-test, ANOVA followed by Bonferroni post-hoc test). If criteria for normality were not met for at least one dataset of a group then data were analysed with non-parametric methods (medians with upper/lower quartiles, comparisons of 2 medians with Mann-Whitney U test, Kruskal Wallis). One Way Anova on Ranks followed by Dunn's pairwise comparison method for three or more medians. Correlations between the applied amount of Melocont®-Pilzgerste and the observed Beauveria densities were analysed with a Spearman rank order test. All tests were done with Sigmastat 3.0 for Windows.

Results and discussion

Efficacy study
Results from these trials documented that Melocont®-Pilzgerste is an effective agent for a sustainable control of M. melolontha if used as part of an intelligent IPM strategy. In this context, the importance of an appropriate timing of mechanical treatments (planting of potatoes and application of Melocont®-Pilzgerste) was demonstrated. Planting and application of the BCA at the time when Melolontha larvae were most frequent in the upper soil increased the percentage of healthy potatoes from 20% to above 90%. By correlating mortality data for Melolontha and Beauveria densities in the soil, a median lethal concentration (LC50-value) of $1.1 \times 10^5$ cfu Beauveria per gram soil dry weight were calculated. Furthermore, the dose of Melocont®-Pilzgerste calculated to kill 50% Melolontha larvae (LD50) was 56 kg ha$^{-1}$ (Fig. 1). This corresponds well to the recommended application dose of 50 kg ha$^{-1}$ per year. Results underline the postulated efficacy of B. brongniartii for the control of the European cockchafer and demonstrate the benefits resulting from the use of the product as required for the registration in the European Union.

Assessment of biodiversity
Changes in the abundance of 15 common soil fungi were estimated. These “fingerprints” of soil microbiota revealed no negative changes of fungal and bacterial diversity that could be attributed to the introduction of Beauveria brongniartii into the system (Figs 2 & 3). No signs of a competitive displacement of indigenous microorganisms were found despite high BCA densities of up to $10^7$ cfu per gram soil dry weight.
Figure 1. Dose response relationship between *Melolontha* mortality and *Beauveria* density in the soil. Solid squares show empirical means, solid line shows sigmoid regression with (dotted lines) 95% confidence limits (see also Laengle *et al.*, 2005).

Figure 2. Two dimensional scatter plot of case factor coordinates calculated from fungal diversity data by Principal Component Analysis. Each data point results from analysis of 24 different variables, representing the abundance of studied fungal genera in the soil. Percentages indicate the amount of variance explained by a PCA Factor.

Data from community physiological profiling revealed a tendency towards distinct groups. This trend was clarified by discriminant function analysis, which clearly separated groups representing different soil treatments (Fig. 3). No specific carbon sources could be identified to have caused this separation individually.

A further statistical analysis of these groups showed a significant correlation ($R^2 = 0.835$, $p < 0.001$) between the distribution of groups and could be assigned to the amount of barley kernels that had been applied to the respective trial plots (Fig. 3).
Figure 3. Canonical discriminant function of bacterial carbon utilisation data obtained from soil samples drawn from trial fields after two consecutive years of treatments. Ellipses represent 95% confidence limits.

**Toxicity assessment of oosporein – Seed germination and emerging**

Oosporein did not inhibit seed germination of any of the plants tested to date, but was mildly phytotoxic during early seedling growth of sugar beet (EC$_{50}$ = 2.6 mM), onion (EC$_{50}$ = 1.3 mM) and wheat seedlings (EC$_{50}$ = 11.6 mM). No toxicity was detected for seedlings of lettuce, tomato and carrot, not even at oosporein concentrations close to the solubility limit. To cause a theoretical inhibition of 99% for onion and wheat a solution of more than 25% oosporein (w/v) would be needed.

The risk of oosporein causing toxic effects under natural conditions was found to be negligible because the metabolite potentially released by the BCA up to a concentration of at least 24 g per square meter will be inactivated by irreversibly binding to the soil. This equals 3290 kg Melocont®-Pilzgerste or 106000 mycosed Melolontha cadavers per square meter. Therefore, oosporein cannot be considered “stable outside the micro-organism” as defined in Council Directive 91/414/EEC. This means that oosporein is not a “relevant metabolite” with respect to the registration in the European Union, because it does not fulfil the conditions that trigger further data requirements as stated in Council Directive 91/414/EEC.

*Beauveria brongniartii* was used as a model organism to test the applicability of the ERBIC Risk Index (Van Lenteren *et al.*, 2003) and identify where adjustments are necessary in this regard. Suggestions for adaptation have been made accordingly. It was demonstrated that the ERBIC Risk Index can be effectively used for the assessment of inoculation fungal BCAs as well as fungal BCAs in general. The application of the ERBIC Risk Index to *B. brongniartii* resulted in a Risk Index of 15 on a scale of 125 (Laengle, 2005). This confirms other findings by our group which postulate the safety of *B. brongniartii* and underline industry demands to push the registration of *B. brongniartii* as the only safe and effective agent available for the control of *Melolontha* species.

**Acknowledgements**

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References


Preliminary survey for insect pathogenic fungi in Greenland

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Abstract: Data from a preliminary survey on natural occurrence of entomopathogenic fungi in Greenland are presented. The presence of Entomophthorales on adult flies is documented, as well as the presence of Ascomycetes (asexual stages) in soil samples.

Key words: Entomophthora, Strongwellsea, Beauveria, Paecilomyces, insects, natural occurrence

Introduction

In temperate and tropical regions of the world, insect pathogenic fungi play an important role in natural regulation of insect populations. Little is known about natural occurrence of these fungi in the Arctic environments. The fauna in Greenland includes a range of insect species from several orders, for example Diptera, Lepidoptera, and Coleoptera. Some insects, like dipterans, may occur in high numbers during the short Arctic summer. A survey on the occurrence of insect pathogenic fungi in different parts of Greenland was initiated.

Materials and methods

Two sites were studied for the possible presence of entomopathogenic fungi in Greenland: the Zackenberg Field Station (NE Greenland) and Disco (situated in NW Greenland) (Fig. 1). At the Zackenberg Field Station, we sampled (sweep-net and water traps) and diagnosed adult dipterans, especially Muscidae. We also looked for the presence of insect pathogenic fungi in soil samples from different plant communities in Zackenberg and Disco. Soils samples of 1-2 dl were taken by a spoon, and small stones, etc. were removed. Galleria or Tenebrio larvae were introduced to the soil samples as baits and fungal infection diagnosed after one and two weeks.

Results

Infection in adult flies

In adult Muscoid flies, Entomophthora muscae s.l. and a probably new species of Strongwellsea were found at Zackenberg. Indeed, in the latter case, morphological data of primary conidia from infected Spilogona sp. suggested that the fungal species is undescribed.

Resting spores of these fungal pathogens from flies have not yet been found in the material from Greenland. It is, however, fascinating that a fungus from the genus Strongwellsea occurs at 74 °N. Fungi from this genus seem to be intimately linked to the host species and must be able to survive the long Arctic winter.
Soil samples
At Zackenberg, we found *Paecilomyces fumosoroseus* in several soil samples from different plant communities, while *P. farinosus* was found in one soil sample only. In the soil samples from the Disko area, we founded *Beauveria bassiana, P. farinosus* and possibly also *Tolypocladium* sp. All the documented species are also commonly found in soil samples from temperate areas, for example in Denmark, and species from these genera are known to infect a range of insect hosts. Due to the sampling method (baiting laboratory insects), we are so far not able to document the natural host species of these fungi in Greenland.

Conclusions and perspectives
The following conclusions and perspectives could be drawn from this preliminary survey in Greenland: 1) insect pathogenic fungi occur naturally in Greenland, and have so far been found in adult dipterans and in soil samples, 2) additional characterization (morphological, pathobiological, genetical) is needed to confirm the presence of novel species or isolates, which are specially adapted to the extreme Arctic conditions, and 3) additional sampling will be performed over the coming years to obtain a more complete picture of the significance of insect pathogenic fungi in Greenland. In 2005 we plan to analyze soil samples from NW and E Greenland.

Acknowledgements
We thank Verner Michelsen for determining *Spilogona* species and Nette Levermann for assisting in the soil sampling.
Laboratory studies to assess the effects of the fungus
Lecanicillium lecanii on the aphid Schizaphis graminum

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Abstract: The pathogenic effects of Lecanicillium lecanii ITEM 3757 towards winged and wingless adult morphs of the species Schizaphis graminum were investigated under laboratory conditions. Fungus was applied to aphids in the form of ground dried cultures grown in solid state fermentation on rice kernels. This formulation affected aphid survival and reproduction, and it interacted differently with winged and wingless aphid morphs. Scanning Electron Microscope observations pointed out a higher amount of adhering formulation and faster rates of fungal germination and sporulation on the winged cuticle. Our results suggest that the strain might be a good candidate for a programme of biocontrol against S. graminum and other aphids species.

Key words: entomopathogenic fungi, biocontrol, insect, survival, fecundity

Introduction

The application of fungi and their metabolites as biopesticides (Butt et al., 2001) promises to reduce the need for chemical agents with high environmental impact. Among the fungi studied, Lecanicillium lecanii (Zimm.) Gams & Zare [syn. Verticillium lecanii (Zimm.) Viegas] is one of the most investigated pathogen of arthropods, including aphids (Sugimoto et al., 2003). It has long been applied especially in greenhouses for biocontrol of some species of aphids (Rabasse & van Steenis, 1999), and two strains have found applications as commercial products (Hall, 1984). In these terms, L. lecanii, characterised by a wide intraspecific variability of pathogenicity, host range and morphological and physiological traits, is a good candidate for the selection of strains to be used as mycoinsecticide in the field, since recent studies have shown that the morphology and physiology of a given strain are important tools for adaptation to the highly variable environmental conditions in the field (Cortez-Madrigal et al., 2003). The aims of this study were to analyze the effects of L. lecanii on survival and reproduction of the aphid Schizaphis graminum Rondani, one of the most important pests of cereal crops, and to try to identify the mechanisms that form the basis for the antagonistic effect of the fungus using scanning electron microscopy (SEM) and histological methods.

Material and methods

Test organisms
Specimens of S. graminum were reared in the laboratory on wheat plants for several generations in a thermostatic chamber at 20°C under 16/8 h day/night photoperiod to induce parthenogenesis. Trials were carried out with winged and wingless adult morphs. Winged offspring were obtained in the laboratory by crowding.
**Lecanicillium lecanii** ITEM 3757 was isolated from wingless aphids of the species *Brevicoryne brassicae*. The fungal culture was maintained in the ISPA fungal collection (ITEM; http://www.ispa.cnr.it/Collection/). The fungus was applied to aphids in the form of ground dried cultures grown in solid state fermentation on rice kernels. To obtain the formulation the fungus was cultured on 200 g of autoclaved rice kernels previously adjusted to above 45% moisture in a 500 mL Erlenmeyer flask and inoculated with 10 mL aqueous suspension containing approximately $10^7$ conidia/mL. The culture was incubated at 25°C under 12/12 h-day/night photoperiod for 4 weeks. The harvested material was dried in a forced draft oven at 35°C for 48 h, finely ground in a Molino Cyclone LMLF (PBI International) to a grain size 0.2 mm and stored at 4°C until use. The CFU/g of the fungal formulation was $2.18 \times 10^8$.

**Bioassays**

Trials were carried out with winged and wingless adult aphids that had moulted within 24 h. Aphids were inoculated with *L. lecanii* by dipping in a suspension containing 25 mg/mL fungal formulation in 0.01% Triton X-100 as wetting agent for 10 seconds. A second group of winged morphs was treated with a 25 µg/mL suspension. The first rate is a value falling between those used in previous studies on aphids; the second is the lowest rate known to be used on aphids. Suspensions had been shaken for 20 minutes. As controls, aphids of both morphs treated with 0.01% Triton X-100 and winged ones treated with a suspension containing only ground rice at 25 mg/mL in 0.01% Triton X-100 were used. Aphids were then transferred onto Petri dishes and reared on wheat leaves. Each treatment, consisting of 10 specimens per replicate, was replicated five times within a trial, and the entire experiment was conducted twice. Leaves were changed every two days. Dishes were maintained under humidity conditions at saturation in a thermostatic chamber at 20°C under 16/8 h-day/night photoperiod. Insects were checked every 24 h to record the number of dead specimens and cadavers were removed. Newborn aphids were counted and removed daily. For each replicate the net reproductive rate ($R_0$) over the days following treatment was calculated as $R_0 = \Sigma l_x m_x$, where $l_x$ is the probability of surviving and $m_x$ the average number of offspring produced by an individual on day $x-1$. In addition, a trial was carried out treating winged specimens whose wings had been removed, with 25 mg/mL suspension of fungal formulation. Specimens with wings removed and treated with 0.01% Triton X-100 were taken as controls.

**Scanning electron microscopy**

Winged and wingless morphs, moulted within 24 h, treated with a suspension containing 25 mg/mL *L. lecanii* formulation in 0.01% Triton X-100 were fixed 12, 24, 48 h after the start of the trial in 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4°C. Ten specimens were used for each group and two replicates were run. An equal number of control aphids was employed. Specimens were dehydrated through a graded ethanol series, critical point dried with CO$_2$ in a Critical Point Dryer 010 (Balzer), mounted on aluminium stubs and sputter-coated with gold to a thickness of 10 nm in a Coating Unit E 500 (Polaron). Observations were carried out under a Philips XL-30 SEM at the “Centro Interdipartimentale Grandi Strumenti” of the University of Modena and Reggio Emilia.

**Histological analysis**

Winged and wingless morphs, treated with a suspension containing 25 mg/mL *L. lecanii* formulation in 0.01% Triton X100, were fixed at 48, 72, 120 h in Duboscq and Brasil’s fluid, dehydrated through a graded ethanol series, embedded in Paraplast Plus (Sigma) and cut into 3 µm sections. Sections were treated with Gridley stain, Bauer variant, for fungi or stained with standard haematoxylin eosin. Slides were analysed with a Leitz Diaplan microscope under differential interference contrast (DIC).
Statistical analysis
Data on aphid survival, after logarithmic transformation, were analysed daily with one-way ANOVA and compared with SNK test; paired data were compared with the two-tailed t-test for independent samples. The R₀ data after logarithmic transformation were analysed with one-way ANOVA and compared with the SNK test or with the two-tailed t-test.

Results and discussion

The higher rate of L. lecanii formulation significantly influenced the survival of winged morphs and the lower rate was significant only from the fifth day up the end of the trial (P<0.01). Survival was not affected by ground rice suspensions (Fig. 1A). Statistical analysis revealed a significantly shorter survival of wingless treated at the higher rate than controls only from the fourth day after the start of the trial (P<0.05) (Fig. 1B). The L. lecanii formulation suspensions significantly affected the reproduction of winged and wingless morphs (P<0.01) (Fig 2, A and B).

![Figure 1](image.png)

Figure 1. 1A: Survival of winged morphs of S. graminum treated with 25 µg/mL (○) and 25 mg/mL (□) L. lecanii formulation suspensions, 25 mg/mL ground rice suspension (*) 0.01% Triton X100 (control) (■). 1B: Survival of wingless morphs treated with 25 mg/mL L. lecanii formulation suspension (▲) 0.01% Triton X-100 (Δ).

Preliminary findings on winged morphs whose wings had been cut, treated with the higher rate showed that survival of treated aphid was significantly lower than that of controls only four days after the start of the trial, and thereafter survival of treated specimens decreased dramatically, with survival data similar to that observed for specimens with wings.

The results suggest that wings might play an important role in the first steps of fungus-insect interactions. Moreover, a different interaction between fungus and winged and wingless morphs has been pointed out by SEM observations that revealed a different distribution of the fungal formulation over the integument of the two treated morphs, with a higher amount of adhering formulation and faster rates of fungal germination and sporulation on the cuticle of winged morphs (Fig. 3, A and B).

Thus the greater susceptibility to the formulation of the winged with respect to wingless morphs could be explained, in part by the presence of the wings but also by the differences in the cuticle, which seems to play a crucial role in initial spore adhesion, retention, germination and sporulation.
Figure 2. 2A: $R_0$ of winged morphs of *S. graminum* dipped in: 0.01% Triton X-100 (1); ground rice suspension (25 mg/mL) (2); *L. lecanii* formulation suspension (25 µg/mL) (3) (25 mg/mL) (4). Different letters indicate different values at $P<0.01$ (SNK test). 2B: $R_0$ of wingless morphs dipped in: 0.01% Triton X-100 (1); formulation suspension (25 mg/mL) (2). Different letters indicate different values at $P<0.01$ ($t$-test).

Figure 3. Winged or wingless morphs of *Schizaphis graminum* treated with *L. lecanii* formulation suspension (25 mg/mL). 3A: Winged morph at 24 h, with hyphae developed on cuticle and conidiophore hyphae producing conidia. 3B: Wingless morphs at 48 h, with hyphae on the cuticle. SEM photographs. Bars 5 µm.

Histological analysis revealed that hyphae invaded the host hemocoel of a limited number of winged and wingless specimens, suggesting that the lethal action of fungal formulation might be expressed independent from fungus penetration. In terms of route of penetration, the analysis revealed that the strain entered only through the spiracles and tracheae, which they crossed to enter the haemocoel (Fig. 4).

In conclusion, our results suggested that the strain *L. lecanii* ITEM 3757 is a good candidate for a programme of biocontrol against *S. graminum* and other aphid species. Additional studies are needed to confirm whether laboratory results reflect performance of this strain in the glasshouse or in the field.
Figure 4. *Schizaphis graminum* winged specimen 48 h after treatment with *L. lecanii* formulation suspension (25 mg/mL). Hyphae in the hemocoel (h) (arrows). Trachea with an hypha (arrow head). Light micrograph of longitudinal section stained with haematoxylin eosin stain. Bar 10 µm.

References


Visualizing the infection process of the entomopathogenic fungi

*Beauveria bassiana* and *Pandora neoaphidis* in aphids

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**Abstract:** *P. neoaphidis* and some *B. bassiana* isolates are pathogens of aphids. These fungi posses a great potential for microbial control of aphids, and knowledge about the infection processes can assist in the development towards biological control. The objective of this recently initiated study is to visualize and elucidate the infection process, especially penetration and colonization, of aphids by these fungi, using Confocal Laser Scanning Microscopy (CLSM). The aphid exoskeleton has autofluorescent properties and can easily be excited by ultraviolet or visible light in a CLSM. Experiments were done attempting to stain the fungal isolates with cfda (carboxy fluoresin di-acetate), Nile Red and Calcofluor white. In addition, *gfp*-tagging of *B. bassiana* using *Agrobacterium tumefaciens*-mediated transformation was conducted. *gfp* was inserted into two *B. bassiana* strains. Because the selective media were not sufficiently inhibiting the fungal growth, the selection for transformants was difficult and only a few transformants of each strain was found. We will continue our work with CLSM, staining and *gfp*-tagging to hopefully obtain 3D registration of the infection processes by entomopathogenic fungi in aphids in situ.

**Key words:** aphid, entomopathogenic fungi, microbial pest control, microscopy, CLSM, transformation, *gfp*.

**Introduction**

Aphids (Hemiptera: Aphididae) are serious pests of crops in many countries. Control of most aphid species has until now primarily been done using chemical insecticides. Given the extensive use of chemical pesticides has caused problems for the environment, there is a general wish and interest to minimize their use and to find environmentally more friendly alternatives such as microbial control for pest regulation.

The entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Pandora neoaphidis* (Remaudière & Hennebert) Humber are regarded as promising species in the development of practical aphid biological control agents (Butt et al., 2001; Pell et al., 2001). *B. bassiana* is a generalist fungus which has a wide host range. Commercial formulations of *B. bassiana* already exist and are used as microbial insecticide in some countries for the control of e.g. whitefly, aphids, thrips, psyllids, weevils, mealybugs, leafhoppers and mites, primarily in green houses (Laverlam Intl. Corp., 2005). *P. neoaphidis* is a specialist fungus, restricted to aphid species, and able to induce regularly spectacular epizootics, which make this pathogen a potential biological control agent. However, no *P. neoaphidis* products have yet been developed and commercialized.

Knowledge and visualization of the infection processes by entomopathogenic fungi could help in the selection of virulent strains for use in pest control programmes and could elucidate yet unknown details about the infection processes.
The material making up the arthropod exoskeleton has autofluorescent properties and can easily be excited by ultraviolet or visible light (Klaus et al., 2003; Neville, 1975; Zill et al., 2000). In our preliminary investigations, using CLSM, we found that the exoskeleton of the English grain aphid *Sitobion avenae* (F.) is indeed autofluorescent and is excited by ultraviolet light. Stacks of optical slices can be reconstructed into 3D objects over time, visualizing the infection process of aphids by selected isolates of *B. bassiana* and *P. neoaphidis*. To make this possible it is crucial that the entomopathogens are also visible in CLSM at the same time. Staining of the fungi is an option and was investigated using ceda (carboxy fluoresin di-acetate), Nile Red, Calcofluor white and Syto-13.

There are often difficulties with staining fungi properly, such as fading of the probe over time when exposed to excitation light and there can be problems in getting the stain deep into the tissue of the specimen. Transformation of the fungus with a fluorescent protein, such as the green fluorescent protein (GFP) could be a solution to the above-mentioned problems regarding fungal staining. Transformation of fungi with *gfp* has been successful in plant pathogens (Sawyer et al., 1998; Spear et al., 1999). Also, a few species able to infect insects, such as *Verticillium dahliae*, *Absidia glauca* and *Mucor circinelloides*, have been transformed with *gfp* (Lorang et al., 2001). Several problems with the stability of transformants, resistance to antibiotics etc. have been a challenge for the researchers using the different transformation methods; electroporation-method and protoplast-PEG method (Michielse et al., 2005).

The *Agrobacterium tumefaciens*-mediated transformation method was first applied in 1998 by De Groot et al. (1998) and has since then successfully been applied to many fungal species. The last couple of years there has been research published using the method to transform the entomopathogenic fungi *Beauveria bassiana* (Fang et al., 2003; dos Reis et al., 2004) and other entomopathogenic fungi that are members of the classes Ascomycetes and Zygomycetes (Michielse et al., 2005). To our knowledge, no fungi belonging to the order Entomophthorales (Zygomycoytina), such as *P. neoaphidis*, has yet been transformed using the *A. tumefaciens*-mediated transformation method. As a part of this project, the aim is to insert the *gfp* gene together with an antibiotic resistance gene into the genomes of *B. bassiana* and *P. neoaphidis* using the *A. tumefaciens*-mediated transformation method.

Prior to an efficient genetic transformation method, it is a prerequisite to find a good selection system for the transformants. Antibiotics are commonly used for the selection of transformants. When an antibiotic that inhibits fungal growth is identified, the *gfp* gene will be inserted together with the antibiotic resistant gene. The antibiotics hygromycin B, phosphinothricin and benomyl have all been used in selective media for transformed entomopathogenic fungi and were tested in this study prior to the transformation.

In the present study we combined CLSM and staining with vital fluorescent dyes and/or *gfp*-tagging of *B. bassiana* and *P. neoaphidis* to monitor the fate and behaviour of the living fungal inoculants infecting aphids *in situ*.

**Material and methods**

**Aphids and fungal isolates**

Four isolates of *B. bassiana* (KVL 9820, KVL 0390, ARSEF 2879, ARSEF 3385) and one isolate of *P. neoaphidis* (KVL 633), all known to be pathogenic to aphids, were used in the experiments.

A clone of the English grain aphid *Sitobion avenae* used in the experiments was isolated from a Danish cereal field and reared in cages in the laboratory, 22°C, 16:8 (light:dark), feeding on wheat plants.
Confocal laser scanning microscopy
A fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and a Confocal Laser Scanning Microscope (CLSM) (TCS SP2, Leica Microsystems, Wetzlar, Germany) were used to study the aphids infected with the stained entomopathogenic fungus. The fluorescent microscope was primarily used to check if the fungi were stained properly and for the selection of gfp transformants. CLSM was used to take high resolution images and make 3D recordings of the fungal development on and inside the specimen over time.

Selection for antibiotic resistance and Agrobacterium tumefaciens-mediated transformation introducing gfp into B. bassiana
The five fungal isolates (B. bassiana KVL 9820, KVL 0390, ARSEF 2879, ARSEF 3385 and P. neoaphidis KVL 633) were tested for resistance against the antibiotics hygromycin B (50, 100 150, 200 and 300 µg/mL), phosphinothricin (20, 30, 40, 50, 60, 100, 200, 300, 500 µg/mL) and benomyl (1, 5, 8, 10, 12.5 µg/mL).

The gfp gene was introduced into two of the B. bassiana isolates using the Agrobacterium tumefaciens-mediated transformation method. The A. tumefaciens strain used was AGL1. The Ti plasmid pPZP-201-BB-GG (11-12 kb) (donated by S. Christiansen) contained the phosphinothricin resistance gene and the gfp gene. Both genes contained the constitutive Aspergillus nidulans glyceraldehydes-3-phosphate (gpd) promoter facilitating expression in many filamentous fungi. A. tumefaciens and germinating spores of B. bassiana were co-cultivated and incubated for a few days. Thereafter, they were transferred onto selective media to facilitate the growth of the transformants only.

Results and discussion

Confocal laser scanning microscopy
The vital dyes Nile red, cfda (carboxy fluoresin di-acetate) and Calcofluor white M2R were used for staining the fungal isolates P. neoaphidis KVL 633, B. bassiana KVL 9820 and ARSEF 3385. So far good images have been obtained using Nile Red and Calcofluor white M2R which stained the lipids and β-polysaccharides, respectively (Figs 1-4). Calcofluor white: excitation at 351 and 364 nm, emission recorded at 400-500 nm, Nile Red: excitation at 543 nm, emission recorded at 580-620 nm.

Aphids infected with P. neoaphidis was been observed in situ in CLSM (Figs 5-6). It was possible to see hyphal growth in and on the aphid. Future work will include visualization of conida that germinate and penetrate the aphid cuticle and colonize the aphid.

Selection for antibiotic resistance and Agrobacterium tumefaciens-mediated transformation introducing gfp into B. bassiana
The five fungal isolates (B. bassiana KVL 9820, KVL 0390, ARSEF 2879, ARSEF 3385 and P. neoaphidis KVL 633) were tested for resistance against the antibiotics hygromycin B, phosphinothricin and benomyl and gave the results shown in Table 1.

No growth inhibition of P. neoaphidis was observed on any of the selective media, whereas two of the B. bassiana strains showed reduced growth on the selective media with 50-100 µg/mL phosphinothricin. Based on the results from the resistance test, phosphinothricin was used as the selective antibiotic in the transformation work. The fungal isolates B. bassiana KVL 9820 and B. bassiana ARSEF 3385 were chosen to be the first to be transformed.

The Agrobacterium tumefaciens mediated transformation method was used to insert gfp into B. bassiana KVL 9820 and B. bassiana ARSEF 3385. Based on the resistance test, phosphinothricin was added to the selective medium at 70 µg/mL.
Figures 1-4. 1: top left) CLSM transmission image of *P. neoaphidis* conidia. 2: top right) Corresponding CLSM fluorescence image of *P. neoaphidis* conidia stained with Calcofluor white M2R. 3: bottom left) CLSM transmission image of a single *P. neoaphidis* conidium. 4: bottom right) Corresponding CLSM fluorescence image of a single *P. neoaphidis* conidium stained with Nile red.

Figures 5. (left) - 6 (right). Fluorescence microscopic images of *S. avenae* infected with *P. neoaphidis*. The fungus is stained with Calcofluor white M2R.
Table 1. Test for antibiotic resistance. The fungal isolates *B. bassiana* KVL 9820, KVL 0390, ARSEF 2879, ARSEF 3385 and *P. neoaphidis* KVL 633 were tested for resistance against hygromycin B, phosphinothricin and benomyl. The highest levels of resistance are indicated with *.

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Antibiotic</th>
<th>Hygromycin B</th>
<th>Phosphinothricin</th>
<th>Benomyl</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bassiana</em> KVL 0390</td>
<td>No growth inhibition</td>
<td>Some reduced growth at 50-100 µg/mL</td>
<td>Some reduced growth at &gt;12.5 µg/mL</td>
<td></td>
</tr>
<tr>
<td><em>B. bassiana</em> KVL 9820</td>
<td>No growth inhibition</td>
<td>* Reduced growth at 50-100 µg/ mL</td>
<td>Some reduced growth at &gt;12.5 µg/ mL</td>
<td></td>
</tr>
<tr>
<td><em>B. bassiana</em> ARSEF 3385</td>
<td>No growth inhibition</td>
<td>* Reduced growth at 50-100 µg/ mL</td>
<td>Some reduced growth at &gt;12.5 µg/ mL</td>
<td></td>
</tr>
<tr>
<td><em>B. bassiana</em> ARSEF 2879</td>
<td>No growth inhibition</td>
<td>Some reduced growth at 50-100 µg/ mL</td>
<td>Some reduced growth at &gt;12.5 µg/ mL</td>
<td></td>
</tr>
<tr>
<td><em>P. neoaphidis</em> KVL 633</td>
<td>No growth inhibition</td>
<td>No growth inhibition</td>
<td>No growth inhibition</td>
<td></td>
</tr>
</tbody>
</table>

After 7 days of incubation, the Petri dishes containing the fungal cultures were analyzed under a fluorescent microscope, using GFP2 and GFP3 filters with 480/40 and 470/40 excitations filter and barrier filter 510nm LP and 525/50nm LP. Both transformants and non-transformants were observed; the hyphae of the transformants emitted green light under the fluorescence microscope. The highest number of transformants was found with the strain KVL 9820, which then is the best result of this transformation experiment.

As indicated in Table 1 and in Fig. 7, the selection pressure with phosphinothricin was not 100%, which is probably the reason the Petri dishes contained mixed cultures of transformed and non-transformed *B. bassiana*.

Manual selection for transformants is required to separate them from non-transformants. This was performed under fluorescent microscope by manually picking up the transformants.
and placing them onto new selective plates. At present, 17 selected transformants of *B. bassiana* KVL 9820 and 12 of *B. bassiana* ARSEF 9355, was recovered and frozen in 10% glycerol and -80°C. A further selection of the transformants will take place by making single spore isolates of each transformant. Then these will be tested for their morphology, growth and ability to infect aphids with the aim of selecting stable transformants that resembles the wild types.

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Naturally occurring *Beauveria bassiana* in *Hypothenemus hampei* populations in unsprayed coffee fields

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**Abstract:** Three unsprayed coffee fields in Nicaragua were studied for the natural occurrence of the insect pathogenic fungi *Beauveria bassiana* in *Hypothenemus hampei* populations throughout the rainy season in 2004. Infection levels were varying considerably throughout the season and between locations. The highest *B. bassiana* infection level observed at one location was 60%. This was observed in November at the end of the rainy season. Three moist chamber methods were used in the survey: a water agar based method, a filter paper based method and a plastic bag based method. The plastic bag method resulted in the highest and most frequent observation of *B. bassiana*. It is probably also the most reliable method when it comes to cross contamination.

**Key words:** Coffee, *Hypothenemus hampei*, *Beauveria bassiana*, entomopathogenic fungi, biological control

**Introduction**

The coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae) is the most important pest of coffee throughout the world, (CIRAD, IRD, CCCR, IICA, PROMECAFE, 1999). In America, *H. hampei* was observed for the first time in Brazil in 1913 and now it has been observed in all Central American countries and Colombia, Mexico and the Dominican Republic (Guharay & Monterrey, 1997; MAG 2000; Ramirez & Reyes, 2000). The only country in Central America where the pest has not been reported yet is Panamá (Villacorta, 2001). The insect pathogenic fungi *Beauveria bassiana*, a natural enemy of *H. hampei*, is considered to be an important biotic mortality factor of this pest insect (De La Rosa et al., 1997), and it is the most frequently found fungal species infecting *H. hampei* (Bustillo et al., 2002). *B. bassiana* is also used as an inundative microbial control agent against *H. hampei* in several countries in Latin America. So far, *B. bassiana* is the most promising and most studied entomopathogenic fungus for microbial control of *H. hampei* (CABI, 2003).

Natural infection of *B. bassiana* in *H. hampei* is reported in all countries where the pest is present. Life table studies on *H. hampei* in Caldas, Colombia, which has a continuously humid climate, shows that natural *B. bassiana* infection levels causes up to 80% mortality of adults, being the largest biotic mortality factor of this pest under these conditions (CABI, 2003). In several coffee growing regions in Brazil, *B. bassiana* have been reported to cause up to 20% mortality in *H. hampei* adults (Alves et al., 2002). Also in Nicaragua, natural *B. bassiana* infections in *H. hampei* populations are found (Lacayo, 1994), and infection levels up to 18% have been reported under humid climatic conditions, at farms where more than 40% of berries bored by *H. hampei* were present (Bustamante, 1993). To our knowledge there are, however, few systematic studies on *B. bassiana* infection levels in *H. hampei* populations worldwide.
The aim of this study was therefore to conduct a systematic study where the natural occurrence of *B. bassiana* on three different unsprayed coffee farms were observed on a two weeks basis throughout the rainy season.

**Materials and methods**

The study was carried out from July to November 2004, at three different coffee farms, in the north coffee growing zone of Nicaragua. The farms were located about 700 and 800 meters above sea level at 13° 08' N, 85° 44' W. In farm 1 and 2 shade levels were between 60 and 70%, while in farm 3 the shade level is 50% and shade regulation is performed. Farm 1 was located about 200 m from farm 2, and farm 3 about 2 km from farm one and two. Neither synthetic pesticides nor *B. bassiana* had been used the last 5 years in any of the farms.

Two hundred bored berries were collected every second week at each field from July to November 2004. The berries were cleaned and surface-sterilized with 75% ethanol in the laboratory before berries or insects were placed in moist chambers for observation of fungal growth. Three different moist chamber methods were used: (1) The water agar method: Berries were opened and *H. hampei* adults were picked out, surface sterilized and placed individually in 30 mL vials with 2% water-agar. (2) The filter paper method: Berries were opened and *H. hampei* adults were picked out, surface sterilized and placed individually in 30 mL vials with moist filter paper. For method 1 and 2, the insects were checked for *B. bassiana* every second day. (3) The plastic bag method: Berries were placed individually in plastic bags with moist cotton. After three weeks of incubation, berries were opened and *H. hampei* adults were picked out and checked for *B. bassiana* infection. Fungal growths observed on the insects, were isolated and morphologically identified according to relevant literature. For the water agar and filter paper methods the infection level was estimated by dividing the number of dead infected insects (adults) by the total of live insects (adults) found in the samples at the collection date. For the plastic bag method the number of dead infected insects (adults) were divided by the total of live insects (adults) found in the berries at the dissection day. *B. bassiana* infection levels were used to estimate the Area Under Disease Progress Curve (AUDPC), and analysis of variance was conducted for comparisons between methods and between farms. The prevalence of *H. hampei* was estimated by counting the number of bored berries and total berries in 160 branches per farm. The data of bored berries were arcsine transformed and a two-way analysis was performed in order to compare percentages of bored berries between farms.

**Results**

*Moist chamber methods*

Using AUDPC, to compare the effect of moist chamber methods on infection level, the plastic bag method resulted in a significantly (P>0.01) higher AUDPC, indicating a higher observed infection level than for the two other methods. The lowest infection level was observed when using the water agar method.

*B. bassiana* infection levels

The *B. bassiana* infection level in *H. hampei* populations varied considerably throughout the season. Using AUPDC, no consistent significant differences in infection level were found between farms however. In coffee farm 1, *B. bassiana* infected *H. hampei* were found at six out of 10 sampling dates. The highest infection level observed in this farm was 25.0% at the end of the survey in the mid of November (Fig. 1). In coffee farm 2, *B. bassiana* infected insects were found at seven out of 9 sampling dates. Only at three sampling dates, in July and
September, no infections were observed. The highest infection level observed was 18.8% in mid August. In coffee farm 3, *B. bassiana* infected *H. hampei* were found at 8 out of 9 sampling dates. The highest infection level observed on this farm was 60.0% in the samples collected November 11.

![Figure 1](image.png)

Figure 1. Numbers of *Hypothenemus hampei* per 50 branches and percentages of *H. hampei* infected with *Beauveria bassiana* on three coffee farms using three different methods.

**H. hampei populations**

*H. hampei* populations were high in all three farms. The analysis of variance showed significant differences in *H. hampei* between all three farms (*p*≤0.0001). The highest *H. hampei* population was observed in coffee farm 1 and ranged from 56.2 to 95.1 insects per 50 branches, and the lowest was observed in farm 3 and ranged from 4.7 to 44.4 insects per 50 branches. Although there were not significant differences between sampling dates, the highest *H. hampei* population was observed in October in farm 1 and in September in farms 2 and 3. In farms 2 and 3, *H. hampei* populations decreased at the end of the season. In farm 1, however, the population was high through the season.
Discussion

The highest infection levels were observed by using the plastic bag method. High relative humidity inside the bored berries in the plastic bag probably favoured the fungal growth. This method is probably also the most reliable method when it comes to cross contamination. High levels of contamination may be a problem when using the water agar and filter paper methods because the berries needs to be opened to observe for fungal growth on the insect.

No significant differences in *B. bassiana* infection levels were found between farms. If we look at infection peaks at single dates, however, the highest peak (60.0%) was observed in mid November at farm 3 followed by farm 1 (25.0%) in mid November and farm 2 (18.8%) in mid August. The high infection level found at the end of the season could be due to optimal conditions for fungal growth at this part of the season. *B. bassiana* infection this late in the season is probably important for *H. hampei* survival in fallen berries. Surprisingly, in farm 3, where the highest infection peak was observed, the lowest *H. hampei* population was also found, and we cannot document any density dependence. Relatively sunny conditions and a high relative humidity provided by medium levels of shade in coffee fields favours *B. bassiana* (Féliz, 2003). In farm 1 and 2, high shade levels might, however have been unfavourable to *B. bassiana*.

The differences in *H. hampei* population levels observed between farms might be due to differences in coffee management practices. In farms 1 and 2, where the highest *H. hampei* population levels were seen, there are several coffee varieties and a high level of shade (60-70%). This is known to favour high *H. hampei* populations (Guharay & Monterrey, 1997). In farm 3 there is just one coffee variety and the shade level is about 50%. This probably allows the sun to reduce the activity of *H. hampei* females.

References


Activity and molecular characterization of *Metarhizium anisopliae* isolates, of potential use for Moroccan locust control

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**Abstract:** *Dociostaurus maroccanus* is an important pest that can reach in Italy an alarming population level, causing extensive and severe damage to the crops. International programmes which were set up for biological control of locusts and grasshoppers, led to the isolation in Niger identification of a fungus as *Metarhizium anisopliae* var. *acridum*. This fungus became the most promising agent for biological control in Africa, and was later on tested in Europe, together with other species of fungi, such as *Beauveria bassiana*. During these trials a different isolate of *M. anisopliae* was found on locusts sampled in Gargano National Park, in Italy. The aims of this research were therefore: a) to test in the laboratory the effectiveness of two *M. anisopliae* varieties (i.e. *acridum* and *anisopliae*) against *D. maroccanus*; b) to characterize a representative strain of these two varieties by using ITS sequence analysis. The results were as follows: a) a higher mortality of locusts due to *Metarhizium anisopliae* var. *anisopliae* in respect of the var. *acridum*; b) the analysis of the ITS region of the two *M. anisopliae* varieties demonstrated its overall conserved character in this fungus. Nevertheless, some variability was detected within a portion (about 600 bp) of the rDNA gene complex, thus allowing the identification of the variety *M. anisopliae* var. *acridum* and *M. anisopliae* var. *anisopliae*.

**Key words:** *anisopliae*, *acridum*, ITS, behavioural thermoregulation.

**Introduction**

The Moroccan locust, *Dociostaurus maroccanus* Thunberg (Orthoptera: Acrididae), is widespread in the Mediterranean region. This pest feeds on wild grasses in pasture and uncultivated areas, then on cultivated plants when grasses are scarce. Over the last few years some of the locust oviposition areas have been localised in Apulia, in the provinces of Bari and Foggia. The chemical applications have been scheduled just in these areas, during the hatching period, when locusts are still very small and aggregate. It has thus been possible to apply low-persistence insecticides, such as the pyrethroids, on quite small areas. Treatments on large areas have been prevented, because some of the involved areas fall within National Parks. With the perspective of using low environmental impact products, some trials to control *D. maroccanus* were realized, using an entomopathogenic fungus: *Metarhizium anisopliae* (Ascomycota, Hypocreales). During the Spring 2000, a first experiment was realized with *M. anisopliae* var. *acridum* (IMI 330189), supplied by CABI Bioscience of Ascot (UK), in the laboratory of the “Dipartimento di Biologia e Chimica agro-forestale e Ambientale (DiBCA)”. During this experiment other isolates of *Metarhizium* spp. were collected from untreated dead locusts; morphological characterization of these isolates allowed their identification as *M. anisopliae* var. *anisopliae*. Based on these results, it was planned to compare the effectiveness of the two varieties of *M. anisopliae* in controlling locusts. Moreover, molecular characterization studies were started in order to clearly differentiate the two *M. anisopliae* varieties.
Material and methods

Laboratory bioassay with two strains of Metarhizium anisopliae
The trial was run in 2004, in the DiBCA laboratory, by testing the strain IMI 330189 (var. acridum: subsequently named M1) and the strain isolated in Apulia (var. anisopliae: M11) on D. maroccanus.

The locusts were collected in the infested areas of Gargano and transferred to the laboratory. The conidia of each strain were suspended in a glycerol mixture (70% distilled water and 30% glycerol), at the concentration of $2.5 \times 10^7$ conidia ml$^{-1}$. Each conidial suspension was supplied by a micropipette, at the rate of 0.5 µL/insect, to 90 locusts, divided into three cages. Each cage contained 30 nymphs. Another group of 90 samples was left untreated. The metal cages were equipped with a mosquito-mesh. The cages were supplied with a 60W bulb, to allow locusts behavioural thermoregulation. The internal temperature of the cages was registered all day long during the period of the trial.

The trial lasted 21 days and every day the insects were fed with fresh grass, and the dead individuals were removed. The latter were counted and transferred onto Petri dishes on a filter paper disk where they were kept dry for one day. Starting from the second day, the filter paper disk was regularly wetted with sterilised water to favour any pest fungi getting out of the locust body.

Molecular characterization and ITS analysis
In order to characterize and differentiate the two M. anisopliae varieties (acridum and anisopliae) molecular analysis by Random Amplified Polymorphism DNA (RAPD) was performed. For each varieties five isolates, collected from dead locusts, were compared to the strain M1 (M. anisopliae var acridum), used as reference isolates. Subsequently, amplification and sequencing of the Internal Transcribed Spacer (ITS) region, including the 5.8S gene, was accomplished on some representative isolates of the two varieties.

Results and discussion

Laboratory bioassay
A higher mortality of locusts due to M11 was observed. The results were analysed with $\chi^2$ test. We had a significance per $P \leq 0.01$ between the different mortality due to M11 and the untreated control ($\chi^2 = 32,047$) and between M1 and M11 ($\chi^2 = 23,733$), but there was no significance between the mortality due to M1 and the untreated control ($\chi^2 = 1,765$) (Table 1).

A low mortality was observed in the first 10 days after the treatment, whereas a high mortality resulted both in the test with M1 and M11 in the last days of the essay (Fig. 1). In the untreated control we observed only on the 17th day the increasing of mortality.

In the test with M1, the mortality was lower than in the untreated control. An increase of mortality was observed at the end of the essay, reaching a pick on the 21st day.

The first pick of mortality was observed after 10 days in the M11 treated nymphs, then after 19 and 21 days. Moreover the mortality due to fungi was very high in the M11 thesis (88%). About 60% of dead locusts showed fungal infection in the M1 thesis. In the untreated control about 40% of dead locusts were infected by fungi, as Beauveria spp. and saprophytic fungi.

The locust mortality due to fungi is delayed in the time, in fact a fungus based treatment has a lower speed of action than a chemical insecticide. Generally locust mortality takes about 10 days under relatively constant temperature conditions (Blanford & Thomas, 2001). Moreover when the locusts can thermoregulate the time of fungi action is much more slow and the mortality is drastically reduced (Ouedraogo et al., 1997).
Table 1. Nymph mortality in *Dociosaurus maroccanus* 21 days after treatment with strains of two varieties of *Metarhizium anisopliae*, var. *acridum* (M1) and var. *anisopliae* (M11).

<table>
<thead>
<tr>
<th></th>
<th>Total mortality %</th>
<th>Dead locusts with sporulation %</th>
<th>Dead locusts without sporulation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>41.1 B</td>
<td>56.6</td>
<td>43.4</td>
</tr>
<tr>
<td>M11</td>
<td>66.7 A</td>
<td>88.3</td>
<td>11.7</td>
</tr>
<tr>
<td>Untreated control</td>
<td>44.5 B</td>
<td>42.5</td>
<td>57.5</td>
</tr>
</tbody>
</table>

During the rearing period the average day temperature was 27°C, that is good both for locusts and for fungi development, the average night temperature was 18°C. M1 growth optimal temperature is 27-30°C (Thomas & Jenkins, 1997). So during the day it may develop, but not during the night. But during the day orthopterans are active behavioural thermoregulators, so they can maintain their body temperature close to 38-40°C (Blanford & Thomas, 1999). Moreover the locusts can elevate the body temperature till 42-44°C for a “fever" as a reaction to the pathogen infection (Inglis *et al.*, 1999). In this way the fungal growth is largely inhibited and Elliot *et al.* (2002) observed that some locusts, treated with *M. anisopliae* var. *acridum*, were able to moult, mature and reproduce. The fungus action is slower in thermoregulating insects, which show a low sporulation of the fungus.

Figure 1. Daily average mortality of *Dociosaurus maroccanus* treated with strains of two varieties of *Metarhizium anisopliae*, var. *acridum* (M1) and var. *anisopliae* (M11).
Molecular characterization by RAPD and ITS analysis

RAPD-PCR patterns were reproducible between repeated amplifications and subsequent DNA extractions. Among the primers tested, H2, H3, and H8 allowed the identification of amplicons which appeared specific for the two fungal varieties. Two uniform groups of isolates with the same banding patterns were identified; the first group included isolates identified as *M. anisopliae* var. *acridum* whereas the second included isolates of *M. anisopliae* var. *anisopliae*. The length of ITS1-5.8S-ITS2 region resulted 575, 566, and 531 bp for isolate M1, M5 and M11, respectively (Fig. 2). The primers used to amplify regions of the repeat unit proved that the ITS1-5.8S-ITS2 region was very conserved within the isolates of *Metarhizium*, exhibiting also a high degree of similarity to several other mitosporic fungi. ITS1 region was highly conserved in *M. anisopliae* var. *acridum*, providing limited information on isolate sequence differentiation. By contrast, the isolate M11 *M. anisopliae* var *anisopliae* displayed some degree of variability, allowing the identification of significant sequence variations by CLUSTAL analysis (Thompson *et al.*, 1994). The preliminary molecular data presented here showed that isolates of *M. anisopliae* var *acridum* are distinguishable from *M. anisopliae* var *anisopliae*, and demonstrated the occurrence in Apulia of a local population of the latter variety with an appreciable antagonistic activity against locusts. Isolates M1 and M5 completely matched the diagnostic sequences reported by Driver *et al.*, (2000) for the ITS1 and ITS2 regions of *M. anisopliae* var *acridum*. Instead, significant variations were found for isolate M11, *M. anisopliae* var *anisopliae*. The molecular analysis of a larger number of isolates collected in Apulia would confirm the variability within the variety, thus allowing the identification of strain-specific sequences, useful for monitoring and environmental tracking of the promising biocontrol agent *M. anisopliae* var *anisopliae*, strain M11.

Figure 2. Agarose gel electrophoresis showing the ITS regions amplified with ITS1 and ITS2 primers. MM= Molecular Marker (100 bp DNA ladder), M1, M5 = *M. anisopliae* var *acridum*; M11= *M. anisopliae* var *anisopliae*. 
Acknowledgements

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References


Entomopathogenic fungi in riparian soils of the Ofanto river valley (Apulia region, Italy)

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Abstract: The natural occurrence of entomopathogenic fungi was investigated in different riparian biotopes of the Ofanto river valley situated in the center of the Apulia region (Southern Italy). During the period between September 2003 and June 2004, seventy-three different sites were sampled using the larvae of Galleria mellonella and Tenebrio molitor as bait insects. Fungi were isolated from 69 soil samples (94.5%) with 238 isolated strains belonging to 7 taxa.

Key words: Beauveria bassiana, Metarhizium anisopliae, soil, Southern Italy

Introduction

Entomopathogenic fungi are distributed in a wide range of habitats including aquatic, forest and agricultural areas. Some of them are used as biological control agents of different insect pests. The aim of this work was to survey the valley of the Ofanto river to study the occurrence of entomopathogenic fungi in the Mediterranean area, specifically in the center of the Apulia region (Southern Italy).

In particular, the abundance of entomopathogenic fungi was evaluated for the first time in 3 different riparian biotopes of this watershed: bank, high-water bed and cultivated soils (about 100 m far from the bank). The importance of investigating the Ofanto river valley is due to the fact that this river is the only one in the region and since the earliest traces of human life has been used as water stock for irrigation and for livestock.

Materials and methods

A total of 73 soil samples were collected during the period between September 2003 and June 2004 following transects that lie perpendicular to the water current 100 m into the river-bed (Fig. 1). Approximately 2 kg of soil were gathered for each sample by pooling 3 to 4 subsamples taken at depths of 10 to 20 cm from an area about 20 m². Isolation of insect pathogenic fungi was attempted using the “Galleria bait method” first developed by Bedding & Akhurst (1975) for trapping entomopathogenic nematodes and later adapted by Zimmermann (1986) for trapping entomopathogenic fungi. For the surveys last-instar G. mellonella (Lepidoptera: Galleriidae) and T. molitor (Coleoptera: Tenebrionidae) larvae were used as insect bait. The soil of each sample was transported in sterile plastic bags to the laboratory, passed through a sieve (mesh, 10mm).

As soon as external fungal growth became visible on the cadavers, attempts to isolate the fungus were made by transferring spores to potato dextrose agar in Petri dishes. Inoculated Petri dishes were then checked every day and the tubes with pure culture were subcultured in potato dextrose agar medium and stored at 8°C. Identification of isolates was carried out by Gisbert Zimmermann’s laboratory using standard procedures.
Results and discussion

A total of 238 fungal strains belonging to 7 taxa were found in 94.5% (69 of 73) of the soil samples. The reliability of the bait insect method for detecting entomopathogenic fungi in soil was confirmed (Barker & Barker, 1998; Bedding & Akhurst, 1975; Zimmermann, 1986); in particular, *G. mellonella* demonstrated to be a better bait insect than *T. molitor* did (mortality rate of 93% versus 35%, respectively).

*Beauveria bassiana* (Balsamo) Vuillemin was the most abundant species (51.7% of the total isolates), followed by *Metarhizium anisopliae* and *Fusarium* sp. (13.4%), *Aspergillus* sp. (3.36%) and *Penicillium* sp. (2.94%), and *Aspergillus niger* (1.7%). *Paecilomyces* sp. and *Phycomycetes* were only isolated once from the soil samples. Because a few of the strains were morphologically atypical, identification of the species was not achieved in some cases, as reported by Tarasco et al. (1997). In 12.6% of the samples, other species of fungi were isolated but not identified (Fig. 2).

It is interesting to underscore the simultaneous presence of 2 or more fungal species in soil samples, up to a maximum of 5 in 4.1% of the samples (Fig. 3).

The frequency of occurrence of fungi in the 3 different biotopes was as follows: 30.7% in the bank, 32.8% in the high-water bed and 36.5% in different cultivated areas 100 m into the
bank. More precisely, the respective frequencies of occurrence of entomopathogenic fungal species from each biotope are presented in Table 1.

Figure 2. Frequency of occurrence (%) of fungal species isolated in sampling locations in riparian soils of the Ofanto river valley.

![Pie chart](image)

- **Beauveria bassiana**
- **Metarhizium anisopliae**
- **Fusarium sp.**
- **unknow**
- **Aspergillus sp.**
- **Penicillium sp.**
- **Aspergillus niger**
- **Paecilomyces sp.**
- **Phycomycetes**

Figure 3. Percentage rates of soil samples with multiple fungal isolates from riparian soils of the Ofanto river valley.

![Bar chart](image)

There was no significant difference in the occurrence of total fungal isolates from the different biotopes (ANOVA, \( P > 0.05 \)). This finding shows that the isolation rate of entomopathogenic fungi was slightly affected by the soil moisture of the various biotopes (Ali-Shtayeh et al., 2002).
Table 1. Frequency of occurrence of fungal species in soils from different habitat types of the Ofanto river valley.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Bank</th>
<th>High-water bed</th>
<th>Cultivated soils</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td>0.4</td>
<td>0.8</td>
<td>0.4</td>
<td>1.7</td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td>0.4</td>
<td>2.1</td>
<td>0.8</td>
<td>3.4</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>14.3</td>
<td>17.2</td>
<td>20.2</td>
<td>51.7</td>
</tr>
<tr>
<td><em>Fusarium</em> sp.</td>
<td>3.8</td>
<td>5.0</td>
<td>4.6</td>
<td>13.4</td>
</tr>
<tr>
<td><em>Metarhizium anisopliae</em></td>
<td>6.7</td>
<td>2.1</td>
<td>4.6</td>
<td>13.4</td>
</tr>
<tr>
<td><em>Penicillium</em> sp.</td>
<td>0.4</td>
<td>0.8</td>
<td>1.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Phycomycetes</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td><em>Paecilomyces</em> sp.</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>unknown</td>
<td>4.6</td>
<td>4.6</td>
<td>3.4</td>
<td>12.6</td>
</tr>
<tr>
<td>Total</td>
<td>30.7</td>
<td>32.8</td>
<td>36.6</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Conclusion

In conclusion, the results have demonstrated that entomopathogenic fungi are very common inhabitants of the soil in the Ofanto river valley; however, only a few species occurring frequently: *B. bassiana*, *M. anisopliae* and *Fusarium* sp. have been obtained from about 77% of the isolates. This small biodiversity, but substantial intraspecific richness, may be attributed to the constant climatic characteristics of the river valley, which maintains a uniform temperature and high humidity throughout the year. *B. bassiana* was the most frequently isolated species in all of the samples studied, in accordance with the results obtained by Tarasco *et al.* (1997) for Southern Italy. Despite both studies being the same, the simultaneous presence of more fungal species in each sample was extremely interesting and deserving of further investigation.

References

Insect pathogenic fungi found in rosy apple aphid (*Dysaphis plantaginea*) and green apple aphid (*Aphis pomi*) in Norway

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**Abstract:** The natural occurrence of insect pathogenic fungi in *Aphis pomi* and *Dysaphis plantaginea* populations in apple orchards in south east Norway were studied throughout the summer 2002 and 2003. Four entomophthoralean species were observed to infect both apple aphid species: *Entomophthora planchoniana*, *Neozygites fresenii*, *Pandora neoaphidis* and *Conidiobolus obscurus*.

**Key words:** insect pathogenic fungi, rosy apple aphid, green apple aphid, *Dysaphis plantaginea*, *Aphis pomi*, *Entomophthora planchoniana*, *Neozygites fresenii*, *Pandora neoaphidis*, *Conidiobolus obscurus*

**Introduction**

The rosy apple aphid (*Dysaphis plantaginea*) is considered a serious pest on apple. It damages the fruit, which become small and malformed and ripen prematurely. The leaves are severely curled and shoots are stunted and twisted. The green apple aphid (*Aphis pomi*) is most harmful to young apple trees and damages the trees by stunting new growth (Alford, 1984). The honeydew from the aphids provides a medium for black sooty mould. Sooty mould can affect photosynthesis in leaves and reduce fruit quality (Gullan & Cranston, 2000). Insect pathogenic fungi are important natural enemies of aphids, and fungi in the order Entomophthorales are known to cause epizootics in aphid populations (Latgé & Papierok, 1988; Hajek, 1997). Few studies have, however, investigated insect pathogenic fungi in *D. plantaginea* and *A. pomi*. The aim in this study was to investigate the natural occurrence of Entomophthorales in the two aphid species, and to find the infection level through two successive seasons.

**Material and methods**

Aphids were collected weekly from one conventional and four organic apple orchards in southeastern Norway during the summer 2002 and 2003. In the laboratory, aphids were transferred individually to small apple leaves embedded in 1.5 % water agar in 30 mL vials, and incubated at 20° C, 16 hours light and 70 % relative humidity (RH). Aphid mortality was recorded daily for a week except in the weekends, and dead aphids were placed on microscope slides in moist chambers at room temperature. These were checked daily for fungal infection for 3-4 days. Cadavers of fungal infected aphids were mounted in lactophenol cotton blue and examined under the microscope. Identification of fungal species was based on morphological characters described by Keller (1987, 1991) and Balazy (1993).

**Results and discussion**

Four species of Entomophthorales were found in both aphid species. These fungal species were *Neozygites fresenii*, *Entomophthora planchoniana*, *Pandora neoaphidis* and *Conidiobolus obscurus*.
obscurus. All fungal species are found in earlier studies in Norway (Klingen et al., 2002) but this is the first time they are reported from A. pomi and D. plantaginea. N. fresenii was the most frequently found fungus. Draganova & Simova (2001) found also that N. fresenii was the most frequently fungal pathogen in A. pomi followed by Pandora neoaphidis.

In our study the highest mortality caused by fungi (all four species) was 39.6% in A. pomi and 33.3% in D. plantaginea. Fungi were found from late June and throughout the season in several locations. N. fresenii caused an epizootic in a A. pomi population in one organic location in 2002. Epizootics due to that species were known to occur regularly in populations of the cotton aphid (Aphis gossypii) (e.g. Silvie & Papierok, 1991; Steinkraus et al., 1995). Resting spores of N. fresenii were found in A. pomi the 15th of July 2002.

Table 1 shows mean percentages A. pomi and D. plantaginea infected by entomophthoralian fungi at five locations throughout 2002 and 2003. One individual of D. plantaginea and two A. pomi had a double infection (N. fresenii and E. planchoniana), and one individual of A. pomi had a triple infection of fungi (E. planchoniana, P. neoaphidis, C. obscurus). Ekbom & Pickering (1990) found double infection with Neozygités sp. and E. planchoniana in the blackmargined aphid (Monellia caryella Fitch).

Table 1. Percentages of A. pomi and D. plantaginea infected with Entomophthorales at five locations in south east Norway throughout 2002 and 2003.

<table>
<thead>
<tr>
<th>Aphid species</th>
<th>Location</th>
<th>2002</th>
<th>2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. pomi</td>
<td>Hurum, organic</td>
<td>22.3 (412)</td>
<td>5.7 (140)</td>
</tr>
<tr>
<td></td>
<td>Gjennestad, organic</td>
<td>4.3 (207)</td>
<td>0 (169)</td>
</tr>
<tr>
<td></td>
<td>Lier, organic</td>
<td>0 (82)</td>
<td>0 (89)</td>
</tr>
<tr>
<td></td>
<td>Ås, conventional</td>
<td>0 (22)</td>
<td>36.4 (11)</td>
</tr>
<tr>
<td>D. plantaginea</td>
<td>Hurum, organic</td>
<td>16.2 (68)</td>
<td>0 (39)</td>
</tr>
<tr>
<td></td>
<td>Gjennestad, organic</td>
<td>9.7 (41)</td>
<td>0 (29)</td>
</tr>
<tr>
<td></td>
<td>Lier, organic</td>
<td>0.5 (216)</td>
<td>0 (119)</td>
</tr>
<tr>
<td></td>
<td>Ås, conventional</td>
<td>0 (27)</td>
<td>0 (86)</td>
</tr>
</tbody>
</table>

Acknowledgements

We thank Dr. S. Keller (Switzerland) for confirming our identification of C. obscurus.

References


The effect of medium and selected metal ions on growth of the entomopathogenic fungus *Beauveria bassiana*

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**Abstract:** The effects of several culture media and selected metal ions (Cd, Pb and Zn) on growth of a few strains of *B. bassiana* was investigated on solid media in Petri dishes. Linear growth was found to be dependent on the medium as well as on strains. The highest growth occurred when strains were cultured on media without any addition of metal ions. Effects of addition of metal ions depended on the ions and on the media. Cadmium had negative impact on the linear growth rate whatever the medium used. A slighter effect was observed when adding lead or zinc.

**Key words:** *Beauveria bassiana*, strains, growth, culture medium, metal ions, cadmium, lead, zinc

**Introduction**

The entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill., which produces the so-called “white muscardine” in insects, is commonly found in soils in Poland. It was known that different species of entomopathogenic fungi produce unlike effects on different culture media as regards both their growth and successive pathogenicity (Kmitowa, 1978). This paper aimed at finding out the effects of standard culture media on growth of several *B. bassiana* strains isolated from the Colorado beetle (*Leptinotarsa decemlineata*, Coleoptera: Chrysomelidae) in different sites. Moreover, given the presence of several metal ions in in Polish soils (Kabata-Pendias & Pendias, 1993), cadmium, lead and zinc were added to culture media to determine their influence on linear growth rates of the fungal strains.

**Material and methods**

We used six *B. bassiana* strains isolated from the Colorado beetle in different locations and kept at the collection of the Institute of Ecology, Polish Academy of Sciences (Table 1).

<table>
<thead>
<tr>
<th>Labelling in collection</th>
<th>Year of isolation</th>
<th>Site of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bb 5</td>
<td>1971</td>
<td>France</td>
</tr>
<tr>
<td>Bb 17</td>
<td>–</td>
<td>USA</td>
</tr>
<tr>
<td>Bb 57</td>
<td>1985</td>
<td>Lodz, PL</td>
</tr>
<tr>
<td>Bb 64</td>
<td>1986</td>
<td>Nowy Sacz, PL</td>
</tr>
<tr>
<td>Bb 66</td>
<td>1986</td>
<td>Tuchola, PL</td>
</tr>
<tr>
<td>Bb 71</td>
<td>1988</td>
<td>Dziekanow Lesny, PL</td>
</tr>
</tbody>
</table>
The four following standard media containing glucose and peptone were used: Martin medium, Waksman medium, Sabouraud medium and the MC medium (as made at the Institut Pasteur, Paris) (Kmitowa, 1978).

Certain metal ions (Cd, Pb and Zn) were added to the media at the amounts corresponding to their average concentration in Polish soils according to the literature (Kabata-Pendias & Pendias, 1993). Metal ions were applied to the media as the following salts and concentrations: for cadmium, cadmium chloride CdCl₂ (0.2 Cd mg dm⁻³), for lead, lead nitrate Pb(NO₃)₂ (18 Pb mg dm⁻³), and for zinc, zinc chloride Zn Cl₂ (40 Zn mg dm⁻³).

Fungal strains were cultured on Petri dishes. After inoculation the fungi were incubated in thermostats at 21°C. The growth was estimated by measuring daily the diameter of cultures. Results were subjected to one factor ANOVA and the means were differentiated by Duncan tests on the significance level p=0.05.

Results and discussion

The experimental results are presented in Figures 1 to 4. When no metal ion was added, linear growth rates appeared to vary according to strains and culture media.

![Figure 1. Colony diameter (mm) of 6 strains of Beauveria bassiana on Martin standard medium supplemented with metal ions (Cd: cadmium, Pb: lead, or Zn: zinc) or not (control). Means marked with the same letters do not differ statistically at the level p=0.05.](image)

The Martin medium appeared to be the less favourable medium for the culture of the 6 strains. The highest growth occurred when strains were cultured on media without any addition of metal ions. Furthermore, growth was shown to be strain dependant only when using the Sabouraud medium (Fig. 3). Effects of addition of metal ions to the medium depended on the ion and of the medium as well. For instance, cadmium inhibited linear growth of all the strains when added to any of the 4 standard media. Lead had effect on the 6 strains when added to Martin medium (Fig. 1) but Waksman and MC media (Figs 2 & 4),
whereas zinc had a general effect only on the Waksman medium (Fig. 2). Further research would be needed to determine a possible adverse impact of such metal ions when mass-producing the fungus or their possible adverse impact on the fate of the fungus in the soil.

Figure 2. Colony diameter (mm) of 6 strains of *Beauveria bassiana* on Waksman standard medium supplemented with metal ions (Cd: cadmium, Pb: lead, or Zn: zinc) or not (control). Means marked with the same letters do not differ statistically at the level p=0.05.

Figure 3. Colony diameter (mm) of 6 strains of *Beauveria bassiana* on Sabouraud standard medium supplemented with metal ions (Cd: cadmium, Pb: lead, or Zn: zinc) or not (control). Means marked with the same letters do not differ statistically at the level p=0.05.
Figure 4. Colony diameter (mm) of 6 strains of *Beauveria bassiana* on MC standard medium supplemented with metal ions (Cd: cadmium, Pb: lead, or Zn: zinc) or not (control). Means marked with the same letters do not differ statistically at the level p=0.05.

References


Entomopathogenic fungi found on *Ips typographus* in Georgia

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**Abstract:** The spruce bark beetle, *Ips typographus* L. (Coleoptera: Scolytidae) is very common throughout Borjomi gorge forests in Georgia and causes considerable damage in stands of spruce trees (*Picea orientalis*). During a survey performed in 2000-2004 in several Georgian forests, the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium* sp. have been found on this insect.

**Key words:** Entomopathogenic fungi, *Ips typographus*, *Beauveria bassiana*, *Metarhizium*

**Introduction**

The spruce bark beetle, *Ips typographus* L. (Coleoptera: Scolytidae) is very common throughout gorge forests of Borjomi in Georgia and causes considerable damage in stands of spruce trees (*Picea orientalis*). The outbreaks of this pest insect often develop after cutting of trees, storm damages or snow breaks, followed by warm and dry climatic conditions.

In the last years numerous investigations were focused on the complex of pathogens of bark beetles, their natural occurrence, their impact on insect population dynamics, and their potential as biological control agents (e.g. for *Ips typographus*: Balažy, 1966; Matha & Weiser, 1985; Wegensteiner, 1992, 1996, 2000; Weiser et al., 2003; Kreutz et al., 2004).

Unfortunately there is presently very few informations on insect pathogens in Georgia. The aim of this study was to investigate the species spectrum of entomopathogenic fungi in *Ips typographus* populations in this country.

**Material and methods**

Survey for presence of fungal pathogens in Georgian populations of *Ips typographus* were conducted in 2000-2004. The larvae, pupae and adults of bark beetles were sampled from two locations in Borjomi and Bakuriani regions (1000-1200 m above sea level) and from one location in Shovi resort (Caucasian mountain, 1200-1400 m above sea level).

The basic survey procedure was to inspect bark beetle habitats thoroughly, and to look for adult beetle cadavers showing apparent mycosis symptoms, such mummification or fungal growth. Infected and no infected specimens of insect were collected and brought to the laboratory for further investigations. The material was studied according to the generally accepted methods in insect fungal pathology (Weiser & Briggs, 1970; de Hoog, 1972; Humber, 1997). The fungi were identified with the help of microscopic preparations made directly with pieces of mycelium developing on dead bark beetles.

Fungal isolates originated from field collected, infected samples according to standard methodology (Evlakhova & Velickaia, 1961; Poinar & Thomas, 1984) and cultivated on the tree following isolation media, malt extract agar (MEA), potato extract agar (PEA) and Beauveria Selectivmedium (BSM) for 14 days at 25°C until they developed features permitting their identification at the species or genus level.

Furthermore, adult bark beetles fresh collected from trap trees and pheromone-baited traps were inoculated by dipping in fungal spore suspension. In an additional experiment the
healthy bark beetles were placed on spruce-bark pieces (5-10 – 12-25 cm) treated with spore suspension. The beetles of each variant with some spruce bark pieces were incubated at 25°C in humid chambers.

**Results and discussion**

Infected adult bark beetles were found under bark of trees or in galleries. As a result of our investigations, the hyphomycetous entomopathogenic fungi *Beauveria bassiana* (Bals.) Vuill., *Metarhizium* sp. have been identified. Mixed infection by these two fungal species occurred in some specimens. Furthermore, numbers of mites were observed under the bark of spruce trees. Their potential role in the dissemination of fungal spores could interestingly be subject for further investigations.

Several strains were isolated and cultivated on the different media for further investigation.

In experimental infections with strains of *Beauveria bassiana* and *Metarhizium* sp., high percentages of beetles inoculated with spore suspension died infected within 14 days following the treatment.

This is the first report of the presence of entomopathogenic fungi in populations of *Ips typographus* in spruce stands in Georgia.

**References**


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Researches on entomopathogenic bacteria and applications for fly pest control in a Mediterranean region (Sardinia, Italy)

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Abstract: During the last few years, investigations were carried out in Sardinia (Italy) to isolate naturally-occurring soil bacteria and detect their toxicity against medfly (Ceratitis capitata), olive fruit fly (Bactrocera oleae) and housefly (Musca domestica) as well as some of their pupal parasitoids. Strains of Bacillus thuringiensis were isolated and then comparatively studied with strains of other geographic origin. Morphological (presence of parasporal inclusions) and genetic (16S rRNA gene sequence, detection of cry genes) observations and protein profile analysis by SDS-PAGE were used for identification and characterization of the Bacillus thuringiensis isolates. Among about 300 different bacterial isolates, 4 were significantly toxic to housefly adults, 1 to housefly larvae, 11 to adult of olive fruit fly, 20 to olive fruit fly larvae, 4 to adult of medfly, and 22 to medfly larvae. No significant toxicity was detected against the Hymenoptera Opius concolor and Muscidifurax raptor (olive fruit fly and housefly pupal parasitoids, respectively). Experimental treatments in olive crops against olive fruit fly adults and in livestock against both housefly adults and larvae were carried out with formulations of the most toxic bacterial strains, encouraging the introduction of microbiological control in the integrated pest management of these fly species.

Key words: biological control, Bacillus thuringiensis, medfly, olive fruit fly, housefly

Introduction

The Mediterranean fruit fly or medfly, Ceratitis capitata Wied., is a species originated in the Mediterranean region of Europe and North Africa. Due to their wide polyphagy, the larvae represent one of the world’s most destructive fruit pests. The olive fruit fly, Bactrocera oleae Gmelin, formerly Dacus oleae, is an important pest of olives around the Mediterranean sea. Its monophagous larvae feed on olives, tunnealing in the fruit and causing an economically important lost of production. The housefly, Musca domestica, is a very common and worldwide species of medical and veterinary importance. Larvae develop in different organic substrates whereas adults, with their behaviour, are potential vectors of diseases.

Integrated pest management programmes for the control of these fly species usually require the use of chemical pesticides, but the risk for the environment and the appearance of insect resistance suggest the use of alternative and more ecologically compatible methods. However, entomopathogenic bacteria have not really found place in the management of these pests, although different investigations have demonstrated the toxicity of Bacillus thuringiensis against M. domestica (Hodgman et al., 1993; Zhong et al., 2000), C. capitata (Satta et al., 1998) and B. oleae (Alberola et al., 1999).
Among Diptera, most studies have been developed on mosquitoes and particular importance had the discovery of *B. thuringiensis* var. *israelensis* (Bti) (Goldberg & Margalit, 1977) and highly toxic strains of *Bacillus sphaericus* (Bsp) (Singer, 1974). The toxicity of *B. thuringiensis* is related to its ability to produce insecticidal proteins contained in parasporal crystals (Cry) that after ingestion by the insect, are solubilized and activated in the midgut, and after binding to specific receptor of the epithelium, insert in the cell membrane causing the formation of a pore responsible of an irregular flux of ions and water which bring the cell to the disruption. Cry genes are normally localized on plasmids and it facilitates recombination between toxin genes, resulting in a wide range of strains with different host specificities. Other bacterial protein toxins have also been found (eg. Cyt, Vip, Bin, Mtx) (de Maagd *et al*., 2003).

The aim of our research project was to build a collection of soil occurring entomopathogenic bacteria and to test their toxicity on some important fly pest both in laboratory assays and field applications.

**Materials and methods**

*Isolation, collection and characterization of strains*

About 300 different bacterial isolates were collected from soil of Europe, Asia, South America, Africa. Colonies with morphology similar to those of *B. cereus-B. thuringiensis* were plated in CCY agar, incubated until complete sporulation and then examined by phase microscope to detect for the presence of parasporal crystals. The crystal producing isolates were stored in nutrient broth plus 15% glycerol at -80°C.

A rapid protocol for protein extraction was used to check by SDS-PAGE for the protein content in the spore-crystal mixture used in bioassays (Alberola *et al*., 1999). Furthermore, all isolates were analyzed by PCR for their cry gene content and 16S rRNA gene sequencing was used for specific identification of various bacterial isolates. General primers were designed on highly conserved region of some cry genes encoding for protein toxins active against Diptera (*cry*2, *cry*4, *cry*11), Lepidoptera (*cry*1, *cry*2) and Coleoptera (*cry*8). Multiplex PCR was performed using different mixtures of primers. *B. thuringiensis var israelensis* ONR60 and *B. thuringiensis var kurstaki* HD-1 were used as positive controls for the detection of *cry*1, *cry*2, *cry*4, *cry*11 and cyt genes (Bravo *et al*., 1998). The presence of large number of *cry*1 and *cry*2 genes, as revealed by multiplex PCR, was followed by further characterization using primer pairs specific for these genes (Ceron *et al*., 1994).

**Laboratory bioassays**

Bioassays were performed on larvae and adults of the three fly species reared in the laboratory. A suspension containing a mix of spores and crystals of the different strains at a standard concentration of at least $10^9$ spores-crystal/mL was incorporated in their diet. The bacterial culture supernatant was as well used in toxicity test after being treated at 121°C for 15 minutes, to check for the presence of the thermostable and unwanted β-exotoxin. Larval experiments were performed in an incubator at 25 ± 1°C and relative humidity next to saturation. Groups of 10-20 larvae of the same age, were kept into Petri dishes and fed with the same diet used in the rearing, mixed with the bacterial suspension. Adults were kept in boxes at 25 ± 1°C and daily fed by capillary tubes containing the spore-crystal suspension diluted 1:1 with a 60% sucrose solution in distilled water (Alberola *et al*., 1999). Separate controls were run using water or *B. thuringiensis var. kurstaky* HD-1 and mortality was assessed daily.
Similar methods were used to feed with spore-crystal suspensions of the most fly toxic strains, the Hymenoptera, *Opinus concolor* Szepl. and *Muscidifurax raptor* Girault and Sanders, olive fruit fly and housefly parasitoids, respectively.

**Field applications**

Formulations containing some of the most toxic bacterial isolates were prepared in fermenter as a fluid paste and used in application trials in olive crops and cattle farms during the summer seasons.

A mixture of *B. thuringiensis* spore-crystal (5%) and Buminal (1%) was applied in the south-east side of olive trees at a dosage of 1,3 L/tree. The treatments were repeated weekly for a total of 10 times. Adult fly abundance and olive infestation were used as parameters to evaluate the efficacy of the treatments, comparing the treated areas to the control ones.

Solid candied food made of the bacterial formulation well mixed with sucrose in the ratio of 1:5 and at a total dosage of 2.5 Kg three times a week, was released in a cattle farm. Treatments were repeated for a 4 week period. In the meanwhile housefly management in a control farm was based on commercial food baits containing metomil.

The bacterial formulation was also spread on the paddock surface at a concentration of 10% and a dosage of 2L/ m². A similar not treated area was used as control. Fly abundance in treated and control areas were estimated by both adult trap monitoring and standard core sampling of manure in the paddok for juveniles.

**Results and discussion**

The results obtained show a great difference among isolate toxicities, even when the same *cry* genes were detected. It suggests the presence of other toxic factors. Although sometimes the unwanted and non specific thuringiensin (β-exotoxin) activity was recorded, in many cases toxicity was associated only to the spore-crystal mixture and never to the culture supernatant.

Multiplex PCR indicated the presence of 43 strains with the joint occurrence of *cry1-cry2* genes, 16 strains with *cry1* genes, 6 strains harbouring *cry2* genes, 2 strains *cry4* genes, 6 strains *cry8* genes and 8 strains *cry11* genes. Genes encoding for Cyt toxins were not detected in any of our strains. SDS-PAGE analysis in many cases corroborated the *cry* genes detection, but some strains that didn’t give any PCR amplifications showed as well the production of crystal proteins. These strains are being characterized using other primer pairs (data not shown). These results are encouraging as we can suppose that the toxic strains could contain new *cry* genes species or new protein combinations active against the fly species assayed. Some results, when using general and specific PCR primers, are summarized in Table 1 for the most active strains.

Toxicity test showed a general major susceptibility of larvae than adults. The isolates were often toxic on both larvae and adults, but in many cases they were either toxic to one stage or the other (Table 2).

Field applications gave general positive results. Nevertheless, treatments were not able to cause significant reductions in adult fly populations whereas a stronger effect was produced against housefly juvenile development rate.

**Conclusion**

This research project has permitted to set up a precious collection of entomopathogenic soil occurring bacteria and further work is planned for their characterization and the study of their toxicity against other target insects.
Interesting strains have also been found to be toxic to different fly species and the results of the first field applications encourage to study strategies of fly pest management involving the use of entomopathogenic bacteria.

Table 1. *Cry* gene detection in several of the most toxic *B. thuringiensis* strains.

<table>
<thead>
<tr>
<th>Susceptible insect</th>
<th>Bacterial isolates</th>
<th>Detected <em>cry</em> genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>General primers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Specific primers</td>
</tr>
<tr>
<td></td>
<td>SS29</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 1B, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>SS81</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>SS136</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 1B, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>SS137</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>SS141</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 1B, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>SS145</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 1B, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>SS152</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 1B, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>AF33</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ad, 1B, 1C, 2Ab</td>
</tr>
<tr>
<td></td>
<td>AF42</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 1Ad, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>AF66</td>
<td><em>Cry</em> 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Ab</td>
</tr>
<tr>
<td><strong>Ceratitis capitata</strong></td>
<td>JC120</td>
<td><em>Cry</em> 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Ae, 1Ia, 1Ac</td>
</tr>
<tr>
<td></td>
<td>SS152</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac</td>
</tr>
<tr>
<td><strong>Bactrocera oleae</strong></td>
<td>SS41</td>
<td><em>Cry</em> 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab</td>
</tr>
<tr>
<td></td>
<td>SS46</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
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<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 1Ad, 1B, 1C, 2Aa, 2Ab</td>
</tr>
<tr>
<td></td>
<td>SS47</td>
<td><em>Cry</em> 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cry</em> 1Aa, 1Ab, 1Ac, 1Ad, 1B, 1C, 2Aa, 2Ab</td>
</tr>
</tbody>
</table>

Table 2. Bacterial isolates with a significant toxicity against medfly, olive fruit fly and house fly.

<table>
<thead>
<tr>
<th>Species</th>
<th>N° assayed isolates</th>
<th>Significantly toxic isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Larvae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adults</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Mean % mortality (min-max)</td>
</tr>
<tr>
<td><strong>Ceratitis capitata</strong></td>
<td>140</td>
<td>22</td>
</tr>
<tr>
<td><strong>Bactrocera oleae</strong></td>
<td>133</td>
<td>20</td>
</tr>
<tr>
<td><strong>Musca domestica</strong></td>
<td>50</td>
<td>1</td>
</tr>
</tbody>
</table>

Acknowledgements

This study was supported by Italian Ministero dell’Università e della Ricerca Scientifica (Research Program: “Biotecnologie innovative per il controllo di insetti nocivi mediante l’impiego di agenti microbiologici”. Coordinator: Prof. Ignazio Floris, Sassari University - Italy).
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Microbiological control of lepidopterous defoliators in Sardinian cork oak forests

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Abstract: Mediterranean oak forests are exposed periodically to infestations of gypsy moth (Lymantria dispar). This insect, alone or in association with other lepidoptera, such as Malacosoma neustrium and Tortrix viridana, can cause the total defoliation of thousands of hectares in one year. From 1990 to 1995, aerial spray trials using several preparations of Bacillus thuringiensis var. kurstaki against gypsy moth were performed in Sardinian cork oak forests to determine the efficacy of the preparations and the most suitable modalities for a Mediterranean environment. Based on these preliminary trials, a microbiological control program to limit the damage caused by L. dispar and M. neustrium was implemented in approximately 45000 hectares from 2001 to 2004.

Key words: Gypsy moth, common lackey moth, Quercus suber, Bacillus thuringiensis kurstaki

Introduction

In Sardinia, severe infestations of the gypsy moth, Lymantria dispar (L.) (Lepidoptera: Lymantriidae), and the common lackey moth, Malacosoma neustrium (L.) (Lepidoptera: Lasiocampidae), have become increasingly frequent in cork oak forests degraded to woodland pasture, resulting in the total defoliation of large forested areas (Luciano & Prota, 1995). These infestations cause a progressive decay in the health conditions of the oak, which threatens not only the quantitativ-quality production of cork, but also the conservation of the forests (Luciano et al., 2003).

In response to the ever-increasing awareness of the need to control the gypsy moth infestations, in the period 1990-95, large cork-oak forest areas were subjected to microbiological control tests using several preparations of Bacillus thuringiensis var. kurstaki (Btk), in order to determine to determine the efficacy of the preparations and the most suitable modalities for a Mediterranean environment (Lentini & Luciano, 1995). Based on these preliminary trials, a microbiological control program to limit the damage caused by L. dispar and M. neustrium was implemented in approximately 45000 hectares from 2001 to 2004. Here we report the main results achieved in the preliminary sperimentation and in the control programme.

Microbiological control tests

The following products used were: 1) Bactucide P (Caffaro), wettable powder, distributed at low volume (LV) in 1 Kg/ha dosage; 2) Bactucid S (Caffaro), aqueous flowable concentrate formulation, distributed at ultra low volume (ULV) in 7 L/ha dosage; 3) Bactospeine (Siapa), wettable powder, distributed at LV in 2 Kg/ha dosage; 4) Foray 48 B (Novo Nordisk), aqueous flowable concentrate formulation, distributed at ULV in 2.5 L and 5 L per ha dosage; and 5) MVP (Mycogen) distributed at ULV in 8 L per ha dosage.

Treatments were carried out by helicopter equipped with a 12-m bar carrying 52 hydraulic nozzles for LV distribution or 4 rotary nozzles (Micronair) for ULV distribution.
Although it was not possible to compare exhaustively the effects of each of the four products in various cork-oak environments during the same year, useful information was obtained concerning their efficacious employment (Table 1). Application at 16 BIU/ha (Bactucide P) was insufficient to provide adequate foliage protection, whereas ca. 60 BIU/ha (Foray and Bactucide S) caused more than 80% mortality even when 30% of the phytophagous populations already consisted of III instars, guaranteeing effective control against the highest infestations. Accurate timing is essential: in the case of low infestations (2 larvae/branch tip), when at least 90% of the larval population consists of I and II instars, 32 BIU/ha determines more than 80% mortality. An important advantage is obtained from the application of the undiluted product at ULV, thereby providing greater aircraft productivity and reducing ground support requirements. Another important factor is the question of climate: as seen in the results, lowering of spring temperature can reduce treatment efficacy and necessitate a second application.

Table 1. Gypsy moth larval densities in B.t.k. treated and untreated cork oak plots (Sardinia, 1990-1995).

<table>
<thead>
<tr>
<th>Year</th>
<th>Treatment</th>
<th>BIU/ha</th>
<th>N. larvae per 30 cm branch tip</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>1990</td>
<td>Foray 48B</td>
<td>63.50</td>
<td>5.2 a**</td>
</tr>
<tr>
<td></td>
<td>Bactucide P</td>
<td>16.00</td>
<td>5.1 a</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td></td>
<td>4.9 a</td>
</tr>
<tr>
<td>1991</td>
<td>Foray 48B</td>
<td>31.75</td>
<td>1.6 a</td>
</tr>
<tr>
<td></td>
<td>Bactucide S</td>
<td>56.00</td>
<td>1.4 a</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td></td>
<td>1.5 a</td>
</tr>
<tr>
<td>1992</td>
<td>Foray 48B</td>
<td>31.75</td>
<td>0.6 a</td>
</tr>
<tr>
<td></td>
<td>Bactospine</td>
<td>32.00</td>
<td>0.8 a</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td></td>
<td>0.9 a</td>
</tr>
<tr>
<td>1993</td>
<td>Foray 48B</td>
<td>31.75 x 2#</td>
<td>1.7 a</td>
</tr>
<tr>
<td></td>
<td>Foray 48B</td>
<td>63.50</td>
<td>1.7 a</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td></td>
<td>2.1 a</td>
</tr>
<tr>
<td>1995</td>
<td>Foray 48B</td>
<td>63.50</td>
<td>6.6 a</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td></td>
<td>6.8 a</td>
</tr>
<tr>
<td>1995</td>
<td>MVP</td>
<td>4.1 a</td>
<td>2.0 b</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>4.5 a</td>
<td>4.2 a</td>
</tr>
</tbody>
</table>

*Days after treatment: A = 7; B = 14; C = 21.
** Values reported in the same line folwed by the same letter have no significant difference (P<0.05).
TD = total defoliation.
# The total distribution of 63.5 BIU/ha was applied in 2 equal doses a week apart.

In our experiments, *Bacillus thuringiensis* did not prove to have any direct effect on the beneficial insects, whose incidence seemed to be dependent on host densities in treated and
untreated areas (Lentini & Luciano, 1995). The lower mortality incidence, due to Braconids, observed in the treated areas, is attributable probably to the death, due to the treatment, of the young instars possibly already parasitized. Also *B. intermedia* showed a higher percentage of parasitism in the controls, the adults preferring these sunnier, defoliated areas (Luciano et al., 1991). The Ichneumonids (Hymenoptera) can be attracted by the relatively higher *L. dispar* population densities in the controls, as may be indirectly demonstrated by some cases of greater Sarcophagids (Diptera) emergence from the pupae than in the treated plots.

Microbiological control can have a negative impact on non target lepidopterous fauna in cork oak forests but observations carried out in Sardinia suggested that a treatment with Btk at a high rate has an effect on the richness and abundance of lepidoptera population similar to the one caused by the competition for food during the outbreak of *L. dispar* (Luciano & Lentini, 1999).

**Microbiological control program**

In Sardinia, the results of the experimentation with Btk have enabled a microbiological control program to limit the damage caused by *L. dispar* and *M. neustrium* which began in 2001, and involved approximately 45,000 hectares of cork-oak in the period 2001-04. The insecticide treatments were performed when the populations consisted of II-III larval instars. In all cases, 4 liters per hectare (equal to 42.4 BIU/ha) of Foray 48B (Valent BioSciences Corporation), were sprayed at ultra low volume with a helicopter equipped with 4 micronairs.

The treatments resulted in a very high larval mortality in all areas. The mortality in *M. neustrium* was generally higher than that in *L. dispar* populations in areas with mixed populations of the two species and in other treated forest areas with the lymantriid larvae at the II-III instars. In fact, 7-12 days after treatment, the average mortality of *L. dispar* was between 57.4 and 97.12% (Table 2), while the mortality of *M. neustrium* varied between 88.8 and 98.4% (Table 3) (data partly published in Luciano et al., 2003). The generally higher mortality of *M. neustrium*, compared with *L. dispar*, could be due not only to the higher susceptibility of *M. neustrium* to Foray 48B but also to the behavior of its larvae: in the early instars, they build their nests and feed mainly in the highest part of the crown.

Microbiological control has been particularly effective in Sardinia, resulting in adequate protection of the spring budding of cork-oaks and thus indirect protection of cork production.

The funds necessary for the implementation of these interventions were mainly provided under the «Programma d’Iniziativa Comunitaria (PIC) Interreg III A Francia-Italia “Isole”, Sardegna-Corsica-Toscanà». All resources were used for the purchase of bioinsecticides, the hire of the helicopter and flying crew, and for the collaboration of the University of Sassari in consultations and assistance in the planning and carrying out of the treatment. The cost per hectare varied from 43.31 to 48.45 €. However, the effective cost proved to be significantly higher: the indicated figures do not include the hire of backup (e.g. tankers) or payment for the work carried out by the Servizio Antinsetti delle Province, the Corpo Forestale e di Vigilanza Ambientale and the Ente Foreste della Sardegna. In fact, these corps provided labor and machinery free of charge, thereby enabling the defense of an area of forest significantly larger than that which would have been possible with the available resources. In this way, an effective synergy between public corps in pursuit of a common good was achieved, to the approval of public opinion and in particular that of the cork-oak growers.
Table 2. Gypsy moth egg-mass and larval densities in Sardinian cork oak forest treated with B.t.k.

<table>
<thead>
<tr>
<th>Year</th>
<th>Treated area</th>
<th>Hectares (No.)</th>
<th>Egg-masses on 40 trees (No. ± SD)</th>
<th>Larvae per 30 cm branch tip (No. ± SD)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 day before treatment</td>
<td>7-12 days after treatment</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>Berchidda (SS)</td>
<td>5,000</td>
<td>459 ± 525</td>
<td>2.28 ± 1.44</td>
<td>81.7</td>
</tr>
<tr>
<td></td>
<td>Buddusò (SS)</td>
<td>5,000</td>
<td>337 ± 136</td>
<td>2.60 ± 1.13</td>
<td>97.1</td>
</tr>
<tr>
<td>2002</td>
<td>Ardara (SS)</td>
<td>800</td>
<td>312 ± 273</td>
<td>3.63 ± 0.69</td>
<td>75.1</td>
</tr>
<tr>
<td></td>
<td>Sarule (NU)</td>
<td>700</td>
<td>391 ± 433</td>
<td>2.39 ± 1.03</td>
<td>85.5</td>
</tr>
<tr>
<td></td>
<td>Sorgono (NU)</td>
<td>1,900</td>
<td>1334 ± 2184</td>
<td>3.15 ± 1.26</td>
<td>79.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>Villanova (SS)</td>
<td>800</td>
<td>140 ± 312</td>
<td>6.67</td>
<td>75.6</td>
</tr>
<tr>
<td></td>
<td>Ploaghe (SS)</td>
<td>1,500</td>
<td>97 ± 56</td>
<td>2.10 ± 1.45</td>
<td>86.9</td>
</tr>
<tr>
<td></td>
<td>Sorgono (NU)</td>
<td>3,000</td>
<td>200 ± 160</td>
<td>2.18 ± 1.32</td>
<td>81.3</td>
</tr>
<tr>
<td></td>
<td>Abbasanta (OR)</td>
<td>2,000</td>
<td>317 ± 448</td>
<td>3.82 ± 2.17</td>
<td>91.8</td>
</tr>
<tr>
<td></td>
<td>Samugheo (OR)</td>
<td>2,000</td>
<td>29 ± 6</td>
<td>2.61 ± 2.77</td>
<td>91.4</td>
</tr>
<tr>
<td>2004</td>
<td>Bosa (NU)</td>
<td>2,000</td>
<td>17 ± 3</td>
<td>1.80 ± 2.16</td>
<td>82.6</td>
</tr>
<tr>
<td></td>
<td>Abbasanta (OR)</td>
<td>2,000</td>
<td>98 ± 129</td>
<td>1.53 ± 0.87</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>Iglesias (CA)</td>
<td>2,000</td>
<td>154 ± 115</td>
<td>7.88 ± 5.86</td>
<td>57.4</td>
</tr>
</tbody>
</table>

Table 3. Common lackey moth egg-mass and larval densities in Sardinian cork oak forest treated with B.t.k.

<table>
<thead>
<tr>
<th>Year</th>
<th>Treated area</th>
<th>Hectares (No.)</th>
<th>Egg-masses on 40 branches (No. ± SD)</th>
<th>Larvae on 40 branches (No. ± SD)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 day before treatment</td>
<td>7-10 days after treatment</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>Bono (SS)</td>
<td>3,200</td>
<td>17.7 ± 3.9</td>
<td>483.6 ± 274.4</td>
<td>98.4</td>
</tr>
<tr>
<td></td>
<td>Orotelli (NU)</td>
<td>1,700</td>
<td>5.6 ± 3.8</td>
<td>504.8 ± 357.1</td>
<td>98.2</td>
</tr>
<tr>
<td>2003</td>
<td>Mamoïada (NU)</td>
<td>2,700</td>
<td>6.3 ± 3.2</td>
<td>121.3 ± 123.4</td>
<td>88.8</td>
</tr>
<tr>
<td></td>
<td>Thiesi (SS)</td>
<td>700</td>
<td>5.0</td>
<td>57.50</td>
<td>96.6</td>
</tr>
<tr>
<td>2004</td>
<td>Orune (NU)</td>
<td>8,000</td>
<td>5.1 ± 3.6</td>
<td>447.5 ± 343.6</td>
<td>98.1</td>
</tr>
</tbody>
</table>

References


Comparison of the efficacy of AdorGV and chemical insecticides against the Summer fruit tortrix, *Adoxophyes orana*, in commercial apple orchards in the Czech Republic

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² ZD Chelčice, BIOLA Laboratories, Záhorčí 106, 389 01 Vodňany, Czech Republic

Abstract: The field trials of the Summer fruit tortrix (*Adoxophyes orana*) control in three commercial apple orchards were carried out in the Czech Republic in 2002-2004. *Adoxophyes orana* granulovirus, AdorGV (CAPEX 2), etofenprox (TREBON 10 F), flufenoxurone (CASCADE 5 EC), fenoxycarb (INSEGA WP), triflumuron (ALSYSTIN 480 SC), triflubenzuron (NOMOLT 15 SC), phosalone (ZOLONE 35 EC), deltamethrine (DECIS EW 50) or acetamiprid (MOSPILAN 20 SP) were applied against the overwintering larvae or larvae of the first generation of the pest. From the tested insecticides, CASCADE 5 EC was the most effective against *A. orana*. CAPEX 2 had similar efficacy as CASCADE 5 EC except the first years of application. Efficacy of the other tested insecticides was insufficient against *A. orana*. When CAPEX 2 is used first time in locality, it is necessary to apply it 2-times against the overwintering larvae and then to made 2 applications against the larvae of the first generation (all in interval 7-10 days). The sequence of CAPEX 2 application given above reduces the population density of the 2nd generation of *A. orana* and in the 2nd year of treatment it prevents completely the fruit damage.

Key words: *Adoxophyes orana*, AdorGV, Capex, etofenprox, flufenoxurone, fenoxycarb, triflumuron, triflubenzuron, phosalone, deltamethrine, acetamiprid

Introduction

The Summer tortrix moth, *Adoxophyes orana*, didn’t cause any important damage in the past in the Czech Republic and chemical control wasn’t aimed against this pest. As a matter of fact, populations of this insect were likely reduced by organophosphates and non-selective insecticides used against *Cydia pomonella*. Since 1999 however, *A. orana* caused important damages in apple orchards in Eastern and Central Bohemia. Recently, it caused exceptionally damages on peach trees in Moravia (damage on leaves, not on fruits). Low efficacy of IGRs and neonicotinoids in the field was observed whereas some populations of *A. orana* on apples became resistant to organophosphates. At present, *Adoxophyes orana* granulovirus (AdorGV) is frequently used in the biological control of *A. orana* in fruit orchards in Switzerland and Germany, where the virus is commercially available under the name Capex® 2 (Charmillot, 1992; Andermatt, 1986). In the Czech Republic, registration of Capex® 2 is being prepared.

The aim of this study was to evaluate the efficacy of AdorGV (CAPEX 2) on *A. orana* larvae in comparison with chemical insecticides and ascertain the optimal terms of treatment and frequency of application of this biological product.

Material and methods

Experimental orchards

The experiments were carried out in two apple orchards it the East Bohemia, around the villages Libčany and Svinišťany and in one apple orchard in Slaný in Central Bohemia.
Description of variants of treatments on the experimental plots and used insecticides are given in Table 1.

The insecticides were applied in following rates: AdorGV (CAPEX 2, commercialized by Andermatt Biocontrol AG, and to be applied at 100 mL/ha containing $5 \times 10^{10}$ granules/mL), etofenprox (TREBON 10 F, 0.5 L/ha), flufenoxuron (CASCADE 5 EC, 1.5 L/ha), fenoxycarb (INSEGAR WP, 0.3 kg/ha), triflumuron (ALSYSTIN 480 SC, 0.25 L/ha), triflubenzuron (NOMOLT 15 SC, 0.75 L/ha), phosalone (ZOLONE 35 EC, 2.0 L/ha), deltamethrine (DECIS EW 50, 0.2 L/ha) and acetamiprid (MOSPILAN 20 SP, 0.25 kg/ha). CAPEX 2 was applied 2-times against the overwintering *A. orana* larvae (in 7-10 days interval) and 1- or 2-times against the L1 and L2 larvae of the 1st summer generation.

**Efficacy evaluation method**

The average percentage of shoot damage caused by the overwintering, the 1st and the 2nd summer generation larvae was estimated on ten shoots per tree, at 100-600 trees per plot, as well as the number of the damaged leaves per each attacked shoot. The average percentage of fruit damage was counted from 100 to 2000 fruits per plot, picked by 10-20 per trees. Flight activity of *A. orana* males and its fluctuation were monitored by pheromone traps (BIOLATRAP-delta with diffuser BIOLATRAP-AO during 1999-2001 or Pherobank™ ADOR during 2002-2004) in Libčany and Svinišťany. In Slaný, larvae of *A. orana* were collected from injured shoots 14 days after the treatment in constant time-interval. Number of live larvae and mortality of larvae was evaluated. After 10 days of development of collected larvae in the laboratory, the mortality was evaluated.

Table 1. Description of experimental plots for the comparison of AdorGV and several chemicals against *Adoxophyes orana* in the Czech Republic.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Variant of treatment</th>
<th>Year of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2001</td>
</tr>
<tr>
<td>Libčany</td>
<td>Capex I</td>
<td>No treatment</td>
</tr>
<tr>
<td></td>
<td>Capex II</td>
<td>No treatment</td>
</tr>
<tr>
<td></td>
<td>Untreated control</td>
<td>No treatment</td>
</tr>
<tr>
<td>Svinišťany</td>
<td>Capex I</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Capex II</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Trebon</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cascade</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Untreated control</td>
<td>–</td>
</tr>
<tr>
<td>Slaný</td>
<td>Capex</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cascade</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Alsystin</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Mospilan</td>
<td>–</td>
</tr>
</tbody>
</table>
Results and discussion

Shoot and rosette damage, leaf and fruit damage

In the first year of treatment by CAPEX 2 in Libčany 2002, the injury of shoots was reduced in comparison with untreated variant by 50%. Similarly, the damage of shoots was reduced in the second year 2003 of treatment by CAPEX 2 (Fig. 1). In 2004, the shoots’ damage was nearly zero in all the variants. After three years of treatment by CAPEX 2 in Libčany, AdorGV spread into the surroundings and caused the reduction of population density of A. orana also in the untreated plots. According to Charmillot (1992), AdorGV can persist in orchards between generations, in shady locations where it can multiply in contaminated larvae. In contrast to AdorGV, the persistence of Cydia pomonella granulovirus (CpGV) among the C. pomonella individuals surviving CpGV treatment was shown to be very low (Kundu et al., 2003).

Figure 1. Damage of shoots caused by larvae of the 2nd generation of Adoxophyes orana (evaluated before harvest) in two different regimes of control by Capex 2 in Libčany in 2001 – 2004.

In Svinišťany, the damage of shoots was reduced after 2nd generation by nearly 50% in comparison with the untreated control already in the first year of treatment by CAPEX 2 in 2003 and was lower then in variant TREBON 10 F (Fig. 2). After the application of CASCADE 5 EC, the damage of shoots was even lower than in variant CAPEX 2. In the second year of treatment, the efficacy of all the used preparation CAPEX 2, TREBON 10 F and CASCADE 5 EC was good against the A. orana according to reduction of shoot damage. Similarly as in Libčany, the reduction of population density of A. orana in the untreated plots was caused by the spreading of AdorGV in orchard.

The efficacy of CAPEX 2 in the first year of treatment wasn’t sufficient to reduce the shoot damage (Fig. 3). The shoot damage was considerably reduced in the second and mainly in the third year of treatment by CAPEX 2.

In Slaný, CASCADE 5 EC was the most effective against A. orana when evaluated 14 days after the treatment. Mortality of A. orana larvae reached in this case 70% (Table 2). After 10 days of development of larvae in the laboratory, mortality of larvae after 24 days...
from the application of CAPEX 2 in orchard reached 85% and was the same as the mortality of larvae in the laboratory after 24 days from the application of CASCADE 5 EC in orchard. Total mortality of larvae after the application of ALSYSTIN 480 SC and MOSPILAN 20 SP was much lower (Table 2).

Figure 2. Damage of shoots caused by *Adoxophyes orana* larvae of the overwintering, 1st and 2nd generation after treatment with Capex 2 and two different regimes of insecticide treatment in Svinišťany, in 2003 and 2004.

Fruit injury caused by larvae of *A. orana* evaluated before harvest was the lowest in variant CASCADE 5 EC. Fruit injury in variant CAPEX 2 was high in the first year of treatment and reached 43% (Table 2). In comparison with the totally ineffective insecticide ZOLONE 35 EC, CAPEX 2 reduced the fruit injury in the first year of the treatment by 50%.

**Pheromone traps**
The seasonal capture of males was relatively low in the first year of the monitoring (2000) in Svinišťany (Table 3). In 2001, when no control was carried out, the capture increased 7.5 times in CAPEX II variant. In 2002, decreasing to 22% of the male capture of the previous year was recorded where CAPEX was sprayed 2-times against the overwintering larvae and ones against the 1st summer generation larvae (CAPEX I), but as well in the untreated plot (CAPEX II) to 77% or 10% in the comparison with 2000 and 2001, respectively.
The seasonal capture of males was numerous in 1999 in Libčany (Table 4). The captures decreased by 50% in the following two years (2000-2001) even though no control were carried out. The comparable captures of males to the previous two years was registered in 2002, where two spraying by CAPEX 2 were applied against the overwintering larvae and the 1st summer generation larvae. In extreme hot year 2003, the captures decreased strongly to 17% and 23% of the year 2002 in the both plots, where CAPEX 2 was sprayed 2-times against the overwintering larvae and 2-times against the 1st summer generation larvae or 2-times against the overwintering larvae only. In 2004, two CAPEX 2 sprayings against the overwintering larvae reduced the captures in both plots to eradication like situation, so no treatment against the 1st summer generation was carried out.

Table 2. Long term effect of Capex 2 on Adoxophyes orana larvae in comparison with chemical insecticides in Slaný in 2003. Evaluation in orchard: number of live larvae and mortality of larvae collected in orchard from attacked leaves. Evaluation in lab: number of live larvae and mortality of larvae in lab 10 days after collection in orchard.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Evaluation in orchard</th>
<th>Evaluation in lab</th>
<th>Total % mortality of larvae</th>
<th>% of damaged fruits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nb of live larvae 14 days after treatment</td>
<td>Nb of live larvae 24 days after treatment</td>
<td>% mortality of larvae 14 days after treatment</td>
<td>% mortality 24 days after treatment</td>
</tr>
<tr>
<td>Capex</td>
<td>135</td>
<td>20</td>
<td>0</td>
<td>85.2</td>
</tr>
<tr>
<td>Cascade</td>
<td>40</td>
<td>20</td>
<td>70.4</td>
<td>14.8</td>
</tr>
<tr>
<td>Alsystin</td>
<td>108</td>
<td>81</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Mospilan</td>
<td>118</td>
<td>60</td>
<td>12.6</td>
<td>43</td>
</tr>
<tr>
<td>Decis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zolone</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
When CAPEX 2 is used for the first time in a locality, it is necessary to apply it 2-times against the overwintering larvae (according to advice of the producer) and then make 2 applications against the 1st summer generation larvae (all in interval 7-10 days). It is necessary to start with application of CAPEX 2 against the overwintering L2-L3 larvae. Timing of treatment against the larvae of the 1st generation is in postbloom at the beginning of occurrence of L1 larvae of the 1st generation. The sequence of CAPEX 2 application given above reduces the population density of 2nd generation of *A. orana*, and in the 2nd year of treatment it prevents completely the fruit damage. Several years (2-3 years) of treatment by AdorGV resulted in more stable pest reduction and in sufficient control by application against the overwintering larvae only.

Table 3. Seasonal captures of *Adoxophyes orana* into pheromone traps per 1 trap at two different plots in Svinišťany in 2000 – 2004.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Year of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2000</td>
</tr>
<tr>
<td>Capex I</td>
<td>No trap</td>
</tr>
<tr>
<td>Capex II</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Table 4. Seasonal captures of *A. orana* into pheromone traps at the different plots in Libčany in 1999 – 2004.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Year of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1999</td>
</tr>
<tr>
<td>Capex I</td>
<td>No trap</td>
</tr>
<tr>
<td>Capex II</td>
<td>299.0</td>
</tr>
</tbody>
</table>

Acknowledgements

We thank to Ministry of Agriculture of the Czech Republic for funding this research under the project No. QD1048 and 0002700603.

References


Evaluation of efficacy of *Adoxophyes orana* granulovirus on the reduction of *Adoxophyes orana* populations using PCR

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² ZD Chelčice, BIOLA Laboratories, Záhorčí 106, 389 01 Vodňany, Czech Republic

**Abstract:** The presence of *Adoxophyes orana* granulovirus (AdorGV) in larvae of the summer fruit tortrix (*Adoxophyes orana*) after treatment by AdorGV-based preparation Capex® 2 against *A. orana* was investigated in three localities in the Czech Republic in 2003-2005. A PCR assay was developed for this purpose; it could be used to identify the frequency of AdorGV in the insect populations infected with AdorGV as well as in natural populations. The AdorGV was detected in larvae of *A. orana* from all three localities treated by Capex® 2. The portion of the overwintering larvae positive for AdorGV differed according to locality and ranged from 5% to 80% after the treatment. The AdorGV was detected from 10% to 80% in larvae from localities, where AdorGV was disseminated by special pheromone traps. No AdorGV was detected in larvae collected in the locality without virus treatment. The results suggested a strong persistence of AdorGV in surviving larvae after direct treatment by AdorGV causing high mortality of larvae in next generations. The population density was reduced by AdorGV under damage threshold during two years after the virus treatment.

**Key words:** *Adoxophyes orana*, *Adoxophyes orana* granulovirus, AdorGV, PCR, detection

**Introduction**

The summer fruit tortrix moth, *Adoxophyes orana* (Fischer v. R., 1834) is the most important leafroller in Europe, especially in Belgium, south Netherlands, Austria, south Switzerland, Germany and the Balkans (van der Geest and Evenhuis, 1991). Recently, *A. orana* became the most important leafroller also in the Czech Republic. At present, *Adoxophyes orana* granulovirus (AdorGV) is frequently used in the biological control of *A. orana* in fruit orchards in Switzerland and Germany, where the virus is commercially available under the name Capex® 2 (Charmillot, 1992; Andermatt, 1986; Anonymous, 2002). In the Czech Republic, registration of Capex® 2 is being prepared.

AdorGV was isolated in Switzerland in 1975 from larvae of *A. orana* (Schmid et al., 1983). This virus is a “slow” granulovirus, i.e. the host dies in the final instar regardless of when it was infected (Flückiger, 1982). Recently, the complete nucleotide sequence of the AdorGV DNA genome was determined and analyzed (Wormleaton et al., 2003). The AdorGV genome is composed of 99,657 bp. It is the smallest lepidopteran baculovirus sequenced to date with the highest A + T (AT) content of 65.5% (Wormleaton et al., 2003).

This paper reports the determination, using PCR, of the frequency of AdorGV using PCR in insect populations infected with AdorGV as well as in natural populations of *Adoxophyes orana*. 
Material and methods

Field trials
L3-L4 larvae of *A. orana* were collected for PCR detection of AdorGV presence in four localities were Capex® 2 was applied (Libčany, Svinišťany, Slaný, Těchlovice) and in one locality without AdorGV treatment (Holovousy). Capex® 2 (100 mL/ha containing 5x10^{10} granules/mL) (Andermatt Biocontrol) was sprayed 2-times against the overwintering larvae (pre-bloom) and 1- or 2-times against the first generation larvae (post-bloom) and disseminated by special pheromone stations (Svinišťany, Libčany). In Svinišťany and Libčany, larvae were collected in the first and second year of AdorGV treatment. In Těchlovice, larvae were collected in the second year after AdorGV treatment, and in Slaný, larvae were collected in the year of AdorGV treatment and in the following year post treatment. Plots treated by chemical insecticides, untreated plots and plots with auto-dissemination of AdorGV were in neighborhood of plots sprayed by Capex® 2. Larvae of *A. orana* of the overwintering and first generation were collected 10 – 14 days after Capex® 2 treatment. In Libčany and Svinišťany, larvae of the second generation of *A. orana* were collected, against which no treatment was carried out. AdorGV was detected by PCR also in larvae, pupae and adults of *A. orana* from laboratory colony established from natural *A. orana* population from Slaný.

Virus detection
Genomic DNA of individual larvae was isolated using DNeasy Tissue Kit (QIAGEN) following the manufacturer’s instructions. AdorGV was detected by PCR using primer pairs AoG III/AoG IV targeting a 403bp fragment of “granulin” gene from the complete sequence of *Adoxophyes orana* granulovirus genome (Wormleaton et al., 2003).

Results and discussion

Numbers and percentages of *A. orana* larvae positive for AdorGV from orchards subjected to different treatments were presented in Table 1. The portion of the overwintering larvae positive for AdorGV differed according to locality. The value after the treatment was 15% in Slaný, ranged from 55% to 64% in Svinišťany, and reached up to 80% in Libčany. The presence of AdorGV in 1st summer generation larvae was 20% in Slaný, and ranged from 52% to 80% in Svinišťany. In the 2nd generation larvae in the year of AdorGV treatment, the presence of AdorGV ranged from 22% to 55% and from 70 to 80% in Svinišťany and Libčany, respectively. The presence of AdorGV in *A. orana* larvae was found in 12% (Těchlovice) and in 10%-25% (Slaný) of individuals in the year following post treatment. AdorGV was found in 50% – 60% of larvae collected in orchards in the second and third year of treatment by Capex® 2 (Svinišťany, Libčany). In the orchard without AdorGV treatment (Holovousy), no AdorGV was detected in larvae of *A. orana* in 2004 neither in 2005. AdorGV was detected in larvae (29%) and pupae (23%) of *A. orana* from laboratory colony established from natural *A. orana* population from Slaný. No virus was detected in *A. orana* adults from laboratory colony. The efficacy of Capex® 2 on the reduction of *A. orana* population was high in all three treated orchards. After the second or third year of AdorGV treatment, the population density of *A. orana* was reduced to zero.

Laboratory assays have shown that despite good activity against young larvae, the development of the disease is slow and mortality generally resulted in final stage larvae only (Flückiger, 1982). According to Oho (1975) virus exhibited very slow pathogenicity and newly hatched larvae infected by AdorGV die at fifth instar only. *Adoxophyes orana* granulovirus can persist in orchards between generations, especially in shady locations where it can multiply in contaminated larvae (Charmillot, 1992). Until now, there are any data
available about the persistence of AdorGV in *A. orana* larvae after AdorGV treatment in field. Using highly sensitive PCR based detection tools, we found that the persistence of AdorGV in *A. orana* larvae after AdorGV treatment as well as in next *A. orana* generation was high. In contrast to AdorGV, the persistence of *Cydia pomonella* granulovirus (CpGV) among the *C. pomonella* individuals surviving CpGV treatment is low. CpGV was present in only 15% of the surviving *C. pomonella* larvae in CpGV-treated orchards (Kundu *et al.*, 2003).

In practice the reduction of high populations with Capex® 2 gave varying results with a sometimes unacceptable efficacy (Höhn *et al.*, 1998). However, the application of Capex® 2 has a long term effect on the population of *A. orana* (Sato *et al.*, 1986). In our experiments, the population density of *A. orana* was reduced by AdorGV under damage threshold during two years after the virus treatment. The results suggest a strong persistence of AdorGV in surviving larvae after direct treatment by AdorGV causing high mortality of larvae in next generations.

**Acknowledgements**

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**References**


Oho, N. 1975: Possible utilization of a granulosis virus for control of *Adoxophyes orana* Fischer von Röslerstam (Lepidoptera: Tortricidae) in apple orchards. – JIBP Synthesys, Tokio, 7: 61-68.


Table 1. Number and percentage of *Adoxophyes orana* larvae positive for AdorGV from orchards subjected to different treatments.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Year</th>
<th>Date of collection / generation</th>
<th>Control type</th>
<th>Nb of evaluated larvae</th>
<th>Nb of AdorGV positive larvae</th>
<th>% AdorGV positive larvae</th>
<th>% average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sviníšťany</td>
<td>2003</td>
<td>22 March / overwintering</td>
<td>Capex® 2 - 2003</td>
<td>11</td>
<td>7</td>
<td>64</td>
<td>51.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 May / overwintering</td>
<td>Capex® 2 - 2003</td>
<td>20</td>
<td>11</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 May / overwintering</td>
<td>Trebon - 2003</td>
<td>20</td>
<td>6</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>19 May / 1st generation</td>
<td>Capex® 2 - 2002 + 2003</td>
<td>21</td>
<td>11</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>19 May / 1st generation</td>
<td>Capex® 2 - autodissemination 2003</td>
<td>15</td>
<td>12</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 July / 2nd generation</td>
<td>Capex® 2 - 2002 + 3</td>
<td>20</td>
<td>11</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 July / 2nd generation</td>
<td>Capex® 2 - autodissemination 2002 + 3</td>
<td>18</td>
<td>4</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 July / 2nd generation</td>
<td>Capex® 2 - 2002 + 3</td>
<td>20</td>
<td>11</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2004</td>
<td>22 March / overwintering</td>
<td>Capex® 2 - 2002 + 3</td>
<td>10</td>
<td>6</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Libčany</td>
<td>2003</td>
<td>7 May / overwintering</td>
<td>Capex® 2 - 2002 + 3</td>
<td>20</td>
<td>16</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 May / overwintering</td>
<td>Capex® 2 - autodissemination 2002 + 3</td>
<td>20</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 May / overwintering</td>
<td>untreated control</td>
<td>20</td>
<td>5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 July / 2nd generation</td>
<td>Capex® 2 - 2002 + 3</td>
<td>20</td>
<td>16</td>
<td>80</td>
<td></td>
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<td>20</td>
<td>14</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 July / 2nd generation</td>
<td>Capex® 2 - autodissemination 2002 + 3</td>
<td>14</td>
<td>7</td>
<td>50</td>
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<td></td>
<td></td>
<td>untreated control</td>
<td>20</td>
<td>7</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2004</td>
<td>3 May / overwintering</td>
<td>Capex® 2 - 2002 + 3</td>
<td>20</td>
<td>3</td>
<td>15</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 May / overwintering</td>
<td>Capex® 2 - 2002 + 3</td>
<td>20</td>
<td>4</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 May / 1st generation</td>
<td>untreated control</td>
<td>20</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 May / 1st generation</td>
<td>Capex® 2 - 2003</td>
<td>20</td>
<td>5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>untreated control</td>
<td>20</td>
<td>4</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>untreated control</td>
<td>20</td>
<td>5</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Těchlovice</td>
<td>2005</td>
<td>26 April / overwintering</td>
<td>Capex® 2 - 2004</td>
<td>25</td>
<td>3</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Holovousy</td>
<td>2004</td>
<td>12 July / 2nd generation</td>
<td>untreated control</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td>26 April / overwintering</td>
<td>untreated control</td>
<td>20</td>
<td>0</td>
<td>0</td>
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</table>
Codling moth granulovirus: First indication of variations in the susceptibility of local codling moth populations

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Abstract: This study is part of a BMVEL (German Federal Ministry for Consumer Protection, Food and Agriculture) project for prevention of codling moth damage by long-term population control on large areas. Local codling moth populations were collected in three different orchards in the South of Germany; two of them having been treated with CpGV over many years (Lake Constance II and South Baden) and one since two years (Lake Constance I). The susceptibility of the offsprings of the overwintering larvae for the granulovirus of codling moth (CpGV “Mexican strain”) was investigated in bioassays on artificial diet and compared to a laboratory strain of the codling moth. The results indicated significant differences in sensitivity to the virus between the three local codling moth populations. The determination of the LC50-values showed that South Baden and Lake Constance II were more than thousand fold less susceptible than Lake Constance I and the laboratory strain. The slope of the dose-mortality-regression lines of populations South Baden and Lake Constance II was significantly lower than those of Lake Constance I and the laboratory strain. This indicates a high inhomogeneity in the individual response of the larvae against the virus. At present, it is not possible to judge the significance of these first observations for the practical use of the virus in the field. Therefore, more investigations of other local codling moth populations have been initiated.

Key words: CpGV, granulovirus, baculovirus, codling moth, susceptibility, field population, field tests, integrated plant protection

Introduction

Background of this study was a project on prevention of codling moth (Cydia pomonella) damage by long-term population control on wide areas with codling moth granulovirus (CpGV) funded by BMVEL (German Federal Ministry for Consumer Protection, Food and Agriculture). The codling moth granulovirus (CpGV) is applied in ecologically treated apple orchards in Germany for more than ten years. It is used not only for direct reduction of fruit damage but also to reduce the population density of the subsequent codling moth generation. All the commercially available CpGVs are based on only one genotype. Though, due to the biological characteristics of baculoviruses the development of resistance is rather unlikely (Granados & Federici, 1986) and, so far, there were no reports of resistance of codling moth against CpGV in the field, in the framework of the BMVEL study, the possible presence of resistance to GpGV in some codling moth populations was monitored. Recently, a few apple growers in South Baden started to observe high codling moth infestation in spite of repeated CpGV applications. To find out whether this phenomenon is based on a reduced susceptibility of the codling moth, individuals of this local population in South Baden (orchard planted in fall 1996 on farmland and treated since then with CpGV) and also of two others from the area of Lake Constance (I: treated over 2 years and II: planted 1992 on grassland and treated since then) were collected in 2003. The offsprings of the overwintering generation were examined
in bioassays on artificial diet in the laboratory and compared to a laboratory strain of the codling moth serving as a standard.

**Material and methods**

**Virus**
The granulovirus of the codling moth used in the bioassays is a descendent from the *CpGV* collected in Northern Mexico (“Mexican strain”) (Tanada, 1964). It was propagated in host insects and purified by the method described by Huber (1981).

**Test insects**
Larvae of a laboratory strain of the codling moth served as standard in the bioassays. This laboratory strain has been established more than thirty years ago at the Institute for Biological Control of the BBA in Darmstadt. The rearing method has been described by Bathon (1981).

Wild codling moth larvae were collected with corrugated cardboard bands placed around the trunk of apple trees in the respective orchards and maintained in a frost-free room during winter. In spring they were kept on a sheltered balcony until hatch of the moths at the beginning of May. The moths were kept at room temperature and under natural light conditions in rearing containers for copulation and oviposition.

**Bioassay method**
The bioassays were conducted following the method described by Huber (1981). The virus was incorporated into an artificial diet by thorough mixing of *CpGV* suspensions of different concentrations with codling moth diet (Ivaldi-Sender, 1974) kept at a temperature of 45°C in a water bath. The mixture was dispensed into special boxes (LICEFA, Bad-Salzuflen, Germany) with 50 separate cells (1.5 x 1.5 x 2 cm). One neonate larva was placed in each cell. The boxes were covered with a layer of tissue paper, a polyethylene sheet, and a hard-plastic cover, and fixed with two rubber bands. The boxes were incubated at 26°C, 60-70% RH with an 16 hr photoperiod. Larvae were examined for virus mortality 6 days and 14 days after start of the bioassay. Concentration / larval mortality-correlation was calculated using a probit analysis programme based on a maximum likelihood procedure (MLP 3.08, NAG, Lawes Agricultural Trust, Rothamsted Experimental Station, 1987) in order to obtain LC50 values and other parameters of the dose mortality response.

**Results and discussion**
The neonate offsprings (F1-generation) of the diapausing larvae from the three local field populations (Lake Constance I, Lake Constance II and South Baden) were submitted to a bioassay to estimate their susceptibility for granulovirus. The data were compared with the mortality obtained with a codling moth laboratory strain, showing a normal sensitivity for the *CpGV*.

First results from bioassays evaluated after 6 days indicated significantly lower mortality for the strains Lake Constance II and South Baden than for the strain Lake Constance I (Fig. 1). Even with increased virus concentrations of up to 1x10^7 G/ml medium, larval mortality did not exceed 20%. In comparison, the sensitivity of the strain Lake Constance I was similar to that observed for the laboratory strain. Larval mortality raised from 10% to 100% with an increase in virus concentration from 1x10^5 G/ml medium to 1x10^6 G/ml.

To find out whether the two less sensitive populations (Lake Constance II and South Baden) are able to survive high virus concentrations also for a longer time, the test insects were kept over 14 days. This resulted in higher mortality. However, even larvae exposed to the dose of 10^7 G/ml medium showed 90% mortality only (Fig. 2).
Figure 1. Mortality data obtained from bioassays with different codling moth strains 6 days after incubation. Each symbol represents the mortality recorded from 50 test insects.

Figure 2. Mortality data obtained from bioassays with different codling moth strains 14 days after incubation. Each symbol represents the mortality determined with 50 test insects.

For an accurate determination of differences between the codling moth strains in their susceptibility to the virus, mortality data obtained from bioassays after 14 days were subjected to probit analysis. Important differences in the slope of the regression lines were detected, indicating heterogeneity of the insect populations towards the virus (Fig. 3). The slope of the regression line of Lake Constance I, which corresponded very well to the regression line of the normal, sensitive laboratory strain, was steeper (slope 1.3) than that for the two others, Lake Constance II (slope 0.4) and South Baden (slope 0.5). In addition, LC50 values
calculated by probit analysis (Table 1) differed significantly between some of the four codling moth populations.

![Probit regression lines of the different codling moth populations obtained from bioassays 14 days after incubation.](image)

In a comparison of the data obtained 6 days after start of experiments between the strain Lake Constance I and the laboratory strain, no significant differences in the LC50 values (8.6x10^2 and 5.1x10^2 G/ml) were found (Chi Square Test, p = 95 %). Bioassays for the two strains incubated for 14 days provided also dose response curves with significant parallelism and comparable LC50 values (169 and 341 G/ml). Compared to the field strain Lake Constance I, the LC50 values of the strains South Baden and Lake Constance II were significantly increased by a factor of 1,000 and of 500 respectively. This indicates that the susceptibility for the CpGV is highly reduced in these two field populations.

These results obtained from bioassays confirmed that the local codling moth field populations differ in their susceptibility to the CpGV. The F1 generation originating from the orchards South Baden and Lake Constance II were significantly less sensitive than the strain from the orchard Lake Constance I. These differences in susceptibility are represented by LC50 values being approximately 1,000 times higher than for the normal sensitive strain Lake Constance I. In addition, the lower slopes of the probit regression lines indicate a higher variation in susceptibility among individuals of the populations South Baden and Lake Constance II. The individuals of Lake Constance I showed to be as sensitive as those of the laboratory strain, which is indicated by similar LC50 values and almost the same steep dose response regression lines.

At present it is not possible to decide whether the reduced susceptibility is a result of a selection process or whether these less sensitive populations already existed in the orchards before the beginning of the virus applications. Also an immigration of other local codling moth populations into the CpGV treated apple orchards should be considered.
Table 1. Comparison of the sensitivity of the different codling moth strains estimated in bioassays with neonates after 6 days and 14 days incubation. The slopes of the probit mortality regression lines and the calculated LC\textsubscript{50} values are listed.

<table>
<thead>
<tr>
<th>codling moth strain</th>
<th>incubation time</th>
<th>LC\textsubscript{50}[G/ml diet] (95% conf. limits)</th>
<th>slope (standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>laboratory strain</td>
<td>6 days</td>
<td>5,1 x 10\textsuperscript{3} (4,48 – 5,82)</td>
<td>1,77 (0,126)</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>3,41 x 10\textsuperscript{2} (2,74 – 4,41)</td>
<td>1,61 (0,186)</td>
</tr>
<tr>
<td>Lake Constance I</td>
<td>6 days</td>
<td>8,57 x 10\textsuperscript{3} (6,87 – 10,76)</td>
<td>1,49 (0,130)</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>1,69 x 10\textsuperscript{2} (1,05 – 2,49)</td>
<td>1,29 (0,129)</td>
</tr>
<tr>
<td>South Baden</td>
<td>6 days</td>
<td>– *</td>
<td>– *</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>3,02 x 10\textsuperscript{3} (2,07 – 4,62)</td>
<td>0,52 (0,041)</td>
</tr>
<tr>
<td>Lake Constance II</td>
<td>6 days</td>
<td>– *</td>
<td>– *</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>8,16 x 10\textsuperscript{4} (5,09 – 13,80)</td>
<td>0,41 (0,037)</td>
</tr>
</tbody>
</table>

* Due to the low mortality rates no significant regression lines could be estimated.

**Conclusion**

In recent years, CpGV has been applied in Europe in a large scale, however, the phenomenon of a reduced sensitivity of field populations has not been observed yet. There are several reports about insect strains becoming resistant against insect viruses under heavy selection pressure in the laboratory. For example, Fuxa & Richter (1998) were able to induce resistance to a nucleopolyhedrovirus in a laboratory strain of Spodoptera frugiperda.

Furthermore, it should be considered that in the field only one genotype of CpGV (Mexican strain) was applied over many years. The continued exposure of a homogenous virus to a rather heterogeneous host population may have favoured the development of resistance.

A conclusion on the significance of these first observations for the practical use of the granulovirus is not possible without more data available. Thus, more investigations are required.

The screening of the genetical background of different populations has already started.

**References**


Controlling wireworms (Agriotes spp.) in a potato crop with biologicals

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Abstract: Wireworms (Agriotes spp.), larvae of click beetles, are a serious worldwide soil pest mainly in potatoes (Solanum tuberosum L.), causing damages in summer and early autumn on the daughter tubers. It is an increasing problem in many European countries, like the U.K., Austria, Switzerland, the Netherlands and Italy. Damages consist of round holes on the surface and tunnels running into the tubers. This damage reduces yield but mainly quality of the crop even making a batch unmarketable. Field experiments were carried out in 2001 and 2002, to assess the protection against wireworm in potatoes of different compounds. Treatments were applied as a furrow or broadcast application or as a tuber drench. Compounds used in the trials were biologicals, entomopathogenic fungi and entomoparasitic nematodes or combinations with an insecticide. These compounds were compared with insecticide granulates of ethoprophos applied as a broadcast application at seedbed preparation, or with chlorpyriphos as a furrow treatment at the moment of planting and incorporated.

Assessments focused on the effects of the compounds on the emergence and number of stems per plant, the damage to the mother tubers and the damage to the daughter tubers at harvest. Phytotoxicity was observed from furrow treatments with carvacrol. Furrow applications with carvacrol or cinnamaldehyde or Steinernema feltiae were ineffective at controlling wireworms. Beauveria bassiana or the combined application of B. bassiana (furrow application) with imidacloprid (tuber drench) showed a significant reduction of wireworm damage in potatoes, this was comparable to the etroprophos granulates.

Key words: biological control, wireworms, potatoes, application methods

Introduction

Numerous species of wireworms, i.e. larvae of the click beetles (Coleoptera: Elateridae) are found as crop pests throughout the world (Lefko et al., 1998). In the Netherlands Agriotes lineatus and A. obscurus are common and increasing problems for potato, sugar beet and cereal growers (Dewar, 1993; Huiting & Ester, 2003). In potatoes (Solanum tuberosum L.) problems also occurred in many other European countries, like the U.K., Austria, Switzerland and Italy (Parker & Howard, 2001). High population densities of wireworms are observed in grassland or in arable fields with cereals or grass seed production (Parker & Seeney, 1997). In potato crops, which are the highest value crops in an arable rotation, most damage is observed within three years after ploughed pastures or with Gramineae as previous crops.

In a potato crop, wireworms cause damage to tubers in two ways. First, directly after planting in April-May, the mother tubers are attacked, but plant loss has not been reported. Secondly, the most serious damage is caused in summer and early autumn on the daughter tubers at harvest. Damage consists of round holes on the surface and tunnels running into the tubers. This damage reduces yield but mainly the quality of the crop and can even result in an unmarketable batch. Wireworm tunneling also creates an entry point for certain plant pathogens, which can cause tuber rot.

An effective chemical control is possible with granular organophosphates, but usually high application rates are required. In the Netherlands these products have been withdrawn from use. The granulate insecticide ethoprophos (Mocap 20 MG) is currently used for the
control of wireworms in potato crops but often has an insufficient protection. In addition, the product is relatively expensive and needs to be applied before planting. The organic farmers have no possibilities to control this soil insect with any treatment.

This paper presents the results of a part of a broader project which included the use of chemicals as well as biologicals to control wireworms in a potato crop. The application method of compounds was taken into consideration also. Indeed, the application method may play a role as furrow application or even a tuber drench application could be a more convenient and effective way of controlling wireworms and would be less injurious to other organisms and predators. The amount of product can be reduced by 50 up to 90% compared to the broadcast soil treatment.

Materials and methods

The trials were carried out in 2001 and 2002 at two locations. In Lelystad, centre of the Netherlands, the soil was a marine loam with 19% silt and in Schoonoord, in the north-eastern part of the country, the trial was situated on a sandy soil. In the previous one or two years the fields were used for grass seed production, which is a host plant of the click beetles (Ester & Rozen, 2005). Plots were 4.5 m wide (row distance 75 cm) and 10 m (35 cm in the row in 2001, 20 cm in Lelystad and 33 cm in Schoonoord in 2002) long. The tuber size was 45-50 mm of the cultivar Bintje in 2001 and 35-45 mm of the cultivar Russet Burbank in Lelystad and 28-55 mm in Schoonoord in 2002. The potatoes were planted by machine in mid-May. The experiments were set up as a randomized block design with four replications.

Treatments

The different compounds used in the trials were biologicals such as essential oils, entomopathogenic fungi and entomoparasitic nematodes or combinations with an insecticide and were applied as a furrow or broadcast application or as a tuber drench. References consisted of chlorpyriphos applied as a furrow application and a broadcast application of ethoprophos (4000 grams active ingredient: 4000 g a.i.) granulates and incorporated before planting the potatoes, the latter being common practice in the Netherlands. In addition, an untreated control plot was added.

The broadcast application was carried out by hand at seedbed preparation and immediately incorporated into the soil at a depth of 15 cm. The furrow application was carried out with a manually driven granulate spreader in a 30 cm wide strip at planting and incorporated. Tuber treatment consisted of drenching the tubers in a suspension of the product during two minutes, 8 to 15 days before planting. The rates are expressed in g a.i. per ton tubers or per hectare. Table 1 shows the several compounds and treatments applied each year.

The product Beauveria bassiana consists of dry conidia and is chosen for its high mortality to cabbage root flies (Meadow et al., 1998). The natural compound carvacrol is an essential oil having an antibacterial activity and consists of oil fractions of mainly oreganum (51-84.5%), thyme (45-60%) and savory (26-41%). Due to its strong taste and flavour, application of carvacrol is limited to a certain concentration (Ultee, 2000).

Assessment

Field emergence was determined two and four weeks after planting in 2002 by counting the number of emerged plants in a row of 47 planted tubers. The number of stems per plant were observed six and nine weeks after planting in 2002.

Crop damage by wireworms was assessed on the mother tubers (five and six weeks after planting in 2001) and on the daughter tubers at harvest (1st October 2001 and 27 September 2002). Assessment took place by counting the number of attacked tubers. Twelve plants of each plot were harvested by hand and 100 tubers were selected at random from it, which were
divided into three diameter size classes: < 35 mm, 35-50 mm and > 50 mm. The numbers of affected tubers were examined per size class.

**Statistical analysis**

Data were analyzed by analysis of variance (ANOVA) with the programme Genstat. From the ANOVA analysis means, least significant differences (LSD) and F-probabilities were obtained. LSD’s were calculated with Student’s distribution.

Table 1. Summary of treatments, doses and application methods in two years.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Application method</th>
<th>Formulation</th>
<th>2001</th>
<th>2002</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>furrow</td>
<td>22 WP</td>
<td>440 g/ha</td>
<td>880 g/ha</td>
</tr>
<tr>
<td>Imidacloprid + <em>Beauveria bassiana</em></td>
<td>drench + furrow</td>
<td>350 g/l + 22WP</td>
<td>70 g/ton + 440 g/ha</td>
<td>35 g/ton + 880 g/ha</td>
</tr>
<tr>
<td><em>Steinernema feltiae</em></td>
<td>furrow</td>
<td>545,000 nematodes/m²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>drench</td>
<td>30% ES</td>
<td>150 g/ton</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>furrow</td>
<td>30% ES</td>
<td>600 g/ha</td>
<td>–</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>drench</td>
<td>100% flowable</td>
<td>6 L/ton</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>furrow</td>
<td>100% flowable</td>
<td>6 L/ha</td>
<td>–</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>furrow</td>
<td>480 g/L EC</td>
<td>2.88 kg/ha</td>
<td>–</td>
</tr>
<tr>
<td>Ethoprophos</td>
<td>broadcast</td>
<td>20% MG</td>
<td>4,000 g/ha</td>
<td>4,000 g/ha</td>
</tr>
<tr>
<td>Untreated</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Results**

**Field emergence**

In the first trial in 2002, no significant differences in field emergence between the compound and control treatments were observed (Table 2). The same applied to the number of stems per plant nine weeks after planting.

In the second trial, field emergence of tuber treatment with imidacloprid combined with the furrow application of *B. bassiana* was significantly lower compared with the untreated plots after two weeks. None of the treatments resulted in a lower number of stems per plant than the untreated plots after six weeks. *B. bassiana* applied as a furrow treatment showed a decrease in number of stems compared to the combined treatment of imidacloprid plus *B. bassiana*.

**Efficacy of the compounds**

In the first field trial in 2001, after six weeks all treatments showed a significantly lower number of holes per mother tuber than the untreated plots (Table 3), except the carvacrol tuber treatment which resulted in 100 % plant loss (not mentioned in the table 3). *B. bassiana* applied as a furrow application decreased the attack compared to the cinnamaldehyde tuber treatment. The reference treatment chlorpyriphos as well as the combined treatment imidacloriprid plus *B. bassiana* resulted in a 100% protection of the mother tubers.

In the second field trial, after five weeks ethoprophos showed a significant decrease in damage (number of holes) of the mother tubers.
In the first field trial in 2001, all the treatments in the overall assessment (total) resulted in a decrease in percentage of attacked tubers at harvest, except for the tuber treatment with cinnamaldehyde (Table 4).

Table 2. Emergence (% from planted tubers) and the number of stems per potato plant in two field trials, in 2002.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Application method</th>
<th>Dose</th>
<th>Emergence</th>
<th>Number of stems</th>
</tr>
</thead>
<tbody>
<tr>
<td>First trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>0</td>
<td>82.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Beauveria bassiana</td>
<td>furrow</td>
<td>880 g/ha</td>
<td>93.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Imidacloprid + B. bassiana</td>
<td>drench + furrow</td>
<td>35 g/ton + 880 g/ha</td>
<td>90.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Ethoprophos</td>
<td>broadcast</td>
<td>4 kg/ha</td>
<td>89.5</td>
<td>4.0</td>
</tr>
<tr>
<td>LSD (p = 0.05)</td>
<td></td>
<td></td>
<td>14.55</td>
<td>1.13</td>
</tr>
<tr>
<td>F-probability</td>
<td></td>
<td></td>
<td>0.599</td>
<td>0.803</td>
</tr>
<tr>
<td>Second trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>0</td>
<td>46.1</td>
<td>5.94</td>
</tr>
<tr>
<td>Beauveria bassiana</td>
<td>furrow</td>
<td>880 g/ha</td>
<td>41.1</td>
<td>5.50</td>
</tr>
<tr>
<td>Imidacloprid + B. bassiana</td>
<td>drench + furrow</td>
<td>35 g/ton + 880 g/ha</td>
<td>26.6</td>
<td>6.26</td>
</tr>
<tr>
<td>Ethoprophos</td>
<td>broadcast</td>
<td>4 kg/ha</td>
<td>45.8</td>
<td>5.76</td>
</tr>
<tr>
<td>LSD (p = 0.05)</td>
<td></td>
<td></td>
<td>13.07</td>
<td>0.569</td>
</tr>
<tr>
<td>F-probability</td>
<td></td>
<td></td>
<td>0.035</td>
<td>0.129</td>
</tr>
</tbody>
</table>

Table 3. Number of holes per mother tuber caused by wireworms in two field trials, in 2001.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Application method</th>
<th>Dose</th>
<th>Number of holes</th>
</tr>
</thead>
<tbody>
<tr>
<td>First trial</td>
<td></td>
<td></td>
<td>25 June</td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>0</td>
<td>0.68</td>
</tr>
<tr>
<td>Beauveria bassiana</td>
<td>furrow</td>
<td>440 g/ha</td>
<td>0.13</td>
</tr>
<tr>
<td>Imidacloprid + B. bassiana</td>
<td>drench + furrow</td>
<td>70 g/ton + 440 g/ha</td>
<td>0.00</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>drench</td>
<td>150 g/ton</td>
<td>0.38</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>furrow</td>
<td>2.88 kg/ha</td>
<td>0.00</td>
</tr>
<tr>
<td>LSD (p = 0.05)</td>
<td></td>
<td></td>
<td>0.217</td>
</tr>
<tr>
<td>F-probability</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Second trial</td>
<td></td>
<td></td>
<td>12 June</td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>0</td>
<td>3.31</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>furrow</td>
<td>600 g/ha</td>
<td>2.12</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>furrow</td>
<td>6 L/ha</td>
<td>2.44</td>
</tr>
<tr>
<td>Ethoprophos</td>
<td>broadcast</td>
<td>4 kg/ha</td>
<td>0.06</td>
</tr>
<tr>
<td>LSD (p=0.05)</td>
<td></td>
<td></td>
<td>2.829</td>
</tr>
<tr>
<td>F-probability</td>
<td></td>
<td></td>
<td>0.133</td>
</tr>
</tbody>
</table>
Compared to the untreated tubers *B. bassiana* applied as a furrow application decreased the damage significantly in the size classes 35-50 and > 50 mm. Also the combined treatment imidacloprid tuber drench application and *B. bassiana* furrow treatment had a decrease in damage. Chlorpyriphos furrow treatment reduced the damage significantly.

In the second field trial, only broadcast applied ethoprophos showed a significantly decrease of affected tubers.

In both field trials the percentage of affected tubers of the untreated plots were lowest in the size class 35-50 mm.

Table 4. Percentage of wireworm attack of tubers at harvest of three size classes (mm) in two field trials, in 2001.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Application method</th>
<th>Dose</th>
<th>&lt; 35</th>
<th>35 – 50</th>
<th>&gt; 50</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First trial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>0</td>
<td>26.0</td>
<td>31.9</td>
<td>46.8</td>
<td>38.0</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>furrow</td>
<td>440 g/ha</td>
<td>12.5</td>
<td>17.2</td>
<td>29.9</td>
<td>22.8</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>drench</td>
<td>70 g/ton</td>
<td>15.3</td>
<td>16.4</td>
<td>20.6</td>
<td>18.2</td>
</tr>
<tr>
<td>+ <em>B. bassiana</em></td>
<td>+ furrow</td>
<td>+ 440 g/ha</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>drench</td>
<td>150 g/ton</td>
<td>39.5</td>
<td>37.0</td>
<td>36.2</td>
<td>36.8</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>furrow</td>
<td>2.88 kg/ha</td>
<td>26.4</td>
<td>9.5</td>
<td>18.8</td>
<td>13.8</td>
</tr>
<tr>
<td>LSD (p=0.05%)</td>
<td></td>
<td></td>
<td>21.61</td>
<td>10.63</td>
<td>15.23</td>
<td>10.34</td>
</tr>
<tr>
<td>F-probability</td>
<td></td>
<td></td>
<td>0.116</td>
<td>&lt; 0.001</td>
<td>0.010</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Second trial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>0</td>
<td>13.9</td>
<td>32.9</td>
<td>46.2</td>
<td>39.0</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>furrow</td>
<td>600 g/ha</td>
<td>12.5</td>
<td>24.0</td>
<td>33.6</td>
<td>29.5</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>furrow</td>
<td>6 L/ha</td>
<td>28.6</td>
<td>31.9</td>
<td>49.0</td>
<td>41.0</td>
</tr>
<tr>
<td>Ethoprophos</td>
<td>broadcast</td>
<td>4 kg/ha</td>
<td>9.8</td>
<td>12.3</td>
<td>15.6</td>
<td>14.0</td>
</tr>
<tr>
<td>LSD (p=0.05)</td>
<td></td>
<td></td>
<td>29.70</td>
<td>18.05</td>
<td>16.5</td>
<td>15.34</td>
</tr>
<tr>
<td>F-probability</td>
<td></td>
<td></td>
<td>0.513</td>
<td>0.098</td>
<td>0.005</td>
<td>0.012</td>
</tr>
</tbody>
</table>

In 2002, in the first field trial the combination of imidacloprid drench tuber application and the furrow treatment with *B. bassiana* reduced the damage by wireworms significantly (Table 5). For the other treatments no significant differences with the untreated plots were observed.

In the second field trial, on a sandy soil, none of the treatments showed a reduction of the damage caused by wireworms. Compared to the other three trials, a lower density of wireworms was observed resulting in a three fold lower damage level.

**Discussion**

This research aimed first to develop an alternative for the standard wireworm control with the compound ethoprophos (dose 4 kg a.i. per ha, broadcast applied before seedbed preparation). A second goal was to make an attempt to develop a recommendation to control wireworm pests in organic farming systems.

At the marine loam soil sites, in 2001 as well as in 2002 the basic wireworm populations were at the same high level. At the sandy soil location populations were quite low (Table 5).
Table 5. Percentage of wireworm attack of tubers at harvest of three size classes (mm) in two field trials, in 2002.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Application method</th>
<th>Rate</th>
<th>&lt; 35</th>
<th>35 – 50</th>
<th>&gt; 50</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First trial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated –</td>
<td>–</td>
<td>0</td>
<td>9.8</td>
<td>31.1</td>
<td>31.1</td>
<td>28.5</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em> furrow</td>
<td>880 g/ha</td>
<td>12.1</td>
<td>29.3</td>
<td>37.1</td>
<td>30.8</td>
<td></td>
</tr>
<tr>
<td><em>Imidacloprid + B. bassiana</em> drench + furrow</td>
<td>35 g/ton + 880 g/ha</td>
<td>7.0</td>
<td>7.5</td>
<td>6.5</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Ethoprophos broadcast</td>
<td>4 kg/ha</td>
<td>17.7</td>
<td>33.4</td>
<td>39.5</td>
<td>34.5</td>
<td></td>
</tr>
<tr>
<td><em>Steinernema feltiae</em> furrow</td>
<td>545,000 nematodes/m²</td>
<td>14.0</td>
<td>31.8</td>
<td>33.7</td>
<td>30.5</td>
<td></td>
</tr>
<tr>
<td>LSD (p=0.05)</td>
<td></td>
<td>19.4</td>
<td>14.83</td>
<td>16.35</td>
<td>12.97</td>
<td></td>
</tr>
<tr>
<td>F-probability</td>
<td></td>
<td>0.896</td>
<td>0.016</td>
<td>0.008</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td><strong>Second trial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated –</td>
<td>–</td>
<td>0</td>
<td>9.1</td>
<td>9.3</td>
<td>16.8</td>
<td>10.1</td>
</tr>
<tr>
<td><em>B. bassiana</em> furrow</td>
<td>880 g/ha</td>
<td>12.7</td>
<td>5.5</td>
<td>4.2</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td><em>Imidacloprid + B. bassiana</em> drench + furrow</td>
<td>35 g/ton + 880 g/ha</td>
<td>4.7</td>
<td>7.9</td>
<td>5.6</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Ethoprophos broadcast</td>
<td>4 kg/ha</td>
<td>8.5</td>
<td>4.1</td>
<td>5.3</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>LSD p=0.05</td>
<td></td>
<td>14.91</td>
<td>5.615</td>
<td>12.77</td>
<td>5.858</td>
<td></td>
</tr>
<tr>
<td>F-probability</td>
<td></td>
<td>0.798</td>
<td>0.237</td>
<td>0.114</td>
<td>0.295</td>
<td></td>
</tr>
</tbody>
</table>

Damage to the mother tubers caused by wireworms showed to be a representative indication of the effect of the treatments on the daughter tubers at harvest (Table 3).

After treatment with the entomopathogenic fungus *Beauveria bassiana* (Botanigard 22WP) at doses of 440 g and 880 g per hectare, applied as a furrow application, a significant reduction in damage caused by wireworms was determined at harvest (Tables 4 & 5). Blaser et al. (2004) reported the presence of the entomopathogenic fungus *Metarhizium anisopliae* in a high percentage of soil samples of 30 farm fields in Switzerland. No differences in the incidence of the fungus between organic and conventional farms were observed. Correlation between fungus presence and wireworm damage was not clear. The combined treatment imidacloprid (Amigo 350 g/l SC) with a tuber drench plus *B. bassiana* as a furrow application in doses of 70 g a.i./ha plus 440 g/ton or 35 g a.i./ton plus 880 g a.i./ha respectively shows an excellent protection of 52% (2001) and 75% (2002) against wireworm attack. The standard treatment with ethoprophos 4 kg a.i. per hectare (Mocap 20% MG) applied broadcast before planting has the same level of protection as the combined treatment. The granulate application of ethoprophos has insufficient effect on the control of wireworms when the soil is dry (Table 5), because granulates need some water to be active. The combined treatment shows a delay in emergence, but this did not result in a decrease of the number of stems per plant after four weeks (Table 2).

Treatment with the entomoparasitic nematodes *Steinernema feltiae* applied as a furrow treatment at a dose of 545,000 per m² has no effect on protection of the tubers against wireworm attack.

Cinnamaldehyde at a dose of 150 g a.i. per ton tubers (applied as a drench application) decreases the damage to the mother tubers significantly in the first field trial up to six weeks.
after planting (Table 3). This contrasts with the observed damage of the daughter tubers at harvest even with higher doses applied as a furrow application (Table 4).

The compound carvacrol in a dose of 6 L (100 % flowable) per hectare has no significant effect in protecting the mother tubers after six weeks. In the same field trial this product did not reduce the wireworm damage at harvest as well (Table 4). The drench treatment of the tubers with carvacrol 6 L per ton resulted in a 100% plant loss.

Chlorpyriphos applied at a dose of 2.88 kg a.i. per ha (480 g/L EC, Dursban 4E) applied as furrow application shows a sufficient protection against the wireworm damage (Tables 3 & 4).

Standard treatment, ethoprophos 4 kg a.i. per ha applied broadcast in the second field trial in 2001, showed a significant protection, but in 2002 the decrease of damage was not significant compared to the untreated tubers (Table 5).

Wireworm damage is mostly cosmetic and does not usually reduce gross yield. In the field trials no differences in potatoe yields were observed between the treated untreated plots.

Parker (1996) reported a baiting technique to detect wireworms in the field soil, which consists of a simple cereal-baited trap, being at least as effective as traditional soil sampling methods for identifying the presence of wireworms. A limitation of baiting is that the traps cannot be used to give an estimate of population density per unit area. Damage occurred even in plots where no wireworms were detected, and high damage levels were observed even where only one wireworm was caught in the sampled plot.

Another way in reducing the population density of wireworms, is the use of funnel-traps with pheromones to catch the click beetles. This supervised control system was used in the host crop of the *Agriotes* spp. (Ester et al., 2004; Ester & Rozen, 2005).

In conclusion, the entomopathogenic fungus *Beauveria bassiana* at doses of 440 g and 880 g a.i. per hectare applied as a furrow application has the potential to protect the potato crop against wireworm attack at the same level as the insecticide granulates ethoprophos 4 kg a.i. per hectare. This is an environmentally friendly alternative for conventional as well as organic farmers.

Acknowledgements

We thank Mr. R. Gruppen and Mrs. M. Huisman-de Lange for their assistance in the field trials.

References


The peach flatheaded rootborer, *Capnodis tenebrionis* (L.), and its enemies

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**Abstract**: *Capnodis tenebrionis* (L.) (Coleoptera Buprestidae) is an important and common phytophagous insect of Mediterranean stone-fruit orchards. Current knowledge on its enemies is really scanty; consequently no biological control strategy has been set up. For this reason, a survey was carried out in South Italy areas. Only a bethylid species, *Sclerodermus cereicollis* Kieffer, and some entomopathogenic fungi were found, so confirming the extreme scarcity of *Capnodis* natural enemies.

The susceptibility of the peachborer adults to 2 commercial formulations of *Bacillus thuringiensis* (Berliner) was assessed in laboratory bioassays. Both products proved to be totally inefficacious in controlling the beetles.

**Key words**: peachborer, biological control, stone-fruit, *Bt*, entomopathogenic fungi, bethylids

**Introduction**

The peach flatheaded rootborer, *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae), is widespread in the Mediterranean countries where it is considered as major threat to stone-fruit orchards (Rivnay, 1944; Ferrero, 1987; Sanchez-Capuchino *et al*., 1987; Hmimina *et al*., 1988; Ben-Yehuda *et al*., 2000). Economic damage is due to the endophytic larvae which drill the roots and the base of the trunk between the bark and the wood, leading to the formation of winding, sometimes girdling galleries. Severe larval infestations considerably affect plant growth and fruit production up to cause the death of the trees (Balachowsky, 1962).

Despite its importance, very few data are reported in the literature about *C. tenebrionis* enemies. Ants (*Pheidole pallidula* Nyl.) are known to prey on eggs and pupae (Pussard, 1935); the maggots of a flesh fly (*Sarcophila latifrons* Fall.) were observed feeding on adults (Rivnay, 1947); a tachinid fly, *Billaea adelpha* (Loew) (*Billaea subrotundata* Rond.) was recorded as parasitoid of larvae (d’Aguilar & Féron, 1949); the wireworms of *Melanotus rufipes* (Herbst) and some mites were found associated to pupae and adults in pupal cells (Rekk, 1932); the encyrtid *Avetianella capnodiobia* Trjapitzin was reported emerging from *Capnodis* eggs in Armenia (Alexeyev, 1984). Un unidentified entomopathogenic fungus (Del Guercio, 1931) and the insect parasitic nematode *Steinernema carpocapsae* (Filipjev) (Santos Lobatón *et al*., 1998) were recovered from adults and larvae, respectively. Recently, the effectiveness of EPN species (*Steinernema* sp., *Heterorhabditis* sp.) against newly hatched larvae (Marannino *et al*., 2004; García del Pino & Morton, 2005) has been ascertained in laboratory bioassays.

Resistant rootstocks are not available but some researches report that cyanogenic glycosides of the roots (mainly prunasin) influence the adult and larval biology (Malagón & Garrido, 1990; Mulas & Barbera, 1994; Dicenta *et al*., 2002; Mendel *et al*., 2003).

Thus, the pest management is based on adulticide applications especially during the pre-oviposition period, in late spring-early summer (Colasurdo *et al*., 1997; Ben-Yehuda *et al*., 2000). No recommendation is provided for protecting organic cultivations.
For some years our research group has been studying the ecological and behavioural features of this insect in order to set up an integrated control strategy. As a consequence, a survey on the Capnodis natural enemies in South Italy areas and some laboratory tests with commercial BCAs have been carried out, with the aim to develop environmentally friendly technical measures.

**Materials and methods**

During the last ten years many adults, pupae and larvae of the peachborer have been collected in several localities of Apulia, Basilicata, Sicily and Molise. Their vitality was firstly checked. Endophagous parasitoids were searched by dissecting the individuals in a saline solution and storing dead adults in emergence boxes.

Entomopathogenic fungi were searched in dead individuals, from field and laboratory rearing, which appeared colonized by mycelium. The insects were put on a water agar substrate, then the fungi were transferred to a potato agar substrate and kept for 7 days at 25°C under dark condition. Fungi were identified and compared with the specimens of a local collection and partly submitted to some experts for confirmation.

The efficacy of two Bacillus thuringiensis (Berliner) (kurstaki EG2424 and tenebrionis) commercial formulations against the adults of Capnodis tenebrionis was assessed in a preliminary laboratory test. Twenty adults (10 males, 10 females), reared on fresh apricot twigs (de Lillo, 1998) under laboratory conditions (28-30°C, 40-50% R.H., 16:8 L:D), were treated by injecting 100 µL of product directly deep in their mouth using a micropipette. Such a very large dose was supplied in order to obtain a prompt susceptibility response. The mortality and the feeding activity were recorded daily by 10 days after the treatment. The test was repeated 3 times for each formulation.

**Results and discussion**

No predator was found and no parasitoid was obtained from the individuals either dissected or stored. It is worthy of note the recovering of a small population of larvae, pupae and adults of Sclerodermus cereicollis Kieffer (Hymenoptera: Bethylidae) associated to a parasitized pupa of Capnodis.

Some authors have advanced the hypothesis that some compounds of the jewel beetle hemolymph, commonly referred to as buprestins, might deter the action of predators (Moore & Brown, 1985, 1989; Gullan & Cranston, 1994). Up to now, these substances have been isolated only from adults of a few species of Australian and Central European buprestids (Schramm et al., 2003). It is unknown if they occur in all developmental stages. In addition, no experimental data are available on their biological rule. About the fungi, Beauveria bassiana (Bals.) Vuill., Paecilomyces farinosus (Holm. Gray), Paecilomyces sp. and Scopulariopsis sp. were recovered from larvae, pupae and adults. It could be of interest to study their efficacy against: 1) adults, since overwintering takes place in the soil; 2) neonate larvae, since they have to crawl through the soil before entering the host (Marannino et al., 2004); 3) eggs, since oviposition occurs just beneath the soil surface.

Bt commercial formulations proved to be absolutely ineffective in controlling the beetles. In fact, all treated individuals survived and continued to feed as much as before the test.

**Acknowledgments**

The authors are grateful to Dr. G. Zimmermann for fungi identification, to Dr. Jeroen de Rond for identification of bethylids, and to Mr. M. Poliseno for fungi isolation and culture.
References


Viability of entomopathogenic microorganisms encapsulated in alginate pellets

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Abstract: Several authors have studied the applicability of sodium alginate formulations for different purposes. This kind of formulation seems to be quite suitable to control phytophagous insects living in the soil for at least one phase of their life cycle. Therefore, we devised a method to encapsulate entomopathogenic microorganisms in alginate pellets and evaluated the duration of their viability. Twenty bacterial and twenty-nine fungal isolates from agricultural and forest soils and from insect samples collected in several Italian localities were tested. In the viability tests, we periodically plated alginate pellets and checked the development of colonies on the growth media. The bacterial isolates maintained their viability for five years while the fungal isolates showed shorter survival, with a mean viability of 12-18 months. The results for bacteria were highly positive. However, for fungi, it would be necessary to modify the alginate formulation process to extend the storage period.

Key words: alginate pellets, entomopathogenic microorganisms, microbiological control, Bacillus thuringiensis, Beauveria bassiana, Paecilomyces, Lecanicillium lecanii

Introduction

The effective control of pests in agro-forest environments is closely linked to the chosen method to kill the target species. Knowledge of the biological cycle and the most vulnerable growth stage of a specific insect are also essential. At the same time, it is necessary to keep the phytopathological risk below the damage threshold and the phytosanitary interventions must ensure habitat protection, particularly in forests. Integrated pest management strategies are of major interest in this regard. In particular, the use of microbiological control is less polluting than chemical control, even if slower acting and less effective. Several entomopathogenic microorganisms have been tested in field trials and some of them have achieved good results in the Integrated Pest Management of many ecosystems. However, suitable microbial formulations and application methods are essential for success.

Several authors have studied the applicability of sodium alginate formulations for different purposes, such as myco-weeding, experimental inoculation of soilborne phytopathogenic fungi, and bacterial and fungal control of noxious arthropods (Walker & Connick, 1983; Fravel et al., 1985; Lewis & Papavizas, 1985; Knudsen et al., 1990; Petrolini et al., 1990; Prabakaran et al., 2001; Bextine & Thorvilson, 2002a, 2002b; Thorvilson et al., 2002; Winder et al., 2003). This kind of formulation seems to be quite suitable to control phytophagous living in the soil for at least one phase of their life cycle. Therefore, we tested a method to encapsulate entomopathogenic microorganisms in alginate pellets and evaluated the duration of their viability, which is an important step to achieve when developing biological preparations based on this technology.
Materials and methods

Twenty bacterial (Bacillus thuringiensis or B. cereus) and twenty-nine fungal isolates (Beauveria bassiana, Paecilomyces lilacinus, P. variotii or Lecanicillium lecanii) from agricultural and forest soils and from insect samples collected in several Italian localities were tested (Tables 1, 2 & 3).

Table 1. Entomopathogenic bacterial isolates tested for viability when encapsulated in alginate pellets.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>N° isolate</th>
<th>Recovered from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus thuringiensis</td>
<td>1</td>
<td>Zygaaena sp.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Zeuzera pyrina</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Tortrix viridana</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Elkneria pudibunda</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Phalera bucephala</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Aporia crataegi</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Phalera bucephala</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Thaumetopoea pityocampa</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Cossus cossus</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Agrius convolvuli</td>
</tr>
</tbody>
</table>

Bacillus cereus

<table>
<thead>
<tr>
<th>N° isolate</th>
<th>Recovered from</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hyponomeuta padellus</td>
</tr>
<tr>
<td>2</td>
<td>Arhopalus tristis</td>
</tr>
<tr>
<td>3</td>
<td>Fallow farm soil</td>
</tr>
<tr>
<td>4</td>
<td>Thaumetopoea pityocampa</td>
</tr>
<tr>
<td>5</td>
<td>Sesamia sp.</td>
</tr>
<tr>
<td>6</td>
<td>Elkneria pudibunda</td>
</tr>
</tbody>
</table>

Table 2. Beauveria bassiana isolates tested for viability when encapsulated in alginate pellets.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>N° isolate</th>
<th>Recovered from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beauveria bassiana</td>
<td>1</td>
<td>Beech leaves</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Olive leaves</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Aradius sp.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Pissodes notatus</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Eriophyidae sp.</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Elm leaves</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Thaumetopoea processionea</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Euproctis chrysorrhoea</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Aporia crataegi</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Pissodes piceae</td>
</tr>
</tbody>
</table>
Table 3. *Paecilomyces* spp. and *Lecanicillium lecanii* isolates tested for viability when encapsulated in alginate pellets.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>N° isolate</th>
<th>Recovered from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td><em>Phyllaphis fagi</em></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Resseliella oleisuga</em></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Untilled soil</td>
</tr>
<tr>
<td><em>Paecilomyces lilacinus</em></td>
<td>5</td>
<td><em>Euproctis chrysorrhoea</em></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td><em>Pissodes piceae</em></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Farm soil</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Farm soil</td>
</tr>
<tr>
<td><em>Paecilomyces variotii</em></td>
<td>4</td>
<td><em>Globodera rostochiensis</em></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Thaumetopoea pityocampa</em></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Pear leaves</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>Zygaena</em> sp.</td>
</tr>
<tr>
<td><em>Lecanicillium lecanii</em></td>
<td>4</td>
<td>Forest soil</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td><em>Otiorhynchus sulcatus</em></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td><em>Aporia crataegi</em></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td><em>Lymantria dispar</em></td>
</tr>
</tbody>
</table>

The alginate pellets were produced according to Lewis & Papavizas (1985). Bacterial and fungal isolates were cultured in 250 mL flasks containing 100 mL of NB (Nutrient Broth) for bacteria or 100 mL of PDB (Potato Dextrose Broth) for fungi. Inoculum was added to the culture medium and the flasks were placed in a stirred (100 rpm) thermostat, at 28°C for bacteria and 25°C for fungi, for 7-15 days.

The concentration of microorganisms was evaluated with a Thoma haemocytometer and then adjusted to 7.3 to 11.6 x 10⁶ cfu/mL.

The sodium alginate-kaolin suspensions were prepared by adding 1.33% (w/v) sodium alginate and 13% (w/v) kaolin to sterile water.

Pellet formation was achieved by dripping the continuously stirred mixture of microorganisms, sodium alginate and kaolin into a beaker containing a 0.25M gellant solution of calcium chloride (CaCl₂, 2H₂O) maintained in a slow, constant rotating movement. Pellets were removed from the solution several minutes after their formation and dried on filter paper in a sterile air stream at room temperature for 24 hours. The pellets were stored in screw-capped sterile glass tubes at room temperature (15-25°C).

In the viability tests, we periodically plated alginate pellets and checked the development of colonies on the growth media: NA (Nutrient Agar) and PDA (Potato Dextrose Agar) for bacteria and fungi, respectively. Ten alginate pellets of each isolate were put in Petri dishes every six months for five years. Viability was checked after one week of incubation (28°C for bacteria, 25°C for fungi) and the number of colonies was recorded.

**Results and discussion**

Our tests provided useful information about the long-term viability of the entomopathogenic bacteria and fungi encapsulated in alginate pellets (Figs 1 and 2).
There were no problems with the storage of bacterial pellets: all the isolates maintained viability for five years (each pellet originated a colony). In contrast, the fungi showed shorter survival, with a mean viability of 12-18 months. Only a few isolates survived for a longer period.

Figure 1. Viability of entomopathogenic isolates of *Bacillus thuringiensis* and *Bacillus cereus* when encapsulated in alginate pellets.
**Conclusion**

The formulation (and storage) method in alginate pellets appeared very promising for entomopathogenic microorganisms to be used for biological control of pests. Our investigations demonstrated that this method is very promising for entomopathogenic bacteria. For fungi, however, more research would be needed to extend the storage period, possibly through an optimization of the alginate formulation process.

**References**


Occurrence of pathogens and parasites in *Ips typographus* L. from spruce stands (*Picea orientalis* L.) in Georgia

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² Department of Forest and Soil Sciences, University of Natural Resources and Applied Life Sciences, BOKU-Vienna, Gregor Mendel Strasse 33, A-1180 Vienna, Austria

Abstract: Occurrence of pathogens was investigated in the spruce bark beetle *Ips typographus* from two different sampling plots in Borjomi region at 900m above sea level, collected in September 2004. Investigations brought evidence of one pathogen species only, *Gregarina typographi*, was found in the mid-gut lumen of 48.6% and of 23.2% of adult beetles respectively. In both cases were more male beetles infected than female beetles. Furthermore, unidentified fungal infections were observed in some few individuals. Relatively few beetles had larvae of the Hymenopteran parasitoids *Tomicobia seitneri* and *Ropalophorus clavicornis*. In addition, *Contortylenchus typographi* nematodes were found in the haemolymph of beetles, and not identified nematode larvae in the gut lumen.

Key words: *Ips typographus*, bark beetles, pathogens, *Gregarina typographi*, nematodes, Georgia

Introduction

*Ips typographus* L. is the most harmful bark beetle on spruce *Picea orientalis* L. in several regions of Georgia. The first occurrence of *I. typographus* in Georgia was reported by K.E. Linderman in 1881 (in Zaitsev, 1950), since that beetles migrated and were transported with timber into various regions of Georgia (Lozovoi, 1965).

Generally, *I. typographus* is a secondary pest species, which needs physiologically stressed trees as habitat. A massive outbreak of this species occurs when they have favourable abiotic conditions and unlimited breeding sources (Altenkirch et al., 2002). When the number of attacking bark beetles is high enough to overcome the defensive system of stressed trees, *I. typographus* is also able to breed in standing, “healthy looking” trees. Unfortunately beetles can damage a high proportion of standing spruce trees in certain places up to 70-80%.

In the year 2000 the lack of rainfall caused severe draught problems in the Borjomi region in Georgia. Due to prosecution of drought ecological conditions collapsed for spruce stands, the resistance of trees was reduced continuously and thus a major outbreak of *I. typographus* was induced. In the past, this bark beetle species attacked mainly trees weakened by *Dendroctonus micans* Kugel. in Georgia (Shavliashvili & Zharkov, 1985). Today, *I. typographus* has spread into approximately 7,000 hectares of coniferous forests in the Borjomi region. Therefore, this outbreak resulted in a significant economic loss in timber production. However, it is noticeable, that occurrence and prevalence of this pest species has still a local character.

Control of bark beetles is difficult because these insects live most of the time of their life cycle hidden under the bark. Currently, the control of *I. typographus* is based on a range of unsatisfactory methods, including insecticide treatment of trunks and phytosanitary measures (removal of fresh breeding material or of freshly infested trees), and by monitoring with pheromone traps (Altenkirch et al., 2002).
Biological control by use of predators, parasitoids or pathogens may help with this problem. There is still a lack of knowledge about natural enemy complexes of bark beetles, especially about pathogens, even less is known about their potential manipulation as environmental safe and effective biological control agents (Händel et al., 2003).

The aim of our study was to investigate the occurrence of pathogens in *I. typographus* from spruce stands (*Picea orientalis*) in Georgia, particularly in Borjomi region. Moreover, the percentage of infections in male and female bark beetles was determined.

**Materials and methods**

In September 2004 adult *I. typographus* were collected in Tsagveri (15 km east of Borjomi) in central Georgia at 900 m altitude at two locations, the distance between both plots was about 1 km. The stand consisted mainly of *Picea orientalis* (most of the trees being 80 years old), and was situated in the centre of a slope (approximately 25° slope angle).

Beetles were collected in two different sampling series by cutting log sections of infested trees or by peeling off infested bark or they were collected by hand directly out of their galleries in the phloem, in order to get insects from many different breeding systems. Log sections, bark and beetles were brought to the laboratory and were incubated at 5°C in a refrigerator. Beetles were removed for diagnosis from their galleries every day. Only living adult beetles were dissected. They were examined on microscopical slides in a drop of water. The gonads, the whole gut and parts of the fat body were removed from the carcass (Wegensteiner et al., 1996) and examined under a normal light microscope at magnification 200-1000x. Search for pathogens was conducted on fresh smears. Dry smears were fixed with methanol and stained with Giemsa’s dye, and then the diagnosis was performed.

**Results and discussion**

Pathogen investigation in a Georgian population of *I. typographus* was conducted with beetles from two sampling series. In a first series, 253 adult beetles were dissected (125 male beetle and 128 female beetles). *Gregarina typographi* (Protozoa, Eugregarinida) described by Fuchs (1915) was found in the mid-gut lumen of 48.6% of the beetles; more male than female beetles showed this infection. One beetle had an unidentified fungal infection (Table 1).

Larvae of hymenopteran parasitoids, *Tomicobia setneri* (Hymenoptera: Pteromalidae) and *Ropalophorus clavicornis* (Hymenoptera: Braconidae), were found in very few cases. Nematodes were found free in the haemolymph and in the gut lumen. In case of presence of female nematodes it was possible to identify *Contortylenchus diplogaster* in the haemolymph, this species was already described for *I. typographus* from Georgia (Kakuliya, 1989). All other nematodes were larvae and could not be identified (Table 1).

In a second series, 99 adult *I. typographus* were dissected. *G. typographi* infection was detected in 23.2% of the beetles, but less prevalent compared to the first series; *G. typographi* was present in both genders, but in this series only slightly more males were infected compared to females. Unidentified fungal infections were found in three beetles (Table 1).

*Tomicobia setneri* was found in the second series too, never *Ropalophorus clavicornis*. Not identified nematode larvae were also observed in the midgut of the beetles (61.0%), they occurred in 72.1% of the male beetles and in 63.0% of the female beetles of the second sample (Table 1).
Table 1: Occurrence of pathogens and parasites in *Ips typographus* in two sampling series from *Picea orientalis* stands in Georgia (G.t. = *Gregarina typographi*; fungus = not identified entomopathogenic fungus, parasitoids: T.s. = *Tomicobia seitneri*, R.c. = *Ropalophorus clavicornis*, and insect parasitic nematodes: Nem.hae. = nematodes in the haemolymph, Nem.gut = nematodes in the gut) (in total, in male and in female beetles, and in beetles without determined gender for the second sampling series; in %).

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</thead>
<tbody>
<tr>
<td><strong>First sampling series</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>48.6</td>
<td>0.4</td>
<td>1.2</td>
<td>0.4</td>
<td>13.0</td>
<td>53.0</td>
<td>253</td>
</tr>
<tr>
<td>Male b.</td>
<td>55.2</td>
<td>0.8</td>
<td>2.4</td>
<td>–</td>
<td>16.0</td>
<td>52.0</td>
<td>125</td>
</tr>
<tr>
<td>Female b.</td>
<td>42.2</td>
<td>–</td>
<td>–</td>
<td>0.8</td>
<td>11.7</td>
<td>53.9</td>
<td>128</td>
</tr>
<tr>
<td><strong>Second sampling series</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23.2</td>
<td>–</td>
<td>3.0</td>
<td>–</td>
<td>–</td>
<td>61.0</td>
<td>99</td>
</tr>
<tr>
<td>Male b.</td>
<td>25.6</td>
<td>–</td>
<td>7.0</td>
<td>–</td>
<td>–</td>
<td>72.1</td>
<td>43</td>
</tr>
<tr>
<td>Female b.</td>
<td>21.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>63.0</td>
<td>46</td>
</tr>
<tr>
<td>not det.</td>
<td>2.0</td>
<td>3.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>20.0</td>
<td>10</td>
</tr>
</tbody>
</table>

The present study aimed to describe the pathogen species spectrum in *I. typographus* from two different sampling plots, and it was surprising to not find other pathogen species known from *I. typographus* (Wegensteiner *et al*., 1996). Prevalence of *Gregarina typographi* was different in the beetles from the two sampling series, which underlines the difficulty to get data representative for a local population. It was interesting to find higher infection rates in male beetles compared to female beetles from both sampling plots. Higher *G. typographi* infection rates in male beetles compared with female beetles were also reported by Wegensteiner & Weiser (1996a, b). According to Wegensteiner & Weiser (2004), *G. typographi* is presumed to appear more often in *I. typographus* males because one male is in contact with two to three females within one breeding system, helping all the females to remove their boring dust and faeces, in connection with the external life cycle of *G. typographi*. There is therefore a greater risk for males to get infected in case one or more of the females are infected. However, Gregarines are presumed to be minor virulent pathogens (Tanada & Kaya, 1993), which do evidently not interrupt migration of beetles.

It is not clear presently if and how the *I. typographus* outbreak will develop in the future. In the mountainous region of Borjomi, 61% of forests (22,400 hectares) consist of conifers, from these the major part (18,000 hectares) is spruce (*Picea orientalis*). It is admitted that the number of bark beetles increases permanently, but it establishes new foci of attack, structurally disordered, especially in spruce stands in mountainous regions, due to vertical variation of temperature and with regard to predisposition of stands in connection with local water stress. However, prevalence and multiplication of the pest can be totally different in other geographical situations (Girits, 1975).

The results of our study underlined the strong importance to conduct investigations of bark beetles pathogens in different geographical zones in order to get an accurate knowledge of their diversity and prevalence. Furthermore, the role of the pathogens in the regulation of bark beetle density in nature should be clarified with regard to different phases of population dynamics.
Acknowledgements

We wish to thank Dr. D. Pilarska (Institute of Zoology, Bulgarian Academy of Sciences, Sofia, Bulgaria) for inspiring discussions during the study and A. Stradner (Institute of Forest Entomology, Forest Pathology and Forest Protection, BOKU-University, Vienna, Austria) for technical assistance in beetle dissection.

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Survival of the spruce bark beetles, *Ips typographus*, infected with pathogens or parasites

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**Abstract:** The survival of adult *Ips typographus* beetles infected with four pathogens was tested under laboratory conditions, as well as the type and amount of the food offered to adult bark beetles necessary for optimum infections. Adult beetles were successfully infected with microsporidia, gregarines and nematodes, but viruses.

**Key words:** Spruce bark beetle, *Ips typographus*, *Polymorphotylenchus typographi*, *Gregarina typographi*, *Chytridiopsis typographi*, ItEPV

**Introduction**

The spruce bark beetle, *Ips typographus*, is the most economically important pest of spruce forests in the palearctic region. From adult beetles several pathogens which can be used in the biological control of the bark beetles were described (Wegensteiner & Weiser, 1995; Weiser & Wegensteiner, 1994; Wegensteiner et al., 1996).

To study further virulence and infectivity of these pathogens we need to establish a model system which can be used for long-term assays. The usual way of maintaining bark beetles in the laboratory consists in using the infected logs maintained in insectarium. We tested survival of the adult beetles infected with several pathogens on bark chips and we also made experiments with the consumption of bark to get the best infection results in adult beetles.

**Materials and methods**

To get infection, bark beetles were fed with the following pathogens or parasites: the microsporidian *Chytridiopsis typographi*, the virus *Ips typographus* entomopoxvirus (ItEPV), the eugregarin *Gregarina typographi*, and the nematode *Polymorphotylenchus typographi*. All pathogens have been isolated from infected bark beetles collected in the Sumava outbreak area.

Prior to infection experiments, beetles were collected in populations far away from the outbreak area, where the tested pathogens were absent. The overwintering brown beetles were used in the survival tests. A small chip of the bark (0.5 cm long) with drop of the infectious suspension was offered to beetles *ad libitum*. Five adult beetles were placed in each plastic tube and were fed with piece of bark each two days. They were kept in 26 ± 0.5°C with 16h : 8h (L:D) light regime. 57 beetles were used as a control group (without any contact with pathogens), whereas 100 beetles were submitted to infection with each pathogen.

During the experiment all dead beetles were systematically removed and dissected. The control group of beetles was fed with the piece of bark with the drop of water and the beetles were kept under the same conditions as the infected ones.
We also tested the consumption of the offered bark. Pieces of bark in form of granules, size 5 mm in diameter and 5 mm height, were offered to adult beetles. The granules were cutted from the bark with phloem and 20 µL of distilled water was put on the surface of each granule. Then one granule was added into Eppendorf test tube with one single beetle. The phloem's consumption was calculated by weighting the faeces and the rest of the granule. Fluorescent color was added to the suspension to see under the UV light if the suspension penetrate into the bark from the surface. In this tests we used three types of the adult beetles: brown, dark brown and black beetles.

The survival probability was analyzed with survival package of R language project.

Results and discussion

Adult beetles were successfully infected with the microsporidian C. typographi, the eugregarin G. typographi and the nematode P. typographi, but inclusion bodies of I. typographus entomopoxvirus (Fig. 1).

In the control, some beetles died without any signs of infection. Bacteria were only present in beetles which died after 3 days of experiment. The last beetles died 72 days after the beginning of the experiment. There was no development of bacteria and/or other pathogens in the beetles found died in the control group.

All beetles infected with C. typographi died in 60 days. First infected beetle died 16 days after the infection. Cysts of the microsporidian were present in the gut of dead beetles and we used the infection material for further infection experiments with new beetles.

The eugregarin G. typographi started to kill beetles 17 days after the beginning of the test. Last infected beetles died 41 days after the infection. The midgut of beetles was filled with trophozoites and cysts of gregarines. Not all beetles were infected. The last beetles died 65 days after the start of the experiment.

The nematode P. typographi develops in the body cavity of hosts infected with invasive larvae. In dead beetles we observed larvae of the nematode. First nematodes were identified 5 days after the start of infection. The main development of nematodes occured from day 12 after the beginning of the infection. The last beetle bearing nematodes in the body cavity died 53 days after the exposure. In some beetles the nematode infection did not develop, and the last beetles died 67 days after the infection.

In consumption experiments beetles started to eat immediately. Non active and non feeding beetles were eliminated 2 days after the beginning of the experiment. For brown and dark brown beetles the eating curve is exponential, whereas for the black ones, the curve is sigmoidal (Fig. 2). During the first week, 50% of each granule was eaten in all groups and the majority of the granule was consumed during the next two weeks. The brown beetles survived and eated much better than the black ones but their survival is not so good as evident from tests mentioned above. After 3 weeks, 90% of the phloem was consumed. In this period 100% of granules were consumed. These results are related to the fact that the infectious suspension is not able to soak into the phloem and the majority of suspension remains on the surface of the bark, as observed under the UV light.

Our method allowed us to keep live beetles for two months, which is enough for the development of different pathogens. Owing to that, we succeeded in infecting bark beetles with various natural enemies. The microsporidian C. typographi appeared the most efficient pathogen, killing all exposed beetles, contrary to P. typographi and G. typographi.
Figure 1. Survival (in %) of *Ips typographus* adults infected with the microsporidian *Chytridiopsis typographi*, the eugregarin *Gregarina typographi*, or the nematode *Polymorphotylenchus typographi*. 
We did not succeed with infection of bark beetles with *I. typographus* entomopoxvirus, even if there is an evidence of field infection of adult beetles in logs sprayed with the virus suspension (Pultar & Weiser, 1999). There is no evidence of any transmission of *I*EPV in larval and preadult stages of bark beetles. In any case, the development of the poxvirus in Coleoptera is known to be very long (Goodwin & Filshie, 1969) from internal organs, not from the midgut epithelium, and further investigations are needed to understand the phenomenon.

In conclusion, this contribution could help to investigate biological features (survival, infectivity, virulence, mode of action) of bark beetle pathogens with the aim of developing their use for the biological control of the spruce bark beetles.

**Acknowledgments**

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Occurrence of \textit{Gregarina typographi} (Sporozoa: Gregarinidae) and \textit{Metschnikowia cf. typographi} (Ascomycota: Metschnikowiaceae) in \textit{Ips sexdentatus} (Coleoptera: Scolytidae) from Austria

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\textbf{Abstract:} \textit{Ips sexdentatus} from seven Austrian locations were dissected and inspected with regard to the occurrence of pathogens. \textit{Gregarina typographi} was found in specimens from all seven locations, \textit{Metschnikowia cf. typographi} was found in the beetles from six locations. Prevalence of both pathogen species varied in the beetles from the different locations.

\textbf{Key words:} \textit{Ips sexdentatus}, Scolytidae, pathogens, \textit{Gregarina typographi}, \textit{Metschnikowia typographi}

\section*{Introduction}

\textit{Ips sexdentatus} (Börner 1776) is known to occur in palaeartic region mainly on \textit{Pinus} spp., but sometimes also on other conifers (e.g. \textit{Picea orientalis}) This bark beetle species develops in central Europe on \textit{Pinus sylvestris} and on \textit{Pinus nigra}, and is known to attack fresh logs just after felling and standing trees, suffering from draught or after injury by forest fire (Postner, 1974; Pfeffer, 1995).

Up to now, very little is known about pathogens in \textit{I. sexdentatus} (ref. in Wegensteiner, 2004), only Théodoridès (1960) reported about the occurrence of \textit{Gregarina typographi} (Sporozoa, Gregarinidae) in this insect. Furthermore, \textit{I. sexdentatus} was found to be sensitive against \textit{Malamoeba scolyti} (Rhizopoda, Amoebidae) in laboratory infection experiments (Kirchhoff, 1982). Therefore, our investigations focused on the natural occurrence of pathogens in field collected, living and reproducing \textit{I. sexdentatus}.

\section*{Material and methods}

Adult \textit{Ips sexdentatus} were collected at seven different locations in Eastern Austria, five located in the federal state of Lower Austria (Goldberg, Katzelsdorf, Obermarkersdorf, Pulkau, Hernstein) and two in Burgenland (Forchtenau, Rechnitz), at altitudes ranging from 400 m to 600 m); they were collected by hand, out of their galleries in the phloem of \textit{Pinus sylvestris} and \textit{Pinus nigra} logs, in 2003 and 2004.

Beetles were dissected just after collection or after storage in an incubator at 15 ± 1°C, for one week at maximum. In consideration of the fact that the presence of pathogens was the primary objective, beetles were dissected on microscopical slides in a drop of water and were inspected in normal light microscope for diagnosis (Wegensteiner et al., 1996). After fixation with methanol smears were stained with Giemsa’s dye (Weiser, 1977) and re-inspected in normal light microscope. The presence of pathogens in different organs or free in the haemolymph was recorded, presence of nematodes was noticed.
Results and discussion

In total, 291 beetles were dissected individually, 151 males and 140 females. Two different micro-organisms were found in *I. sexdentatus*, both known from other bark beetle species as pathogens. The protozoan species, *Gregarina typographi* (Sporozoa, Gregarinidae) was described by Fuchs (1915) as a parasite of *Ips typographus*, but Théodoridès (1960) found *G. typographi* also in *Ips sexdentatus* from France. We found *G. typographi* (Fig. 1) in the mid-gut lumen of *I. sexdentatus* from all seven locations in Austria (total n = 84), in female (n = 44) and in male beetles (n = 40) (Table 1).

Infection rates varied in the beetles from the different locations within a wide range, 16.7% to 45.5%; infection rates were relatively similar in male and female beetles but in some cases they were very different (Table 1). It should be noticed, however, that numbers of inspected beetles were rather low in nearly all cases. *G. typographi* was very common in *Ips typographus* populations too and sometimes in high prevalence (Wegensteiner, 1994; Wegensteiner et al., 1996), so it looks that this pathogen species occurs relatively unspecific at least in *I. sexdentatus* and *I. typographus*. In addition, *G. typographi* is presumed to infect other bark beetle species reproducing on Norway spruce (*Picea abies*) (Haidler et al., 2003; Händel et al., 2003), and bark beetle species occurring on *Pinus cembra* (Händel & Wegensteiner, 2005).

Table 1. *Gregarina typographi* infection rates (in %) in *Ips sexdentatus* collected in different locations in Austria in the years 2003 and 2004, and numbers of inspected female and male beetles.

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Gregarania typographi</th>
<th>Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Σ</td>
<td>Fem.</td>
</tr>
<tr>
<td>2003</td>
<td>Goldberg</td>
<td>18.9</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>Katzelsdorf</td>
<td>37.7</td>
<td>38.2</td>
</tr>
<tr>
<td>2004</td>
<td>Obermarkersdorf</td>
<td>25.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Pulkau</td>
<td>16.7</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>Hernstein</td>
<td>44.4</td>
<td>66.7</td>
</tr>
<tr>
<td></td>
<td>Forchtenau</td>
<td>40.0</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td>Rechnitz</td>
<td>45.5</td>
<td>48.0</td>
</tr>
</tbody>
</table>

The fungus *Metschnikowia* cf. *bicuspisdata* (Ascomycota, Saccharomycotina, Metschnikowiaceae), which was first mentioned to occur in *I. typographus* from Finland (Weiser & Wegensteiner, 1998), was later described as *Metschnikowia typographi* in *Ips typographus* and *Ips amitinus* from Finland and Austria (Weiser et al., 2003). Our investigations led to find it for the first time in the cells of the mid-gut epithelium and free in the haemolymph of *I. sexdentatus* (Fig. 2); the fungus was observed in beetles from six locations (total n = 36), in males (n = 23) and in females (n = 13). Infection rates varied in the beetles from the different locations (from 0.9% to 51.4%), but gender dependant differences were not very high in case both were infected (Table 2).

Mixed infections, *M. typographi* together with *G. typographi*, were found in five beetles from Goldberg and in one beetle from Katzelsdorf collected in 2003, and in one beetle from Hernstein collected in 2004.
Table 2. *Metschnikowia typographi* infection rates in *Ips sexdentatus* collected in different locations in Austria in the years 2003 and 2004, and numbers of inspected female and male beetles.

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Numbers</th>
<th>Metschnikowia typographi</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Σ</td>
<td>Fem.</td>
<td>Males</td>
<td>N</td>
</tr>
<tr>
<td>2003</td>
<td>Goldberg</td>
<td>51.4</td>
<td>42.9</td>
<td>56.5</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Katzelsdorf</td>
<td>11.6</td>
<td>11.8</td>
<td>11.4</td>
<td>69</td>
</tr>
<tr>
<td>2004</td>
<td>Obermarkersdorf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Pulkau</td>
<td>0.9</td>
<td>1.9</td>
<td>-</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>Hernstein</td>
<td>22.2</td>
<td>-</td>
<td>33.3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Forchtenau</td>
<td>10.0</td>
<td>-</td>
<td>14.3</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Rechnitz</td>
<td>9.1</td>
<td>8.0</td>
<td>10.5</td>
<td>44</td>
</tr>
</tbody>
</table>

Figure 1. *G. typographi* (associated gamonts) separated from the midgut of *I. sexdentatus*.

Figure 2. *M. typographi* asci in the haemolymph of *I. sexdentatus*.

Presently it is not clear whether *Gregarina typographi* or *Metschnikowia typographi* described from *Ips typographus* are identical with corresponding species found in *I. sexdentatus*. They look very similar in shape and size, and only the use of molecular techniques might help in solving these questions. Both pathogens are presumed to be transmitted horizontally, but *I. typographus* reproduces in Austria on *Picea abies* and *I. sexdentatus* attacks invariably *Pinus* spp. Furthermore, these two *Ips* species normally do not have direct contact among each other in the field. Therefore, horizontal transfer of these two pathogen species from *I. typographus* to *I. sexdentatus* looks to be very unlikely; maybe they get in contact with the *Metschnikowia* species via tree exudates, similar to the situation with *Candida* species found in sap fluxes of trees in Costa Rica (Lachance *et al.*, 2001).

Till now, only relatively few papers concentrated on the occurrence of nematodes in *I. sexdentatus* (ref. in Poinar, 1975; Lieutier & Laumond, 1978; Vilagiova & Mituch, 1991). Interestingly, we found nematodes free in the haemolymph and in the mid-gut lumen and in the hind-gut lumen of beetles from all locations collected in 2003 and 2004. Exact identification of the nematodes was not possible.

**Acknowledgements**

We thank several forest owners and foresters for their help in beetle sampling, and A. Stradner (BOKU-University) for help in dissection of beetles.
References


Taking biocontrol from laboratory into practice: a case study from Kenya

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Abstract: Dudutech is the largest IPM/biocontrol company in Eastern Africa and the only one presently developing microbial pesticides. The first products it successfully developed and registered were natural enemies, which are currently used commercially on a large scale both in vegetable and flower crops; however, the company is presently striving to develop several biopesticides and make them into a commercial reality. Amongst the microbials, the fungi, *Trichoderma asperellum* (for control of soilborne diseases) and *Pochonia chlamydospora* (against *Meloidogyne* spp.), are already in commercial or semi-commercial production, together with the nematode, *Steinernema feltiae*. Other products in the pipeline include *Pasteuria penetrans* and a granulosis virus (*PlxyGV*), as well as some entomopathogenic fungi. Based on Dudutech’s experience, the main factors affecting the development of biopesticides and their successful implementation on commercial crops in Kenya will be briefly discussed.

Key words: Biopesticides, IPM, *Trichoderma*, *Pochonia*, *Steinernema*, *Pasteuria*, natural enemies

Introduction

Dudutech is a biocontrol/IPM company founded in 2001 as part of the Flamingo Holdings Group. Since its inception, the main focus of activities has been the development of alternative pest control solutions and IPM strategies for one of its sister companies, Homegrown, which with almost 200 ha of flower crops and a similar area of vegetables is also the main producer and exporter in East Africa of flowers and vegetables, destined for UK supermarkets. Although Homegrown represent by no means Dudutech’s only client, they are certainly contributing the majority of the income.

The first products developed and registered by Dudutech were natural enemies, which are currently used successfully on a large scale both in vegetable and flower crops. However, subsequently the company started the development of several biopesticides, and have been striving ever since to make them into a commercial reality.

Current status of product development and use

Although this presentation focuses on the development of microbial pesticides, it is worth mentioning the fact that the use of some natural enemies, namely *Diglyphus isaea* and *Phytoseiulus persimilis* has been a great success and represented the inspiration to the subsequent development of microbials. To give an example of this, suffice it to say that the use of *Phytoseiulus* permitted over a period of two years to reduce the use of acaricides on Homegrown flower crops by approximately 85%, which, given the incidence of mites under Kenyan conditions, in turn has meant an overall reduction of 55% in the use of crop protection chemicals (Figs. 1 & 2).

With specific regard to biopesticides, a WP formulate (TrichoTech) has been in production for well over one year and is currently used to treat an area of approximately 80 ha weekly on Homegrown’s farms, with a 90% reduction in the use of chemical fungicides.
previously used to control soil borne diseases. It should be noted that although this product is not yet registered, its large-scale use on commercial crops has been possible thanks to special permission granted to Homegrown by the Kenyan Ministry of Agriculture. A granular formulate for the same strain has also been developed, and Dudutech are now undertaking the registration process for both formulates.

The fungus *Pochonia chlamydosporia* is being produced and tested in commercial scale trials against *Meloidogyne* spp. The production of this nematode parasite was developed in cooperation with a Cuban research institution, and although field data from Cuba indicate that it is effective, the aim now is to prove that it is also effective also under kenyan conditions and that its production and application at the large scale which Dudutech are envisaging is feasible from a practical point of view.

Another line at a pre-commercial stage is the nematode, *Steinernema feltiae*. This is being developed mostly for the control of thrips, and the current production of approx. 10 billion IJ/week is destined entirely for commercial trials.

Other products in the pipeline include some entomopathogenic fungi, as well as *Pasteuria penetrans* (for the control of root knot nematodes) and *PlxyGV* (against the diamondback moth). For the latter two organisms most of the work is presently focused on the development of commercially viable production systems.
Difficulties / challenges encountered

During the development of the biopesticides mentioned above, several logistical, technical and commercial constraints have been encountered, which can be summarized as follows:

**Logistical challenges:**
- Limited local availability of the consumables, equipment, etc. needed.
- Limited access to scientific literature.
- Limited availability of specialised services (e.g. identification of organisms, analysis, technical services, etc.).

**Technical challenges:**
- Scaling up of processes.
- Use of appropriate (= manageable) techniques.
- Lack of specific skills of local personnel.
- Limited knowledge / acceptance of IPM criteria by growers.
- Implementation of strict SOPs.
- Implementation of rigorous and relevant QC procedures.
- Need to develop simple and cheap formulations, but still acceptable to growers.
- Non-amenability of certain micro organisms to production on an artificial media.

**Commercial challenges:**
- Excessive specificity of some biocontrol agents.
- Need to develop integrated control strategies.
- Development of a new and more appropriate legislation for biopesticides.

Some of the above mentioned difficulties are quite obvious and common to the process of developing biopesticides independently of where such process takes place. For this reason, here we would like to spend a few lines commenting on those that are more typical of working in a developing country.

The limited availability of consumables and equipment, which may be lacking completely or found in a regime of monopoly, has obviously the effect of significantly increasing the cost of both development and production. Since direct importation from abroad is quite cumbersome, in many cases it becomes necessary to experiment in order to find substitutes for the items that are not available, with all obvious implications. Similarly, access to recent scientific literature is not so readily available in Kenya, at least not for commercial companies, which, unlike public institutions, are not given cheap access to services such as online databases or journals. The fees which companies are expected to pay for such services can be very high, with the result that sometimes it becomes necessary to “reinvent the wheel”.

With regard to technical difficulties, the development of production processes must take into account the actual conditions under which such processes take place, including factors such as stability of power supplies, quality of the water, etc. In many cases this rules out the use of higher-tech solutions (e.g. liquid fermentation) and for this reason all products developed by Dudutech are produced using solid fermentation technology (with the obvious exception of the obligate pathogens), which is easier to implement but poses considerable problems of scale-up. The very limited availability of local personnel with specific skills in biopesticides, together with the limited knowledge / acceptance of biocontrol and IPM criteria by the growers who are then to use them, have been amongst the main hurdles to overcome. A lot of effort and resources have been put into training of Dudutech’s own personnel as well as personnel of farms willing to use our products. This is reflected in the fact that in the year 2004 a total of 3923 people were trained by our training department, largely to ensure that all growers and farm personnel using biocontrol agents have a proper understanding of their mode of actions and of the way they should be handled and applied (including their integration with chemical pesticides).
On the commercial side, it should be mentioned that in some cases the specificity of some potential biopesticides poses doubts about their commercial viability. One such example is the development of *P. penetrans*, whose specificity may reach species level, since a survey carried out on 5 farms revealed the presence of 4 different *Meloidogyne* species (*M. incognita*, *M. hapla*, *M. javanica* and *M. arenaria*), with some farms having all of them. In this case the only option appears to integrate the use of the biopesticide into a management system which includes other control measures (e.g. crop rotation), which however may not be acceptable to highly specialized growers. Finally, like in most other countries, legislation on (bio)pesticides can be a complex subject also in Kenya. Over the last three years Kenya has finally developed a specific legislation for biopesticides, which should make the registration of products based on indigenous strains considerably easier. To this regard, it should be mentioned the fact that all the microbials developed by Dudutech Ltd. are based on species / strains which are indigenous to Kenya. This was initially done in compliance to the legal requirements and also on the assumption that their registration would be easier than for foreign organisms. The recent registration in what appeared to be a very short time of non-indigenous microorganisms it’s an indication that this may not be necessarily the case.

To conclude with, and in order to avoid giving an unrealistically grim picture of the situation, we should mention that the activities of our company have greatly benefited from the fact that we were given access to strains of some microorganisms which had been isolated and characterized during some research projects carried out by various national and international institutions and funded mostly by DFID. Also, it is a reality that the permission granted by the Ministry of Agriculture to carry out large scale trials and later commercial applications with unregistered biopesticides on the farms of our sister company, Homegrown Ltd., has greatly helped the development of the products which are currently in our front line and for which registration will be sought soon.

**Conclusions**

The very close relationship between a producer of biocontrol agents and their final user represents to the best of our knowledge quite a unique example worldwide, and a model which can greatly favour the practical implementation of biocontrol on large scale commercial crops. The local production of microbials (as well as natural enemies) using appropriate (= adequate to local conditions and low-cost) technologies allows the obtention of effective products at an acceptable cost to the grower. However, training is a vital component of this system and any company engaging in these activities should invest heavily in it. The cooperation and good will of the regulatory / supervisory bodies obviously represents another key-element for success in the development of biopesticides, together with the implementation of a streamlined and clear registration process.

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