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Insect Pathogens and Insect Parasitic Nematodes

"Capturing the Potential of Biological Control"

editor:

P.H. Smits

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IOBC / WPRS

Working group "Insect Pathogens and Insect Parasitic Nematodes"

OILB / SROP

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

7th EUROPEAN MEETING

"CAPTURING THE POTENTIAL OF BIOLOGICAL CONTROL"

at/à

Vienna, (Austria)
22–26 March 1999

Edited by P.H. Smits

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Introduction

This bulletin contains the proceedings of the 7th European meeting of the IOBC/WPRS Working group "Insect Pathogens and Insect Parasitic Nematodes". The meeting was held March 22-26, 1999 in Vienna, Austria under the title "Capturing the Potential of biological Control". The meeting was hosted and organised by Dr. Rudolf Wegensteiner of the Institute of Forest Entomology, Forest Pathology and Forest Protection, University for Bodenkultur (BOKU) in Vienna. On behalf of the Working Group and all attendants I would like to thank him, Elisabeth Koschier and all others that were involved in the organisation for the very pleasant and efficient way in which the meeting was organised, it was an excellent meeting.

This was the last meeting that I acted as convener of the Working Group. After 8 years as convener I felt it was time to hand over this task to someone else with fresh ideas and fresh energy. I am very happy that the Working Group elected Bernard Papierok of the Institut Pasteur in Paris as the new convener, I am convinced the Working Group will continue to thrive under his leadership. I wish to thank the Working Group and all its members for their confidence and support over the years and most of all for their inspirational attendance and participation in the meetings, which I thoroughly enjoyed.

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Forty years of organized international Insect Pathology

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Insect pathology and biological control with insect pathogens is a rather young discipline in its international organization. At its beginning just after the end of the Second World War were several isolated centres organized in different parts of the world. The Laboratory of Insect pathology of Prof. Steinhaus at the University of California, Berkeley, the Laboratory of Insect Pathology of the Canada Department of Agriculture, Sault Ste Marie, in Europe the laboratories of plant protection joined in the International Organization of Biological Control (OILB) with laboratories of insect pathology in La Miniere, France, Darmstadt, Germany and Zurich, Switzerland. In the USSR a laboratory for insect pathology was a part of the Allunion Inst. of Plant Protection, Leningrad. Outside of the professional network of nationally oriented laboratories affiliated to national institutes of plant protection, were research laboratories without this practical affiliation, such as the virology laboratory in Cambridge, the microbiology at the Pasteur Institute, Paris or the Dept. of Insect Pathology at the Biology institute of the Czechoslovac Academy of Sciences, Praha. And here, after my participation at a meeting of insect pathologists during the Montreal Congress of Entomology, the idea started to bring together insect pathologists and organizers of biological control from all important centres in the world to a specialized congress which may give them an opportunity to make contacts, discuss co-operation and form a world-wide network of in developing bio-control. The **Ist Internat. Conference of Insect Pathology and Biological Control** was organized in Praha, August 13-18, 1958 and had 150 official participants from 18 countries, including Canada, USA, China, India, most European countries and USSR. East (60) and West (28) met eventually the first time under conditions of the Iron curtain. 38 Russian and Central Asian workers had the first open window to western science. We have now to celebrate the 40est anniversary of this meeting and, 40 years of international organized insect pathology. If we read the names under the photograph of the participants, we find many names of future leading personalities in insect pathology or biological control and many officials who organized the laboratories and international exchange in the years after the meeting. It was the only time when entomologists working with entomophagous insects met together with insect pathologists on common problems of introductions and use of insects and micro-organisms in biological control. At that time, the specialized interests of the leading laboratories were divided in two main lines: agricultural with the well working system of OILB and that of Berkeley and VIZR Leningrad, and the forestry line which was represented by the Sault Ste Marie laboratory and to some part the laboratories at Darmstadt and Praha. The OILB was an organization of specialized workers in the official laboratories of plant protection, their results were incorporated directly into practice. On the other side, university and research organization laboratories were without possible direct introduction of results into practice.

It is no space to follow the subsequent growth of new laboratories in all parts of the world. But this was a development which resulted in the foundation of the **Journal of Insect,** later **Invertebrate Pathology** and the organization of the **Society of Invertebrate Pathology.** In the Journal and in published abstracts of the SIP meetings, invertebrate pathologists were able to follow the development of the distribution and application of insect pathogens in agriculture and forestry. A special field in invertebrate pathology should be mentioned

separately: the field of **Vector pathology and biocontrol.** This was prepared early in the fifties and was organized as a special discipline of the Special Programme for Research and Training in Tropical Diseases of the WHO, after 1964 with participation of a world-wide community of insect pathologists.

The Praha I. Conference brought many permanent contacts and also a first important news. Prof. Steinhaus announced the first **large scale fermentation** production of *Bacillus thuringiensis,* the Thuricide, and herewith the entry of insect pathology into practical large scale microbial control. It was followed the next year by fermentation of BT in Czechoslovakia with Bathurin on the market based on serotype H-lalb, the Berliner strain 45 isolated in Praha and the same production in France of Bactospeine, with their own BT strain alesti isolated from old rearings of the silkworm and later by Entobacterin of Russian origin to mention only the first formulated materials.

The primary idea of insect pathology was to initiate **natural control,** to complement existing systems of introduction of parasites and predators against major pests in sense of the work with *Rodolia* and *Iceria* in California. Really, the first efficient control of a pest by introduced pathogens was that of the two sawflies, *Neodiprion sertifer* and *Gilpinia hercyniae* in Canada, where the *sawfly NPV* was introduced with parasitoids of *Neodiprion* and *Gilpinia* just before World War Two, and unobserved during the war, the virus took its part in the Maritime Provinces on the Eastern coast, together with granulosis of the fir budworm, *Choristoneura fumiferana.* With efficient production of BT by fermentation and with further results with applications of Deuteromycete fungi produced on solid media as conidia or in liquid fermentations as blastospores, insect pathology concentrated on methods of application of produced material in the same way as insecticides, with the same aparature and in the same formulations. It was a period of search of new, more virulent strains of all different insect micro-organisms and determination of necessary dosages, biounits and times of survival of active propagula in field crops. In agriculture the targets were in annual crops as same as in orchards where a more persistent biotop allowed the long lasting follow-up of the used applications. Analogous productions and applications of *Beauveria* were less efficient and applications of viruses produced on laboratory-reared hosts brought active field control of several pests in agriculture in orchards and in forests. It was evident that only a n introduction of an absent pathogen in an empty niche was not self-spreading as parasitoids were and that **active microbial control** had to be performed again and again, each season, with risks of development of resistence. **Studies of ultrastructures** brought in some disciplines, such as research of viruses or protozoa, details which split old species into a complicated array of new genera and species and eventually explained the differences in host specificity and virulence. The mid fifties brought another tool for insect pathologists: the laboratory rearing of entomophilic nematodes of the codling moth and this remained specialists that there is an older literature on *Neoaplectana glaseri* and *Steinernema* with former history of cultivation and application. The use of the wax moth for laboratory production of *Neoaplectana carpocapsae* brought an easy system of maintaining cultures in the laboratory and their evaluation against pests in the field. The identification of one specific symbiotic micro-organism transmitted by nematodes to insect hosts opened also the door for experiments with mass production of the nemas on fermented *Xenorhabdus* bacteria and this brought a third type of biopesticide to the market: invasive larvae of Steinernematids in a form which enabled their storage and shipment for use in distant areas. The other groups of pathogens, the viruses or protozoa were difficult to manage in direct applications. They need for their massive transmission a complicated production on living hosts in the laboratory and a target in dense populations where the transmission from host to host is easy with close contact of hosts. Even in groups with existing technology of production, some micro-organisms resisted and are

resisting to mass production and application. Among the bacteria it is the group of *Bacillus popilliae, Pasteuria penetrans,* among fungal pathogens the entomophthoracean fungi, where the spores or conidia can be produced, but the material is not infectious.

Modern approach to pathogens with methods of **molecular genetics** and biochemistry helps to explain different toxicity of BT strains in defining the endotoxin as a complex of more than 50 different closely related polypeptides which each is forming a synergistic part in the crystalline complex of BT during sporulation. Analogous investigations helped to improve our knowledge of molecular biology of viruses and the NPV viruses were useful in some systems of gene transformations in tissue cultures.

Proper knowledge of sequences producing the different types of BT endotoxin was important also with the use of **transgenic plants,** where the sequence of genes for production of an individual endotoxin is incorporated in the genome of a plant exposed to attacks of lepidopteran pests. In this way the plant produces the Lepidoptera killing toxin in each cell of its body and kills stages of the insect when they attack and feed on transgenic tissues. So was constructed transgenic corn, tomato, tobacco, cotton or potatoes. The introduction of transgenic plants with BT endotoxin may be compared with the change of films from dumb to sound and from black to color. In this sense BT is a wonderful source of active polypeptide producing sequences and toady's use of transgenic seeds is on over 20 million ha of agricultural yearly crops. It is not yet used in trees. The problem is in formation of resistance in pests, where the dosage of toxin in their food is not reaching the toxic threshold. This is mainly the case of caterpillars which were living on non-transgenic plants up to L-3 or L-4 and later they enter transgenic crops. For these late stages the toxic threshold is rather high and is not reached with normal meals on transgenic plants. This allows the formation of physiological systems of **resistance.** Except bacteria, also **fungal or microbial metabolites** (polypeptides), which usually do not participate in their pathology, could be used for preparation of transgenic plants or another type of biopesticides, but their polypeptidic metabolites are active toxins or immunity modelling substances which are active also in nontarget hosts and may be objected by hygienists. In all other groups molecular genetics may be auxiliary in differentiation of sibling species or complexes and helping to construct philogenetic trees of higher taxa.

What do we need to achieve in **the future:** The tasks for the future are of two main types: projects which are funded and their themes sound attractive to financing bodies and the other complex which cares for the general progress of the field of invertebrate pathology. This is a bad statement but it explains why some types of research are practicized as a new fashion without any support of the older, not yet finished research. We must continue with studies of pathogens in **natural populations** and in improvement of material for direct field application, its production and storage before use. We have to come back to some self-sustaining infections which start the increase of frequency of infection and mortality with the growing density of a pest in outbreaks. We need to analyse populations of pests in outbreak and look for a best fitting application of one or a combination of pathogens. A good way is to concentrate on complexes of pathogens (and parasites) of **specific biotopes:** pests of annual crops, pests of orchards, vectors, pests of stored products, or, pests attacking forests. An example of the last is the work of the host organization, the Institute of Forest Entomology, Pathology and Control.

Molecular biology and nucleotide sequencing is a prominent field especially as source of data for **production of transgenic plants** or **genetic modification and improvement of selected pathogens.** In this specialization we may try to find some limiting factors for Homoptera incorporated in plants, because Homoptera are not exposed to peroral infections with pathogens with contaminated food. Toxic or repellent sequences are expected to be

incorporated into modern transgenic plants. Genetic modification of existing pathogens may increase the virulence and destructive ability of pathogens in bio-pesticides, may improve their spread and survival in the field respecting all criteria of environment protection and public health. **Nucleotide sequencing** is more and more used for improvement of understanding of taxons with minute morphological differences and is expected to help to produce proper phylogenic trees of higher taxons of microsporidia, neogregarines, amoebae or of bacteria, fungi, rickettsiae and other insect pathogens. Available informations matched with morphological data for type specimen bring many questions. They show disorders in old classifications of species in genera and of genera in higher groups. These cases must be reinvestigated. On the other side, it is not clear if just the used sequences in analyses of chromosomes are the most distinct and deciding in characterization of generic or specific differences of different organisms living in the same host and biotope or identical organisms adapted to different hosts and types of environment. The taxonomy of organisms is based on the **Linnean system of binomial nomenclature.** This is based on complex rules which are now hampered by definitions of nucleotide sequencing. Until all will be changed in definitions of living nature we have to respect the type specimen and its morphology as basis for descriptions, differentiation and comparisons of new organisms. For every molecular and sequenced new organism we must seriously try to find a morphological difference other than molecular. And this are some of the requirement of future work in the discipline of **Insect pathology in its fifth decade.**

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Entomopathogenic fungi in the context of pest biocontrol today

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The purpose of this contribution was not to give a comprehensive and updated overview on entomopathogenic fungi in the context of biocontrol of pests. For the author indeed, it seemed more appropriate, at the beginning of the meeting, to draw from all the planned contributions, the most significant trends of the recent research dealing with these beneficial microorganisms, and to underline some new and original results.

The « Fungal part » in the total contributions represented little more than 20% for oral presentations and little more than 30% for posters. It appeared very slightly lower than in previous meetings but still showed a strong interest in fungi.

As it is the usual case, presentations were expected to reveal a high diversity, even if most of them were more or less related to ecological considerations. For the author however, such a diversity made the interest and significance of the meetings of the Working Group, which allowed for instance people working mostly in the field to discuss and share their own experience with people investigating mostly in the laboratory.

The first topic underlined by the author was related to **natural occurrence.** Indeed, during the last years, entomopathogenic fungi were studied in new locations. Aphid-pathogenic Entomophthorales were investigated in Iceland and Slovakia. Diverse fungi were found on mites in Poland. Observations were made on Collembollans in Denmark, including an interesting mention of a species of *Neozygites,* an entomophthoralean genus mostly known as specific to Homoptera. On the other hand, studies on soil inhabiting fungi were recently initiated in Austria. Still regarding these fungi, a special attention had to be paid to *Cordyceps militaris* epizootics observed in Lithuanian *Dendrolimus pini* populations. This was interesting because *Cordyceps* species were mostly investigated in tropical humid areas. Such situations showed that in temperate regions, quite interesting but to date unnoticed phenomena were still to be discovered. Finally, the author mentioned that he was aware of studies carried out during the last two years in southern Europe. The corresponding results were not going to be presented in the meeting but indeed, they showed that both Hyphomycetes and Entomophthorales encountered in ecoclimatic conditions which definitively could be considered as quite unfavourable to fungi.

As regards the **ecology** of entomopathogenic fungi, the interest of recent observations made in Poland had to be emphasized. According to these, temperature could affect results of soil fungi isolation using the *Galleria* bait method. It was then reminded to the audience that in previous meetings, the need for a standardized method in order to accurately compare investigations made in different areas was strongly brought up (see The Proceeding of the Meeting in Copenhagen, 1997, in IOBC/WPRS Bulletin, 1998, 21 (4), 287-289). The last Polish results confirmed the previous concern of the Working Group and still demonstrated the need for further investigations. Another contribution showed that fungal infectivity was reduced in soil highly contaminated with heavy metals. Then the author introduced the tritrophic aspect, i.e. interactions between plant, pest and pathogen. Such interactions were studied for years in the case of parasitoids but were not tackled systematically as regards

entomopathogenic fungi. This underlined the special attention which should be paid to the contribution planned on this subject.

Characterization represented another topic for the author. Even if only one presentation was devoted to the use of molecular tools for characterizing genetic variation within entomopathogenic fungi, such a study helped to progress in this field, especially as regards technical improvement. Concerning virulence and biochemical tools, the attention of the audience was drawn to the two contributions devoted to *Beauveria brongnartii,* their aim being to develop biochemical tests for estimating virulence.

There were planned only a few contributions dealing with **infectivity and virulence.** Some of these investigations were conducted on *Verticillium lecanii,* given the interest of this fungus as a control agent of the important pest, *Frankliniella occidentalis.* As a matter of fact, there was still a need for better understanding the potential of such « well known » pathogens.

Various contributions were devoted to **direct evaluation of the potential of fungi as biocontrol agents.** The author distinguished « classical » laboratory evaluations, as those made with bark beetles or ticks, preliminary field investigations and more integrated approaches, such as those carried out on the possibility to combine a fungus, either an Entomophthorale or a Hyphomycete, and a trap in order to help dissemination of the pathogen. Finally the author mentioned the Czech contribution which introduced the combination of mineral fertilizer and entomopathogenic fungus.

Following his comments on these different topics, the author initiated general reflections on entomopathogenic fungi. First, these microorganisms acted as natural enemies of insects. From this point of view, they presented intrinsic advantages (probably the most important being the ability to provoke epizootics) and shortcomings. On the other hand, entomopathogenic fungi were investigated as potential biocontrol agents, i.e. with the aim to be handled and applied. From this point of view, the most important shortcoming was production and storage feasibility. To date, when considering the world of entomopathogenic fungi, their potential, the fact that some of them were intensively studied for years, and at last, the currently very limited number of commercial mycoinsecticides, one could understand the difficulty to « domesticate » such microorganisms. Such a general consideration should not be regarded as discouraging. Indeed, there were in the audience people who attended for the first time a meeting of the Working Group. New teams started to work with fungi. This had to be considered as a very good point.

As a conclusion, two suggestions were given by the author. The first one was dealing with forest insects, which provided a quite interesting wide field of experience with pathogens in general and fungi especially, in relation to the concern of sustainability. Most of forest insects had a part of their life cycle in soil, whereas soil is the reservoir for most fungal species. The second and last suggestion was related to medical insects. The Working Group was until now mostly involved in agriculture and forest pests. To date, bacteria were used for controlling populations of mosquitoes and blackflies. Given natural enemies of these Diptera comprised among others fungi, nematodes, protozoa, it would be beneficial for the members of the Working Group to extend their concern to the field of medical entomology.

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A researcher's obstacle race: report on the registration of a new bioinsecticide

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Abstract: Scientists, producers, applicators and even the authorities know that there is a need of a world wide harmonisation of the registration requirements for biopesticides. Although they all agree that progress is being made toward achieving harmonisation, differences in detail and in interpretation may undermine these efforts, and continue to raise the hurdles against the development of new biopesticides. In this presentation an overview is given about the registration efforts and strategies to become *Beauveria brongniartii* registered as an biocontrol agent by the Austrian plant protectant legislation.

Keywords: entomopathogen, *Beauveria brongniartii,* BCA, registration

Introduction

The purpose of this report is to discuss the current situation on registration of biological control agents in EEC-member countries. On the basis of an OECD document, which has been prepared for the Pesticide Forum, held in Paris, in November 1998, I want to focus on the numbers of products registered and/or under development in eighteen OECD countries in the last five years. Eleven EEC-member states such as Austria, Germany, Denmark, Belgium, Finland, Germany, Ireland, Italy, Netherlands, Sweden, United Kingdom were included in this OECD report. The following data demonstrate the current registration situation in most EC-countries: Most of them have approved 5 to 30 microbial end-use products during the last five years. The majority of these products are *Bacillus thuringiensis* subsp. - agents with diverse nomenclature to distinguish between different isolates or strains of Bt.. Since the 1993 OECD survey of "Microorganisms registered for plant protection uses", most countries have registered at least two more microbial end-use products, and have approved at least one new microbial agent.

Currently, in EEC-member states eight entomopathogenic fungi are approved (Table 1). Although the same pest problems exist in most European countries only *Verticillium lecanii is* commercially available in more than two states.

This fact demonstrates that the regulatory situation for biopesticides in Europe is in a state of dis-harmony. Scientists, producers, applicators and even the authorities know that there is a need of harmonisation of the registration requirements for biopesticides. Although they all agree that progress is being made toward achieving harmonisation, differences in detail and in interpretation may undermine these efforts, and continue to raise the hurdles against the development of new biopesticides.

Table 1: Entomopathogenic fungi registered for plant protection uses in EEC-member countries and Switzerland (based on 1993-94 survey, OECD monograph no.106 with 1998 updates)

Fungi	Registered uses	Country
Achersonia aleyrodis	white flies *(Trialeurodes vaporariorum, Bemisia tabaci)*	Sweden
Beauveria bassiana 147 spores	maize, treatments of aerial arts, pyrale	France
Beauveria tenella 96 spores	general treatments, soil treatment, may beetle grub *(Holochelus marginalis)*, sugar cane; insecticide	France
Beauveria brongniartii	may beetle grub	Switzerland
Metarhizium anisopliae	*Otiorhynchus sulcatus, Hepialus* spp. for ornamentals and viniculture	Germany, Austria
Paecilomyces fumosoroseus	white flies *(Trialeurodes vaporariorum, Bemisia tabaci)*	Sweden, Belgium
Verticillium lecanii	insects on tomatoes, cucumbers	Netherlands, Switzerland
	aphids and mites	Denmark
	aphids *(Macrosiphum euphorbiae, Aulacorthum solani)*	Sweden
	thrips of tabacco, aphids, *T. urticae*	Switzerland
	ornamental, flower(cut), cucumber, pepper, aubergine, lettuce, bean	Finland, Ireland
Verticillium dahliae	against elm disease on elm	Netherlands

Most of the European countries still have their own evaluation priorities to permit new biological control agents. For example some countries put the emphasis on environmental data collected in big field trials. They want to be informed in detail about the fate and behaviour of the biocontrol agent in the environment. Others are more interested in risk assessment. Detailed information regarding risk of man, stock animals and non-target organisms must be provided.

Currently, in all EEC-countries the registration procedures for biological control agents is based on their national plant protectant legislation. Of course most of the countries apply or will apply soon the microbial requirements and procedures of the EEC directives 91/414 and its Annexes. However, everybody knows that an international harmonisation is still not achieved. Because of lacking guidelines the national authorities are still working on their regulatory control based on precedents or still on standards established for chemicals.

Registration requirements in Austria

In this section an overview is given about the registration efforts and strategies to become *Beauveria brongniartii* registered as an biocontrol agent by the Austrian plant protectant legislation (§ 10 PMG; BGBL. I, Nr. 60/1997). In spring 1998, *Beauveria brongniartii,* a successful antagonist against the cockchafer *(Melolontha melolontha)* has been filed by the company the Austrian F. Joh. Kwizda GmbH for registration. The product name is Melocont®-Pilzgerste. Although the mitosporic fungus *Beauveria brongniartii* has been registered in Switzerland, and conditionally, in France - under the old synonym *Beauveria*

tenella, shown in Tab 1. - field trials in Austria and laboratory studies had to be conducted for more than three years. From the beginning of our research work we were aware to take the big hurdle "risk assessment evaluation". Therefore, from June 1994 all officials were involved to define the requirements for a successful registration of the bioinsecticide Melocont®-Pilzgerste. Additionally, to guarantee the use of fungal colonised barley in organic farming on the basis of EU regulation 2092/91, the representatives of the organic farming associations were also contacted and asked to give statements concerning the use of the biological plant protection from their point of view. It is important to state that all Austrian relevant groups welcomed this biological plant protection method and confirmed also the conformity with the EU regulation.

***Beauveria brongniartii* classified as an "old" active substance**

The first hurdle which had to be taken was to define *Beauveria brongniartii* as an old active substance. Old active compounds do not have to fulfill the rigid registration criteria defined in the EEC-directives for new active compounds. Further, the registration procedure is only based on the national plant protectant legislation. Because *Beauveria bassiana* was already registered in Denmark and France, the Austrian Ministry of Agriculture and Forestry confronted the European Commission (DG VI) with the problem to accept *Beauveria brongniartii* as an old active compound. It was argued that even mycologists have still problems with the systematic of *Beauveria* species. For instance, there are different opinions concerning the separation of the two taxa *Beauveria bassiana and B. brongniartii.* To this day the name B. *tenella,* a synonym of *B. brongniartii,* is still used for registration as a biocontrol agent although only three species of *Beauveria (B. bassiana, B. brongniartii* and *B. alba)* have been described (Domsch & Gams 1980). It was the opinion of the working group on plant protection products (European Commission DG VI) that based on the chemotaxonomic studies carried out on DNA, *B. bassiana* and *B. brongniartii* have to be considered as a "collective" species. However, the morphological differences and the specific quality of the host lead to consider the two species as separate. Since France has authorised both Beauveria strains, the Austrian authorities decided to grade *B. brongniartii* as an existing active substance. Despite the "unsatisfactory" discussion, no EU-member state raised objections against the Austrian arguments and the registration procedure was conducted by the Austrian legislation!

Efficacy study of Melocont®-Pilzgerste

Based on the results of our laboratory and field experiments, official authorities did not doubt the efficacy of the bioinsecticide. This uncritical acceptance was an agreeable surprise because the rate of fungal attack was "only over 20%" after two years of application.

However, over the entire test period of three years it could be demonstrated that the cockchafer population suffered a collapse in the treated fields (effectiveness up to 100 %; data not published). In contrast in the control fields neither the fungus *Beauveria* could be detected, nor could fungal infected larvae be found throughout the entire observation period. This result underscores the dominant opinion of experts that with this biological preventive measure the complete collapse of the cockchafer population in the second generation is to be expected. Further, this will lead to a medium-long-term extensive regulation of the population in the effected region.

Toxicological and environmental behaviour of the fungus

It was clear that there is less information on toxicological and environmental fate and behaviour of the fungus itself and its secondary metabolites excreted into the environment. Secondary metabolites are generally considered as mycotoxins which appear as toxic residues in fungal biopreparations. Authorities declare those substances as undesirable because they may have environmental effects. Therefore, we tried to meet the registration criteria which are defined in the EEC directive 414/91:

(i) Information on the production of secondary metabolites (esp. toxins); (ii) methods to determine and quantify residues such as metabolites and toxins; (iii) Step I - Basic studies (acute toxicity, pathogenicity and infectivity) and (iv) fate and behaviour in the environment.

Many questions concerning the risk assessment of a microorganism and its "mycotoxin" have been answered by our results (Strasser *et al.*, 1998, Strasser, 1999 and Abendstein & Strasser, 1999). However, there are still a few issues which have to be studied in more detail in the future. The Austrian authorities claim to do some more new tests, which can lead to delays in putting the needed biological control product on the market.

- "There is a need of providing more ecotoxicological facts regarding the release of secondary metabolites in the environment (e.g. oosporein) and their impact on the feeding behaviour of birds."

The reason for this reclamation is that an expert demonstrated that if young birds were only be fed with *Beauveria* infected larvae over a long period of time, they could be poisoned with oosporein.

- We were also asked to provide data... "under which circumstances the fungus will produce other toxic secondary metabolites (such as beauvericin, tenellin and bassianin) than oosporein and how the production can be prevented."

The production strains, which were tested over a period of three years, never excreted secondary metabolites other than oosporein (Abendstein & Strasser 1999). Despite these findings, we still have to explain why our production strains do not accumulate the secondary metabolites bassianin, beauvericin and tenellin, which are reported in the literature. It is the opinion of the authorities that *Beauveria brongniartii* must be able to synthesise all of these toxic compounds.

- "Someone has to study the adsorption and accumulation of oosporein in indicator plants of root vegetables (e.g. carrots and potatoes)".

It could be demonstrated that no adsorption or accumulation of oosporein is observable in *Phleum pratense* (grass) and *Lepidium sativum* (garden cress; Abendstein & Strasser, 1999). Second, no negative impacts on plants and fruits have been published. Furthermore, it is common knowledge that the fungus could be isolated in cockchafer infested areas all over Europe and has to be classified as an autochthonous fungus.

Conclusion

It is indisputable that biological control agents should replace or reduce the input of chemical pesticides in agriculture, forestry and horticulture. This goal can only be achieved through the use of highly virulent, newly formulated biocontrol agents. This is fortunately done in accord with the current common European agriculture policy guidelines. In the last decade more grants have been spent by the EC to evaluate biological control agents for their environmental benefits and efficacy to demonstrate that these promising products will be an alternative. There is still more research work necessary. A long list of questions must be overworked in the future such as (i) to evaluate the efficacy; (ii) to improve targeting strategies to maximise the impact of these agents for pest control in different habitats and (iii) to minimise the impact of environment, (iv) to compare biocontrol agents for ecological fitness and virulence, and (v) to study the impact of metabolites which could act as safety risk factors. Some of those questions especially in the field of scarab- and weevil control will be answered within the BIPESCO-project (FAIR6-CT98-4105) during the next three years. Hopefully, we can give support with this project to the European pest management policy. As one consequence our work should lead to a practical application of microbial biocontrol agents in the future.

Additionally, we also have to realise a harmonisation of the regulation by streamlining the regulatory pathways to Commercialisation of biological products. One of today's conclusion is that EU-member states ought to follow the United States and Canadian authorities, which have already achieved harmonisation of biological control guidelines.

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Commercialisation of a microbial pesticide "Challenges and constraints"

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Introduction

Koppert B.V. is the world market leader in the production of biological control agents. The main products consist of parasitic wasps, predatory mites and insects for the control of arthropod pests. Another significant area is the production of bumblebees for natural pollination. The production of microbial control agents is a very small part of the business at present. In addition to the production departments, Koppert has its own research and development department where new developments are constantly being sought. Research focuses on:

- evaluation of new bio-control agents
- design of mass production systems
- development of formulation methods
- testing in real life situations
- registration procedures
- quality control.

Until now, a wide range of new products has successfully been launched onto the market. Although a great deal of research on microbials occurs at research institutes, universities and private R & D departments, most non-chemical crop protection strategies are still based on parasitic wasps or predatory mites and not on microbials.

Why microbial pesticides?

The use of microbials against pest insects has some major advantages:

- A high degree of specificity. Microbials will only control the target insects, and may have a small side effect on other pest insects. Therefore, they can be used together with almost all-beneficial insects.
- Non-toxicity to mammals, as far as we know now.
- No loss in efficacy due to resistance, as often happens with chemicals.
- No phytotoxicity to the crop.
- Environmental safety. Just because the active ingredient is a microorganism, does not mean that these types of pesticides are 100% toxicologically safe and will have no impact on the environment. Research, however, clearly indicates that microbial pesticides are generally much safer than chemicals.
- Ease of use. Most of these products are used for spot-treatments or to support the beneficial insects. Therefore, only a small amount of product is used. The majority of microbial pesticides are wettable powders or liquid formulations, which can easily be divided into small quantities.

Where are the microbial products?

Despite all the benefits, not many available products or new products reach the European market. The current situation in the EU is the following. Before July 1993 when the EU directive EC 91/414 came into effect, 18 different micro-organism were registered in 1 or more EU member states. Since that date 5 dossiers for microbials and 18 for chemical pesticides were accepted for review. At this moment no new microorganism has been added to the positive list (Annex 1). In the USA, on the other hand, 19 new microbials have been registered. The Environmental Protection Agency (EPA) conducts a stimulating policy with regard to microbials and a "Fast Track Registration" procedure for microbials. This makes it less expensive to register them. None of these products have so far reached the European market.

Possible causes:

A certain microorganism may be very pathogenic towards a pest insect and may control it in both bioassays and in (semi-) field trials. This does not immediately imply that it is a possible candidate for a microbial product. The possible cause why most of the microbial products do not reach the European market can be divided into different parts: biological constraints, technical difficulties and economical or market challenges.

1. Biological constraints:

The efficacy of most microbial pesticides (especially fungi based) is largely dependent on the environment. Very often they are hindered by the climatic conditions of the crop. For optimal performance, a relatively long period of high humidity is needed for fungal spores to germinate. Also the influence of, for instance, temperature and UV light (sun) plays a significant role in the performance of such products. Therefore, most of them only perform well in greenhouses, or in protected crops. Moreover, some of the advantages can also be regarded as disadvantages. For instance, the specificity of these products means that they are often only effective against one target. Add to this that the effect takes a relatively long time to show itself, and that the storability is often shorter than for a chemical.

2. Technical difficulties:

The production and formulation of microbial pesticides can be very costly. In order to have an effective product, you need to produce enough infectious bodies (i.e. spores, mycelium fragments, live or dead bacteria) so it can compete with existing (chemical) products. Contrary to the production of chemical pesticides, microbial production is done in batches and is labour intensive. And although easy to use, microbial products are rather labour extensive to apply. Timing of application is also critical because not every insect developing stage is as susceptible as the next. Moreover, spraying has to be repeated to have an optimal effect. Also, the shelf life of microbial pesticides is relatively short and must be maintained under certain conditions (fridge, in the dark in a tightly closed containers etc.) which is not always possible.

3. Economical or market challenges:

High investments are needed to place a new microbial product on the market. The work done by the researchers in the laboratory and field is just a small part of the total costs. Because most of these, relatively expensive, products do not act like chemicals (quick and/or systemic kill), they have to be sprayed more than once to obtain comparable results. Use of these products needs more education, as they are more knowledge intensive.

Even more resources are needed for the registration dossiers and toxicological tests. It is also almost impossible to predict the time-period needed to register the product. Most small enterprises, which are the main producers of microbial pesticides, can not afford these kinds

of investments. Big financially independent companies, often producers of chemical pesticides, are not very interested given the small market and therefore limited profit potential of these products. This discrepancy between registration costs and potential market is regarded as the main hurdle for companies to develop microbial pesticides.

Possible solutions:

Due to the complexity of challenges, it is not easy to have one overall solution. Both institutes/companies and governments can help to overcome the problems. These can be divided into research and governmental solutions.

1. Research solutions:

Finding more effective strains or strains that even work in adverse climatic situations might be a solution to many of the biological problems. Genetic engineering of strains, which of course has its own specific problems, is another option. One aspect that definitely needs more attention is the formulation of the microorganisms into a workable product. By means of a formulation not only the storability and shelf life but also the efficacy of a product can be improved. The application of a product is also largely dependent on the way it is formulated. It is obvious that a formulation is a compromise between all these different aspects and sometimes can not be in favour of all.

Researchers must learn more about the practical situation of how crops are grown in order to adapt the formulation and application methods to the different types of crops. Both researchers and growers must seek out more practical experience with microbial products. In this way they can observe how a product performs in the practical situation, and decide what can be the best way to apply it. Because it takes some time to see an effect, the grower must be trained in what he can expect after one, two treatments. Therefore, specific training must be given to not only the grower but also to the suppliers and extension services.

2. Governmental solutions:

Governments, especially in Europe, can facilitate the registration procedures for microbial products. It is quite obvious that a thorough toxicological study for microbial pesticides is very important. On the other hand, the sub-acute and sub-chronic toxicological studies of the active ingredient are an expensive part of the total cost for the registration dossier. Possible solutions might be a tiered system for toxicological studies and a special, European, fund to finance this. More financial funding for basic research would also provide more information about microorganisms, which would give rapporteurs sufficient scientific knowledge about these kinds of controlling agents. By doing this, it might be feasible to have a fast track system with well-defined time frames and harmonisation of the registration fees. These measures might encourage companies to make more efforts in developing promising microorganisms into new marketable microbial pesticides.

Conclusion:

Microbial products show great potential for controlling pest insects despite their biological constraints. Nevertheless, few new microbial pesticides reach the European market, mainly due to the difficult registration procedures, as these products are not treated differently from conventional chemical pesticides inside the European Union. The development and production of this class of pesticides is mainly carried out by small to medium sized companies. Not only are production and formulation of microbials costly and labour intensive, the registration costs of these products are prohibitive for small companies. This, plus the fact that niche markets or high value crops are the only logical markets for success,

means that fewer and fewer companies are interested in microbial pesticides as the total cost is not proportional to the benefits.

Without future additional support from the European Union, whether as simpler registration procedures or financial assistance, the European Union risks running behind compared to countries such as the USA, where increasingly safer products are registered and available to replace older, more toxic chemicals. Environmentally safe alternatives, such as microbial pesticides, must be actively stimulated if we want to move towards least-toxic management of pests in agriculture in Europe.

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Present use and future potential for biological control of pests and diseases especially in organic production of field crops in Denmark.

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Abstract: In Denmark, the present use of biological control in greenhouses is extensive, whereas biological control on field crops and in orchards is very limited. In recent years there has been progress in the development of biological control agents for field application and the potential for their use in field crops and in orchards is great. However, concerted action is required involving scientists, private companies, extension services and farmers to demonstrate that these control measures can be successfully implemented on a commercial scale. For environmental protection reasons and due to the interests of consumers for plant products produced without input of pesticides, there has been an increase in the number of farms converting to organic production. Biological control could become a key factor in plant protection in organic farming. However, to what extend biological control should be allowed to be used in organic farming is a matter of debate. An increase in the use of biological crop protection in organic farming will therefore depend on a number of scientific, technical, commercial and political aspects.

Introduction

Biological control in Denmark was first introduced more than 25 years ago with the use of predators and parasites in glasshouses and in field trials with *Bacillus thuringiensis* and insect viruses. Since then, the development in glasshouses has been dramatic, with a steady increase in the use of biological control of insects and mites . Now biological control of plant diseases has also reached a state where it is being used commercially in greenhouses. However, the use of biological control on field crops and in orchards is still much more limited despite the high number of pests and diseases which could be targeted using this approach.

A working party was appointed in 1998 by the Ministry of Environment and given the objective to analyse the present status for biological control of pests and diseases in Denmark and to discuss the possible influences on the further development of biological control. Based on that work we would like to focus on biological control in field crops with special emphasis on two aspects: commercial availability of organisms and the development in organic farming.

Materials and Methods

Information about the organisms commercially available was obtained from leaflet material and personal communication with the Danish producers and distributors of biological control agents and .from the list of microorganisms applied for approval to the Ministry of Environment. Information about the development in organic farming was obtained from

personal communication with the Danish Association of Organic Growers and extracted from statistical information from The Ministry of Food, Agriculture and Fisheries.

Results

Biological control agents for sale in Denmark in 1998

Table 1. Number of species available in Denmark in 1998 for biological control in total and the number available for field crops.

(Source: Leaflets and personal communication, companies involved in production and sales of biological control agents (BCAs) in Denmark)

Type	Total number of species	Number for use outdoor
Predatory mites	8	3
Predatory bugs	7	1
Syrphids	1	0
Ladybirds	7	0
Lacewings	1	1
Gall midges	2	1
Parasitic wasps	22	2
Predatory thrips	1	0
Nematodes	4	3
Fungi	5	1
Bacteria	1	1
Virus	1	1

As seen in Table 1, the number of species for biological control is high, but almost solely for indoor use. For glasshouse production, species like *Encarsia formosa* are sold in large numbers. Some of the beneficials for outdoor use are for private garden owners (e.g. lacewings) and are not used in crop production. Species used in commercial crop production are the predatory mites *Typhlodromus pyri* against spider mites in orchards, *Amblyseiulus cucumeris* against mites in strawberries and the nematode *Steinernema feltiae* against sciarids and curculionids in nurseries. The microorganisms available for indoor use are *Bacillus thuringiensis* serovar *israelensis* against sciarids, and *Verticillium lecani* against white flies and aphids, while *B. thuringiensis* serovar *kurstaki* and *Agrotis segetum* AsGV are for outdoor use against several lepidopteran larvae and cutworms, respectively. Other fungal species for biological control include *Trichoderma* spp. for disease control in glasshouse crops. The only fungal species for field use is *Phlebiosis gigantea* which is used to control a root disease in conifers. However, the total area outdoor treated with biological control agents is at present very small.

Development of organic farming

The development of organic farming in Denmark since 1988, when the state approval system was introduced, is shown in table 2.

The total number of farms in 1997 was 63,151, which means that approximately 3.5 of the growers are now under the state guidelines for organic production. Of the 2,228

recognised organic growers in 1998, approximately 700 are members of the Danish Association of Organic Growers (LØJ) and follow their guidelines. Their guidelines do not recommend biological control, but it can be permitted in case by case situations. It should be stressed that the organic growers which are not organised in LØJ are not restricted in their use of biological control.

Table 2. The development of organic farming in Denmark 1988 - 1998.
(Source: The Ministry of Food, Agriculture and Fisheries)

	1988	1994	1998
Number of growers	219	667	2228
Number of hectares	2330	16317	44102

Discussion

The use of biological control of pests in glasshouse vegetable crops is close to the potential level while adequate levels of disease control have not yet been attained. In glasshouse ornamentals, however, the use is still below the potential level both for pest and disease control. The use of biological control in field crops remains negligible. The question is now how biological control can be enhanced.

From a scientific point of view, we can address this question by proving biologically and technically, that biological control can be commercially viable in field crops. With recent initiatives to restrict the use of chemical pesticides, new approaches are needed to tackle diseases and pest problems in field crops and orchards. Successful development of biological control for future pest and disease problems requires further research. Our recommendations are to give priority to investigations on the following subjects: 1) Ecology of introduced biocontrol organisms as well as the ecology of target pest organisms and plant pathogens in experiments simulating the field situation. 2) Interactions between biocontrol agents and target organisms *in vivo*. 3) Soil suppressiveness and natural control and influence of cropping systems on the ability of natural occurring organisms to control plant pathogens and pest species. 4) Applied studies aiming to release organisms in the field and evaluate their potential for biological control. 5) Studies on production, formulation and delivery systems and their compatibility with existing technologies and agricultural practices.

At present, several Danish institutions are involved in co-operation to develop more biological control measures for use in field crops and orchards. However, the reluctant or even dismissive attitude from the association of organic growers represents a dilemma now in crop protection. Should biological control principally be regarded as environmentally friendly and receive the growers support or is biological control merely an artificial introduction of organisms in the environment, which may finally do more harm than give benefit? This subject has mainly been debated amongst organic growers and has yet to be discussed in the wider scientific and political arena. On the other hand there has been an ongoing discussion between scientists and legislators concerning registration of microbial BCAs and how to carry out risk assessments and obtaining efficacy data. It is, thus, very important that scientists do not regard the discussions among organic growers as "only" political and refuse to take part in them. We, as scientists, should give such discussion a sound, biological and ecological input.

A rise in the use of biological crop protection in organic farming systems will thus depend upon a number of both scientific and political aspects.

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Biological control of the garden chafer *Phyllopertha horticola* (L.)

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Abstract: *Phyllopertha horticola* has caused great damages in Europe. Due to its one year life cycle the adult chafer and the larvae causes feeding damages. Currently, the following entomopathogenic viruses, microorganisms and nematodes are recognised as natural enemies of the garden chafer *(Phyllopertha horticola): Entomopoxvirus, Metarhizium anisopliae, Rickettsiella melolonthae, Heterorhabditis* spp. and *Steinernema feltiae* and *S. glaseri.* Of these six entomopathogens the mitosporic fungus *M. anisopliae* is preferred as natural antagonists due to their host specificity and high pathogenicity. The aim of the presentation is to characterise the biology and life cycle of the garden chafer and to discuss the advantages and disadvantages of selected natural antagonists for the control of the chafer.

Keywords: *Phyllopertha horticola,* entomopathogen, *Metarhizium anisopliae, BCA,* antagonists

Introduction

Phyllopertha horticola (L.) - often mistakenly called June-chafer has caused great damage in Europe as well as in areas of East Siberia, Monoplia and Caucasus over the last few years. In Austria mostly Upper Austria, Styria and Tyrol are effected, and isolated cases of damage have been reported from Salzburg and Vorarlberg.

Due to its one-year life cycle the adult cock chafer causes feeding damage in orchards, stock of deciduous trees, roses and other blooming bushes. Early summer, after the eggs have been laid, the growing larvae (three larva stages) eat roots of grasses, cultivated plants and stock of trees, which result in damaged large areas of meadows and lawns, sport facilities, recreation areas, golf courses and reforestation areas. The effected areas cannot only be found in the valley, but reaches regions up to the highlands. For example, from 1993 to 1994 the Styrian agricultural chamber reported approximately 2000 hectare damaged areas (e.g. in the districts of Murau, Knittelfeld, Judenburg, Weiz and Voitsberg), which partly resulted in a complete recultivation of pasture-land.

Biology and lifecycle of the garden chafer

Contrary to the May- and June beetles *(Melolontha melolontha* and *Amphimallon solstitialis)* the developmental cycle of the garden chafer is a one year cycle. The chafer outbreak of the adult chafer is seasonal depending on the climate so that from May to the beginning of July at the latest swarms can be observed. The female chafer lays on the average 30 to 40 eggs individually in cavities in the soil at a depth of approximately five to sixteen centimeters (Milne, A. & Laughlin, R. 1957). The larvae (the first lava stage) hatch in four to six weeks and develop very quickly by feeding on roots. The first larva stage (L1) lasts approximately three weeks. Once the skin has been shed the second larva stage (L 2) begins, which lasts on an average four weeks. After a further shedding the third larva stage begins, usually in the last week of August which is characterised by a eight to ten week feeding period (food intake). The larva immigrate through the soil vertically and horizontally and cause the most damage especially in cultivated fields, pastures and sedge meadows. Whereas the first larva stage can

be detected at a depth of approximately four centimetres, the larva in this third stage can be found at a depth of one to two centimetres. The consumption during the first two larva stages contributes mainly to the growth of the larvae. Conversely the consumption during the third larva stage serves the purpose of storing fat bodies which are necessary for hibernation as well as for production of sperms and eggs. The larva's hibernation begins at the earliest in the third week of October, whereby the larva migrates deeper (approximately 20 centimetres) and the food intake is discontinued until the chafer's swarming. By the end of March the larva pupates, whereby usually a pre-pupa stage (length approx. three to four weeks) precedes a pupa stage. The actual pupa development lasts another four weeks. After the pupa skin breaks open, the chafer remains motionless in the soil for one to seven days. After the cuticle hardens, the chafer requires approximately two days to reach the soil surface. There the chafer remains in the turf in order to await favourable weather conditions for swarming (end of May to the beginning of July). Within three to 38 days (lifespan of the adult chafer) the after-eating commences on deciduous trees, bush bleedings and so on, as well as the impregnation of the female chafer, which in contrast to the May-beetle, usually lay eggs in a new undamaged location. With the exception of overlapping periods more than two stages of the garden chafer can never be observed in the soil next to each other.

Characteristics of the garden chafer

The adult chafer grows to a size of 8.5 to 12 mm. The chafer is characterised by brown to bluish black wings and a green to shiny blue neck breast. The body is covered with thick hair. The larvae can grow as large as 3 cm (L 3). The characteristic construction of the anal segment is used to determine the garden chafer larvae. One can observe a longitudinal row of tiny thorns (consisting of approx. 15 to 20 thorns) on the last abdomen segment (ventrally viewed). Further, the larva possesses a small number of strong shovel like bristles which are bent over in the direction of the anus. The pupa represents a quiet stage in the development of the garden chafer. The garden chafer pupa normally has light skin, coloured creme-brown to ochre-brown, and grows to a length of up to 12 mm. One can already observe in this stage the extremities and wing constructions which lie freely adjacent to the body.

Control strategies for the garden chafer

To this day an acute control of the garden chafer through chemical plant protection agents has had only conditional success. Despite the intensive search for a host specific, chemical contact insecticide (Hexa-agents, arsenic based products), a fully satisfactory preparation has not been found. In addition to the usual high toxicity for beneficial organisms one must also question the environment compatibility of these chemical compounds. A mechanical method of control (e.g. rotary harrow) can only be applied successfully in valley, whereby in any case this method is not adopted due to the high labour and re-cultivation costs. Based on the experience of the biological control of the May-beetle through the imperfect fungus *Beauveria bronghiartii* attempts to find host specific entomopathogens, in order to engage these antagonists preventively against the garden chafer, have been undertaken since the 1980's. A preventive control of the garden chafer, however, is complicated by the fact that the chafer female, in contrast to the May-beetle, does not return to its own breeding place, and instead of flies to new locations every year for each egg laying. Therefore, a successful biological control requires new strategies.

Presently the following entomopathogenic viruses, microorganisms and nematodes are recognised as natural antagonists of the garden chafer:

- Entomopoxvirus (EPV)
- *Metarhizium anisopliae* (imperfect fungus)
- *Rickettsiella melolonthae* (bacteria)
- *Heterorhabditis* spp. (nematode)
- *Steinernema feltiae* and *S. glaseri* (nematode)

Of these five entomopathogens the imperfect fungus *Metarhizium anisopliae* and the three nematode types are preferred as natural antagonists due to their host specificity and high pathogenicity

Habits and effects of the fungus *Metarhizium anisopliae*

Metarhizium anisopliae is a well-known and well-described entomopathogen, among numerous entomopathogenic fungi, with a very large host spectrum (approximately 160 insects). This fungus is an facultative obligate parasite which can be found world-wide in natural soil flora. Since more than ten years the fungus is known as an effective pathogen of the garden chafer. The fungus host specifity, however, has not been satisfactorily researched to this day. Inspite of the garden chafer's numerous appearances (egg, three larva stages, pupa and chafer) *M. anisopliae* has a highly virulent effect on all developmental stages of the garden chafer. The fungus forms on the host's surface a pronounced branching air mycelium and can also colonise the surrounding infected area in the soil many centimetres from the cadaver. The conidiophores are irregularly branched and possess cylindrical to clavate phialides which noticeably become younger toward the end of the hyphen. As mycelia age increases the characteristically cylindrical green spores can be observed.

M. anisopliae would be predestined as an entomopathogen fungus due to its physiological characteristics: quick germination of the conidia, high sporulation rate, formation of metabolic acids, production of exoenzymes and toxic metabolic products distinguish the imperfect fungus. Accordingly, a successful infection of the host not only by conidia but also through direct contact with the fungus mycelium can result. The mycelium or the conidia attach themselves to the host's cuticle by forming appressoria and penetrate through the integument of the host (Butt *et al.,* 1995). Numerous released extracellular enzymes (protease, chitinase, esterase) could be detected which destroy the host's cuticle and allow a penetration into the host hamocoel. Through the excretion of various toxic metabolic products (e.g. zygosporin, cyclodepsipeptide Destruxin A, B, C and D, L-Prolyle-L-Leucin Anhydride, L-Prolyl-L-Valine-Anhydrid and Desmethyl) the fungus accelerates to kill the host. Destruxin (DXT) is characterised by its immune depressive and cytopathological effects (Cavelier *et al.,*1998). In addition the weakening of the immune system, changes in the cell compartments (e.g. mitochondrion, endoplasmic reticulum, ribosome and cell nucleus) can be observed. The optimal growth conditions of *M. anisopliae* are 25°C in a pH-range between pH 3.3 and 8.5. Low temperatures (8°C), high or very little moisture, high $C0_2$- and/or 0_2-concentrations improve the conidia persistence in the soil (virulence can also be detected after 450 days).

M. anisopliae has already been registered for a number of years as a biological control agent in Brazil and in Tasmania since 1995 and approved without market application limitations. The fungus agent is offered under the following product names: e.g. Biocontrol®, Biomax®, Combio®, Metabiol®, Metapol® and Metaquino® and Biogreen®. Pathological and toxicological tests on birds, fish, mice, rats, rabbits, and on humans showed that neither indications of poisoning nor pathological symptoms could be detected. Neither could any

negative effects be observed on rain worms, honey bees, or collembolan. In spite of intensive efforts in the past a biological plant protection agent based on *M. anisopliae* has not yet been approved in the field in Europe. Should it turn out that *M. anisopliae* does in fact have a host-specific effect on the garden chafer, then it should not be difficult, based on the EU-directives (91/414/EEC), to introduce a registered *Metarhizium*-control *agent* on the market.

Nematode effects

By applying the entomopathogenous nematodes *Steinernema glaseri, S. feltiae* and *Heterorhabditis* spp. the garden chafer could be controlled at very high success rate of up to 100 percent in sandy soils (e.g. on golf courses). Tests in the Netherlands showed that the entomopathogenic nematodes colonised and killed more than 50 percent of garden chafer larvae within ten days (Smits *et al.,* 1994). The application of nematodes is only advisable on golf courses, sport facilities, and in green houses due to its high costs, since here only small areas are damaged and managers of such facilities typically are willing to pay higher pest control costs.

A large scale application of these parasites is impracticable, however, due too high production costs (up to thousand times more expensive as standard biological plant protection agents), because the breeding of nematodes must be undertaken on animal tissues or organs. Further, the nematodes can only be applied under climatically favourable conditions because they are very sensitive to dryness. The nematodes have to be absorbed into the soil with a large amount of water in high numbers (> three million nematodes per m^2), in order for them to actively colonise the garden chafer's larvae. Similar to the fungus infection a direct host contact is necessary. The nematodes usually make their way into the intestinal tract of the larvae via oral consumption. They penetrate through the intestinal wall to the hamocoel, in order to produce their virulent effect.

The nematodes are associated with soil bacteria so that they can act in a way as biological "infection needles" and induce bacterial infections.

Larvae in the third larva stage are, according to experience, easier to control with nematodes, because the points of entry for the nematodes are larger than during the preceding developmental stages. It is suspected that the portability of an encounter in the soil increases with the size of the larvae. For this reason a targeted controlling of the garden chafer larvae with entomopathogenic nematodes is recommended in months of August to the end of September.

Conclusion

It is a fact that fungi are more easily applicated and enriched in the infested soils than other organisms. Therefore, our working group started to work with *Metarhizium anisopliae* strains to control the garden chafer. Currently there exist contradictory reports on host specificity of the fungus. Further experiments on a range of target and non-target organisms will show the susceptibility to *Metarhizium* strains of commercial value by using standard biotest methods.

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Capturing the potential of biological control in Italy: Where are we?

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Abstract: The present status of microbial control in Italy is reviewed, and the main factors limiting its implementation are identified and briefly discussed.

Key words: pests, pest control, microbial control, entomopathogens, Italy.

Introduction

Worldwide the utilization of insect pathogens as bioinsecticides has attracted much interest during the last half century. As an example of this it can be mentioned that in the USA the registrations of microbial pesticides totalled 7 in 1980, 14 in 1988 and over 40 in 1995 (Betz, 1995). A similar development, but with a slower pace, took place in Europe although with remarkable differences between countries. However, after a period of great enthusiasm, it seems that the interest for this kind of insecticides has cooled off. Especially at the commercial level often practical results have not met the great expectations of the past. This paper reviews the status of insect pathology and of its practical applications (i.e. microbial control) in Italy, the country where this science has its roots.

Status of the research

Different groups are presently working in this area (Table 1), although their number is rather low in relation to the size of the country. However, more than the total number, what appears really inadequate is their size which in most instances amounts to only a few workers. It should also be noted that for the vast majority (if not all) of the groups indicated the study of insect pathogens constitutes only a side activity. All in all, the work force engaged in this area in the whole of Italy probably amounts to the equivalent of a dozen full-time researchers. Although it is certainly difficult to compare with objective criteria the state of the research in different countries, the simple observation of the directory of the SIP is enlightening: 11 members for Italy, 37 for France and 83 for the U.K.. As much simplistic this comparison may be, it gives a feeling of the scarce attention given to insect pathology in Italy. In fact, this way the gap is most probably underestimated. It is certainly true that Italy is one of the few countries in the developed world with no research institutions (university institute or other) engaged specifically in this area. Such situation has prevented so far the development of long-term research projects and the build-up of specific competences; this in turn discourages the companies in "investing" in research in the country beyond what may be the occasional execution of control trials needed to support the local registration of a given biopesticide. Luckily in recent years there have been some signs of change, and a few higher-profile international research projects have seen the participation also from some of the groups indicated in tab. 1. Those interested in the most recent scientific literature may refer to Sgarzi and Rovesti (1998).

Table 1. Groups presently involved in insect pathology / microbial control.

Name / Organization - Town	Studied organism(s)	Field(s) of activity
Public research groups		
Centro di Studio dei Fitofarmaci Consiglio Nazionale Ricerche - Bologna	entomopathogenic fungi	isolation of species/strains control trials (various target insects)
Centro per la sperim. agraria e forestale Provincia di Bolzano Ora (Bolzano)	*Beauveria brongniartii* *Bacillus thuringiensis* GV of *Cydia pomonella*	control trials (*Melolontha melolontha*) applicn. strategies (lepidoptera on pome fruits) control trials (*Cydia pomonella*)
Dip. di Agrochimica ed Agrobiologia Univ. di Reggio Calabria - Reggio Cal.	entomopathogenic fungi	Identification and ecological studies
Dipartimento di Biologia Università di Milano - Milano	entomopath. nematodes	study of immunitary response of insects
Dip di Biologia Strutturale e Funzionale Università dell'Insubria - Varese	entomopath. nematodes *Bacillus thuringiensis*	study of immunitary response of insects Control trials (*Hyphantria cunea*)
Dip Scienze e Tecnologie Fitosanitarie Università di Catania - Catania	entomopathogenic fungi	strain selection (*V. lecanii, Beauvearia* spp). pathogenicity tests on *Bemisia tabaci*
DI.VA.P.R.A.-Microbiol & Industrie Agrarie Università di Torino -Torino	*Beauveria brongniartii*	control trials (*Melolontha melolontha*) genotypic characterization & ecology
Istituto Agrario S. Michele all'Adige S. Michele all'Adige (Trento)	*Beauveria bassiana*	control trials (*Corythuca ciliata*)
Istituto di Entomologia Agraria Università di Bari - Bari	entomopathogenic fungi entomopath. nematodes	ecology ecology - infectivity tests
Istituto di Entomologia Agraria Università di Bologna - Bologna	*Bacillus thuringiensis* GV di *Cydia pomonella*	control trials (orchards) control trials (*Cydia pomonella*)
Istituto di Entomologia Agraria Università di Sassari Sassari	*Bacillus thuringiensis*	control trials (*Lymantria dispar*) strain isolation & biochem. characteriztn. Strain selection (*B. oleae, Ceratitis capitata*)

Ist. Ric. Controllo Biologico Ambiente	*Bacillus thuringiensis*	strains isolation and biochem. charactztn.
Consiglio Nazionale Ricerche - Sassari		Strain selection (*B. oleae, Ceratitis capitata*)
Istituto Sperim. per la Zoologia Agraria	entomopath. bacteria	strain collection and maintenance
Ministero Politiche Agricole Firenze	entomopathogenic fungi	pathogenicity tests control trials (*Hyphantria cunea*
Istituto Superiore di Sanità Roma	*Bacillus thuringiensis* *Bacillus sphaericus*	control trials (mosquitoes, other vectors)
Servizio Fitosanitario Regione Emilia Romagna Bologna	*Bacillus thuringiensis*	control trials (leafrollers, *Lobesia botrana*)
	entomopath. nematodes	control trials (vineweevils)
	GV of *Cydia pomonella*	control trials (*Cydia pomonella*)
Servizio Fitosanitario Produz. Vegetali Regione Auton. Valle d'Aosta - Aosta	*Beauveria brongniartii*	control trials (*Melolontha melolontha*)
Private / commercial groups		
Abbott - C.A.P.D. Milano	*Bacillus thuringiensis*	testing of new formulations
	Bacillus sphaericus	control trials (mosquitoes)
Bio Integrated Technology Pantalla di Todi (Perugia)	entomopath. nematodes *Bacillus thuringiensis*	product development
Industrie Chimiche Caffaro - B.U.F.	entomopathogenic fungi	control trials
C. E. R. - Galliera (Bologna)		product development
Intrachem Italia Grassobbio (Bergamo)	*Bacillus thuringiensis*	control trials (noctuids)
	GV of *Cydia pomonella*	control trials (*Cydia pomonella*)
	entomopathogenic fungi	control trials (whiteflies, thrips, aphids)
SCAM S. Maria Mugnano (Modena)	GV of *Cydia pomonella*	control trials (*Cydia pomonella*)
Centro Agricoltura Ambiente Crevalcore (Bologna)	*Bacillus thuringiensis* *Bacillus sphaericus*	control trials (mosquitoes)

The pesticide market

The development of microbial insecticides in Italy has constantly proceeded with a large delay compared to most of the other european countries. To this regard, suffice it to say that the first registration of a microbial insecticide (*Bacillus thuringiensis*) took place only in 1984, i.e. a few decades later than in countries such as the UK or France. In 1998 the sales of biorationals represented approx. 3% of the total insecticide market. Of this, approx. 30% is represented by Bt and 40% by pheromones (figs. 1-2). Since Bt is the only entomopathogen registered, it goes along that microbial insecticides really represent a little more than 1% of the total market, in line with the world figures. The italian market for this bacterium is peculiar with regard to the range of crops on which it is mostly used: 38% on grapes, 30% on pome fruits, 15% on stone fruits and only 12% on vegetables (average data of 1996-1998). However, the use on vegetables has been steadily growing after the introduction of formulates more effective against the larvae of noctuids and the overcoming of some limitations for registration imposed in the past by the Ministry of Health.

A good range of commercial Bt products are presently on the market (tab. 2). Remarkable changes have taken place over the last few years leading to an oligopolistic regime. The use of Bt has steadily and significantly increased since its introduction, reaching a plateau in 1993 (excluding a small peak in 1995) (fig. 3). Its use has been practically restricted to agriculture, since the permission for aerial application has never been granted by the registration authority despite the fact that most of the products are registered for use against forest pests. Nevertheless, large scale applications have been carried out during the first part of the present decade as "experimental applications" under the supervision of local authorities to tackle serious infestations which threatened to destroy forests with a high ecological and commercial value (as it was the case with *Lymantria dispar* in cork oak forests in Sardinia and *Thaumetopoea pityocampa* in national parks in Southern Italy). In recent years "forest" applications (including parks, road-lining trees, and other non-agricultural applications) have decreased significantly, both for the impossibility to carry on with such "experimental" applications and due to the natural decline of one of the main non-agricultural targets, *Hyphantria cunea*.

Of the different Bt pathotypes, the lepidopteran-active one is obviously the most widely used representing approx 90% of the total (fig. 4). The consumption of Bti is significant, but after a peak in 1995 its use decreased due to the reduced availability for many local administrations of funds for control programmes against mosquitoes. On the other hand, after a good start immediately following its registration, the use of the coleopteran-active strain (*tenebrionis/san diego*) has decreased down to a nearly negligible level. The high specificity of this strain means that the only target pest of economical importance in Italy is the CPB (*Leptinotarsa decemlineata*); consequently, any factor influencing the appearance of this pest strongly influences also the use of Btt (as it happened in 1995 when due to an unseasonably wet and cool summer the sales of this bacterium dropped to 1/10 of those from the previous year). Besides, very few growers accepted the sight of still living CPB larvae on their crops after spraying with Btt, the majority quickly returning to the use of chemicals with a good knockdown effect.

Theoretically ideal to control soil dwelling insects, entomopathogenic nematodes possess a potentially very large market (see fig. 1). This, together with the ease of introducing them on the market (also in Italy they are allowed for use without the need of a previous registration) these bioinsecticides received much interest during a decade beginning in the mid-eighties. However, due to inconsistent practical results, high cost and difficulties in the distribution to the final user, their present utilization is very limited and presumably current annual sales do not exceed the equivalent of 50.000 US$. Several commercial products are still on the market (tab. 3), although it appears that not all of them are actively commercialised.

None of the other microbial agents (fungi, viruses and protozoa) is presently registered. Some registrations are expected to be granted soon for the GV of *Cydia pomonella*, while only very recently an application has been filed for the entomopathogenic fungus, *Beauveria bassiana*. However, it is no secret to anybody that some of these microbial products (especially viruses) are being used in organic farming together with other biorational pesticides (such as fatty acid potassium salts, antagonistic fungi, etc). Also in case of entomopathogenic fungi, large scale "experimental" applications have been made with *B. brongnartii* in South Tyrol and the Aosta Valley to try and limit the devastating effects of the infestations of *Melolontha melolontha*.

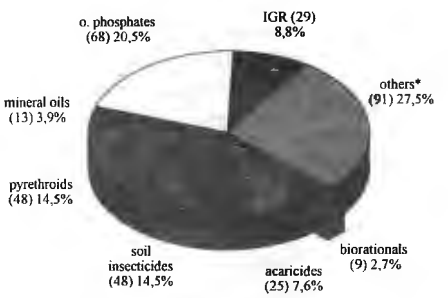

Fig. 1 - The insecticide market 1998 - (billion ITL)

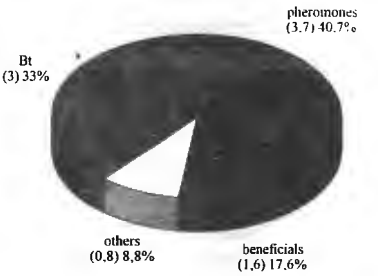

Fig. 2 - Sales of biorationals 1998 - (billion ITL)

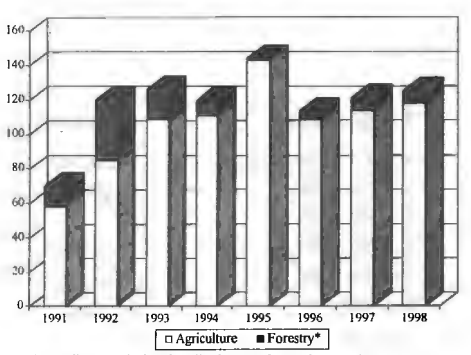

Fig. 3 - Use of Bt (lepidopteran pathotype) in agriculture and forestry (tons)

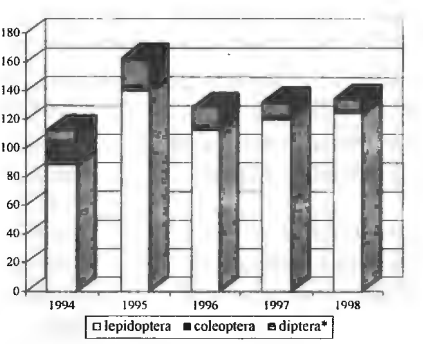

Fig. 4 - Sales of Bt pathotypes (tons)

Table 2 - Bt formulates on the market.

Ssp.	Trade name	Producer	Distributor
aizawai	Agree*	Thermo Trilogy	Suneco
kurstaki	Bactucide WP	-	Industrie chimiche Caffaro
	Biobit HPWP	Abbott	Scam
	Btk 32	-	Agribiotec
	Delfin	Thermo Trilogy	Novartis
	Dipel 2X	Abbott	Sipcam
	Dipel HPWP	Abbott	Bayer
	Jackpot BFC*	Ecogen	Intrachem
	Lepinox*	Ecogen	Intrachem
	MVP**	Mycogen	Rhone Poulenc
	Rapax*	Ecogen	Intrachem
tenebrionis	Novodor	Abbott	Scam
israelensis	Balthas	Abbott	Leica
	Biolarkim 14	Abbott	Colkim
	Skeetal	Abbott	Activa
	Tarin L	Abbott	Copyr
	Turbac CD	Abbott	India
	Vectobac 12 AS	Abbott	Esoform
	Vectobac G	Abbott	Esoform

* Strains obtained by transconjugation. Although in this group, Jackpot is active against Coleoptera.
** Obtained using *Pseudomonas fluorescent* cells coding for the synthesis of the δ -endotoxin of Btk.

Table 3 - Entomopathogenic nematodes on the market.

Nematode species	Trade name	Target pests	Distributor
Heterorhabditis bacteriophora	Bactinem	vineweevils, sciarids, etc.	Intrachem
Heterorhabditis megidis	Larvanem	vineweevils	Koppert
	Nemasys H	vineweevils	*
Heterorhabditis sp.	Nemopak H	vineweevils	Bioplanet
Steinernema carpocapsae	Biovector	vineweevils	Intrachem
Steinernema feltiae	Entonem	Sciarids	Koppert
	Nemasys	Sciarids	*
	Nemopak S	Sciarids	Bioplanet

* Biobest's products, handled by various local dealers.

Table 4 - Pending registrations.

Active ingredient	Registrant	Active ingredient	Registrant
Bacillus thuringiensis var. *aizawai*	Abbott	*B. thuringiensis* var. *kurstaki*	Tecomag
B. thuringiensis var. *kurstaki*	Agrimix	*Beauveria bassiana*	Intrachem
B. thuringiensis var. *kurstaki*	Agrimix	*Cydia pomonella* GV	Intrachem
B. thuringiensis var. *kurstaki*	Fitokim	*Cydia pomonella* GV	N.P.P.
B. thuringiensis var. *kurstaki*	Ital Agro		

The problem of registrations

Unlike several other european countries, Italy has never developed proper ad hoc protocols for the registration of microbial pesticides. Until recently even large multinational companies have been unable to overcome the difficulties imposed by an often unreasonable, "ticking boxes" type, bureaucratic approach by the registration authorities. Hopefully, the introduction of a common regulation system (e.g. the EEC Directive 91/414) will help streamlining the process of registration and ease the introduction of these products, which in many instances are targeted to niche markets and therefore give limited revenues to the registrant company. The development of a tiered testing scheme for the submission of safety data (similar to the one adopted in the USA by the EPA) would certainly be auspicable by reducing the costs of registration. A few years ago the establishment of a commission supposedly with specific competences for microbial pesticides was accomplished, but so far the results of its activity have not met the numerous expectations. It is certainly true that the registration of pesticides is a rather delicate and for certain aspects complicated matter, but some situations (e.g. the length of time needed for the obtention of a new formulation of Bt) are certainly anomalous and can hardly be justified with technical reasons.

The need for education

Despite what mentioned in the previous paragraph, it would be unfair to blame only the legislative bodies for the insufficient development of microbial pesticides. As a matter of fact, most growers still are not prepared, neither technically nor psicologically, for these new tools. The majority of them still rely totally on the potent knockdown effect and long persistence of chemical pesticides, and simple but important measures such as crop scouting are largely ignored. Furthermore the behaviour of most consumers is rather incoherent, demanding on one hand agricultural produces totally free from chemical residues, but on the other hand are not prepared to pay a higher price for that. If much work has already been done from a technical point of view towards the development of reasonably effective and reliable biopesticides, obviously much still needs to be done with regard to the education of the growers and the consumers.

Conclusions

The development of microbial control in Italy has proceeded with difficulty. Nevertheless, microbial insecticides are already a commercial reality and several factors will probably favour their further development. Among these we can mention the quickly increasing level of resistance to chemical insecticides among several important pests, various national and international regulations which significantly limit the use of chemicals (e.g. the EEC regulation 2078/92), the lack of alternatives (e.g.

in case of *M. melolontha* and other soil insects, against which chemical control is often ineffective or too harmful for the environment), the introduction of new techniques (e.g. the use of pollinators such as bumblebees, which is incompatible with that of several chemicals). On the other side, of the factors slowing down such development, the technical obstacles (e.g. product standardization, increased potency, etc.) are probably the most easily solved. The generally poor acceptance by the growers can probably be overcome with the development of an efficient extension service, since at present most growers are totally on their own in the not always easy process of decision-making for crop protection. Probably, the most difficult hurdles to be overcome are those related to the mentioned inconsistence in the behaviour of the consumers and the role of legislative bodies. As for the latter, it is of paramount importance that a more transparent and flexible system be developed, with clearer rules and more effective controls. Significative changes in this direction are needed in order to further the development of this field and to guarantee the protection of the rights of the companies seriously committed to it.

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2. Entomopathogenic nematodes

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Susceptibility of the cockroach, *Blatta orientalis* L. to entomopathogenic nematodes

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Summary: Cockroaches are relatively susceptible on entomopathogenic nematodes but the efficacy depend on insect behaviour in the presence of the nematode and nematode persistence. Females, males and larvae of cockroach, *Blatta orientalis* mortality of two species of nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora* has been studied. In bioassay, we used a filter paper inoculation cocoon with infective juveniles where insects were placed separately. *H.bacteriophora* was less pathogenic to cockroach than *S.feltiae.* Higher doses of IJs caused higher insect mortality. Males and larvae were more susceptible to both nematode species than females.

Introduction

A specific environment of cockroaches considerably limit the possibility of their chemical control, therefore the use of biological means becomes of great importance. The susceptibility of particular development stages of cockroaches, *Blatta orientalis* was tested in relation to two nematode species: *Steinernema feltiae* and *Heterorhabditis bacteriophora.* The development of nematodes within the body cavity of a cockroach was also studied.

Methods

The insects were provided from their mass rearing. The insect diet consisted of granulated nourishment for mice and rats. Tests were conducted with 30-day-old larvae (24 mg mean body weight) and adults (males - 379,7 mg, females - 766 mg). Nematodes, *S.feltiae* (Low87 strain) and *H. bacteriophora* (PL strain) were isolated in Poland. Nematodes was conducted in vivo, using *Galleria mellonella* larvae

Nematodes were applied in 4 doses: 100, 200, 500 and 1000 IJs/insect. To determine mortality of cockroaches, every day dead specimens were collected for their further dissection after 2-3 days. Tests were conducted in 6 replicates of 10 insects each.

Results and discussion

The conducted tests showed that mortality of *B.orientalis* treated with entomopathogenic nematodes was related to various factors such as sex and development stage of an insect as well as species and dose of nematodes (tab. 1). The results obtained indicate that the pathogenity of *S.feltiae* was superior as compared to *H. bacteriophora* nematodes. Results of our research are not in agreement with other reports (Georgies & Gaugler, 1991) regarding *Heterorhabditis* spp. nematodes as more pathogenic to cockroaches than *Steinernema* spp.
The highest mortality of cockroaches was caused by a dose of 1000 IJs *S.feltiae* regardless of insect stage and sex. During the first 12 days of the test significantly more males than larvae

were eliminated by nematodes; females appeared to be the least susceptible (tab. 1). Although cockroach males showed the highest mortality in all test variants with nematodes, their suppression in the control was also very high as compared to larvae and females. Zervos & Webster (1989) obtained high pathogenity of *Heterorhabditis heliothidis* to another cockroach species, *Periplaneta americana.* The same authors found *P.americana* younger larvae much more susceptible to nematodes than adults. Koehler et al. (1992) found *B.orientalis* to be less susceptible than *B.germanica.*

Table 1. Mortality of *Blatta orientalis* in tests with nematodes (data after 12 days of experiment).

	Females		**Males**		**Larvae**	
IJs dose	*S.feltiae*	*H. bacteriophora*	*S. feltiae*	*H. bacteriophora*	*S. feltiae*	*H. bacteriophora*
100	53.3 b,B	36.7 a,AB	95.0 c,B	90.0 c,A	46.7 ab,B	51.7 ab,B
200	81.7 b,C	50.0 a,BC	96.7 c,B	93.3 bc,A	56.7 a,B	60.0 a,BC
500	80.0 b,C	58.3 a,C	98.3 c,B	95.0 c,A	86.7 bc,C	61.7 a,BC
1000	91.7 bc,C	78.3 ab,D	100.0 c,B	95.0 bc,A	96.7 c,c	73.3 a,C
Control	25.0 a,A	21.7 a,A	81.7 b,A	81.7 b,A	18.3 a,A	18.3 a,A

x-Means in rows followed by the same small letters (a,b,c) do not differ at P = 0.01
xx-Means in columns followed by the same capital letters (A,B,C) do not differ at P = 0.01

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The susceptibility of *Dociostaurus maroccanus* (Orthoptera:Acrididae) nymphs to a Spanish isolate of *Steinernema* spp. *(glaseri* group).

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Abstract: The susceptibility of *Dociostaurus maroccanus* fourth instar nymphs to an isolate of an undescribed species of *Steinernema* spp. *(glaseri* group) was studied by the filter paper method. Nymphs mortality was recorded at three intervals posttreatment, 24, 48 and 72 hours. The mortality data 24 hours after treatment was directly related to IJ dosage and gives a LC_{50} of 126 IJ/nymph. The nymphs died at 24 hours after the treatment were used to produce new infective juveniles that maintained the infectivity against fourth instar *D. maroccanus* nymphs. The use of this nematode for the control of the mediterranean locust outbreaks is discussed.

Introduction

The outbreaks control of the mediterranean locust *Dociostaurus maroccanus* (Thunberg) is achieved nowadays by means of broad spectrum chemical insecticides applied at the permanent breeding areas. Nevertheless the environmental concerns ask for a higher safety control measures. Natural populations of *D. maroccanus* are affected by different biotic agents among which the most promising to be included on biological control programmes are the fungus *Beauveria bassiana* (Balsamo) (Hernández-Crespo & Santiago-Alvarez, 1997 a) and the mermitid *Hexamermis serenensis* (Hernández-Crespo & Santiago-Alvarez, 1997 b) although more research is needed to increase the range of useful agents for management of *Dociostaurus maroccanus* outbreaks.

The entomopathogenic nematodes are envisaged as additional biological control agents (Lacey & Goettel, 1995; Baker & Capinera, 1997) although do not naturally parasitize grasshoppers and locusts (Peters, 1996). Moreover, soil is the natural habitat for entomopathogenic nematodes (Kaya, 1990), thus they expands the options for the control of insects, especially those with soil inhabiting stages. Grasshoppers and locusts have at least 1 stage found in the soil; in this case, the egg stage. Eggs are not susceptible to infection, but upon hatching the young locusts must dig to the soil surface and, thereby, are potentially susceptible to attack. The purpose of this paper was to examine the potential of a strain of a non-described *Steinernema* spp. from the *glaseri* group isolated from Spanish soils to infect *D. maroccanus* nymphs.

Materials and methods

The *D. maroccanus* nymphs used in this assay come from a stock colony maintained under controlled conditions 13 : 11h L:D photoperiod, 26°± 4°C T and 40-60% RH and reared by a method developed in our laboratory (Quesada-Moraga, 1998). Wooden cages (50x50x50 cm) were used to maintain populations of nymphs, with a 60 W bulb inside that supplied extra heat during the light period. The locust were fed with dry wheat bran and wheat seedlings.

The nematode used was the strain S2 of an undescribed *Steinernema* spp *(glaseri* group) supplied by Dr. F. Garcia del Pino (UAB) reared in our laboratory according to the method of Dutky *et al.* (1964).

Four graded doses of infective juveniles in 1 ml of deionized water were applied to filter paper in the bottom of plastic cups (40 mm diameter, 20 mm depth). Forty newly moulted four instar nymphs were then individually placed in each cup and incubated at 20°C. As a control the same number of nymphs were exposed to the same volume of distilled water. Mortality was recorded every 24 hours.

The nymphs died at 24 hours after the treatment were used to produce new infective juveniles. Nematode production in *D. maroccanus* nymphs followed the in vivo procedure of Woodring & Kaya (1988). The number of IJ in the suspension was determined by means of McMaster chamber.

The dose-mortality response was calculated by Probit analysis (Finney, 1971) to obtain the Probit regression equation and the LD_{50} by the program POLO-PC LeOra Software (1987). LT_{50} was calculated using the Biever and Hostetter (1971) formula.

Results

Fourth instar *D. maroccanus* nymphs are highly susceptible to the strain S2 of the entomopathogenic nematode *Steinernema* spp *(glaseri* group) as can be seen in Table 1. Mortality by nematodes occurs as early as 24 hours after treatment and continue during the following 48 and 72 hours. Total mortality does not show any differences between IJ doses. The mortality originated 24 hours after the treatment is directly related to IJ doses, that originated 48 after treatment is inversely related to the dose and the mortality originated at 72 hours does not show any relationship to the dose (Table 1 and Figure 1). LT_{50} values decrease as dose increase with a maximum of 1.7 days for 25 IJ/nymph and a minimum of 0.8 days for 200 IJ/nymph (Table 1).

Table 1. Mortality of *Dociostaurus maroccanus* fourth instar nymphs caused by *Steinernema* spp. At different time intervals post-treatment.

Dose (IJ/nymph)	N[1]	Mortality at[2]									LT_{50} (days)
		24h		48h		72h		Total			
		n	%	n	%	n	%	n	%	Abbott	
Control	40	0	0.0	4	10.0	7	17.5	11	27.5	0.0	-
25	40	3	7.5	24	60.0	10	25.0	37	92.5	89.6	1.7
50	40	13	32.5	19	47.5	4	10.0	36	90	86.2	1.4
100	40	16	40.0	15	37.5	9	22.5	40	100.0	100.0	1.3
200	40	25	62.5	10	25.0	4	10.0	39	97.5	96.5	0.8

[1] N= Number of treated nymphs; [2] n= Number of killed nymphs.

The mortality data recorded at 24 hours after treatment were subjected to Probit analysis and the regression equation was $Y=1.71X + 1.4$ ($\chi^2=2.15$; d.f.=2) that gives a LD_{50} Of 126 IJ/nymph.

The production of IJ in *D. maroccanus* nymphs is presented in Table 2. It can be observed that there is a direct relationship between dose and nematode production in the range of 25 to 100 IJ/nymph, but there was a considerable reduction for the highest dose 200 IJ/nymph.

Table 2. Nematode production in *D. maroccanus* fourth instar nymphs after treatment with increasing doses of *Steinernema* spp.

Dose (IJ/nymph)	Number of nymphs	Production (IJ/nymph)
25	3	501.4
50	13	594.5
100	16	800.0
200	25	483.8

The IJ produced in *D. maroccanus* fourth instar nymphs maintained their pathogenicity as can be seen in Table 3. In this case the total mortality is directly related with the IJ doses occurring mostly on a 48 h period and the mortality at 72 hour was negligible (Table 3; Figure 2). Mortality at 24 hours did not reach 50% at any dose so that it was not possible to calculate the LD_{50}. LT_{50} values for each dose, that were higher than in the previous bioassay, decrease as dose increases ranging from 2 days to 1.4 days (Table 3).

Table 3. Mortality of *Dociostaurus maroccanus* fourth instar nymphs caused by *D. maroccanus* produced *Steinernema* spp.

Dose (IJ/nymph)	N[1]	Mortality at[2]									LT_{50} (days)
		24h		48h		72h		Total			
		n	%	n	%	n	%	n	%	Abbott	
Control	40	2	5.0	4	10.0	0	0.0	6	15.0	0.0	-
25	40	6	15.0	14	35.0	0	0.0	20	50.0	41.2	2
50	40	10	25.0	20	50.0	0	0.0	30	75.0	70.6	1.5
100	40	12	30.0	23	57.5	1	2.5	36	90.0	88.2	1.4
200	40	16	40.0	22	55.0	0	0.0	38	95.0	94.1	1.4

[1]N= Number of treated nymphs; [2]n= Number of killed nymphs.

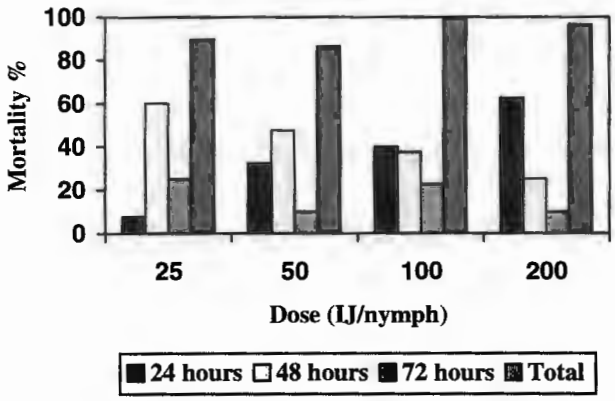

Figure 1. Dose related mortality of *D. maroccanus* nymphs treated with *G. mellonella* produced *Steinernema* sp.

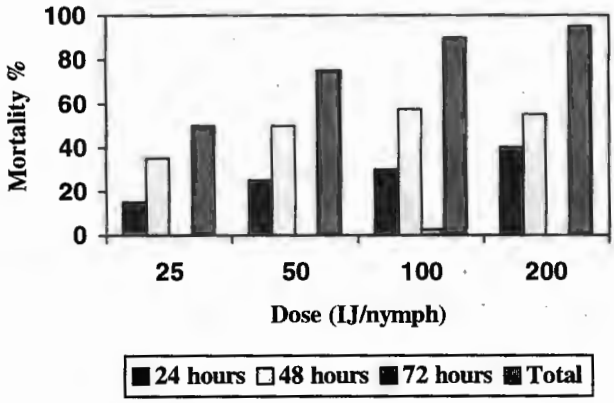

Figure 2. Dose related mortality of *D. maroccanus* nymphs treated with *D. maroccanus* produced *Steinernema* sp.

Discussion

Our results show clearly that the strain S2 of the undescribed *Steinernema* spp. *(glaseri* group) reared in *G. mellonella* has a good insecticidal activity against *D. maroccanus* fourth instar nymphs.

Insecticidal activity of Steinernematids has been tested successfully in other acridids. In *Melanoplus sanguinipes*.(F) *Steinernema carpocapsae* Weiser and *S. scapterisci* Nguyen & Dmart caused 93% and 29% of mortality at 120 hours after application respectively (Nicolas *et al.*, 1995). Moreover *Heterorhabditis megidis* and *Steinernema feltiae* turned out to be successful antagonists of the locusts *Locusta migratoria* L. and *Schistocerca gregaria* (Forsk.) whose adults died 30-35 hours after oral application of 50 IJ/nymph nematodes and between 48 and 96 hours after exposure to nematodes in sand (Sambeek & Wiesner, 1999). The parasitation of these locust by both nematodes is correlated with the inhibition of insect phagocytes (Sambeek & Wiesner, 1999). Our *Steinernema* spp. strain, as indicated by the LT_{50} values, seems to be more virulent than the above species although the physiological mechanism implicated in the defence response of *D. maroccanus* may be also involved in this difference.

In other way, our *Steinernema* spp. strain can reproduce in dead *D. maroccanus* fourth instar nymphs and it can originate a new generation of IJ that keeps the biological activity. The viability of *Steinernema* spp. IJ produced in *D. maroccanus* nymphs has a good interest for it maintenance in the soil environment.

The soil environment affect the nematode performance as biological control agents. It has been determined that the infectivity of *S. carpocapsae* and *S. scapterisci* against *M. sanguinipes is* directly related to soil humidity from 0 to 12% (Nicolas *et al.*, 1995). The usefulness of nematodes against locusts would be limited to areas with moderate-to-high soil moisture levels near the soil surface. This could occur at particularly wet seasons of the year, in crops or natural areas with a dense canopy. *D. maroccanus* breeding areas in the Iberian Peninsula have a dense canopy and a moderate soil moisture in wet years in spring where the hatching of *D. maroccanus* occurs (Cañizo & Moreno-Márquez, 1950). Nevertheless, more research is needed to study the effect of the soil humidity on the insecticidal activity of our *Steinernema* spp. strain against *D. maroccanus* nymphs.

Different species of grasshoppers may differ greatly in the susceptibility to infection by nematodes at hatching depending on the depths of the egg-pod and the shape of the foam plug, which provide a path through the soil that is followed by the young locust as it seek to attain the soil surface (Ewer, 1977). Our experimental design does not point out the effect of *Steinernema* spp. against young nymphs at hatching so that it is necessary to design a soil bioassay container (Nicolas *et al.*, 1995) to test the efficacy of our *Steinernema* spp. strain at field like conditions. Although the nematodes alone would not likely be adequate for *D. maroccanus* outbreaks suppression, this could be an additional strategy to the use of other entomopathogens and to the sound chemicals.

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Pine weevil biocontrol: compatibility of entomopathogenic nematodes and stump-degrading fungi in laboratory conditions

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Abstract: Pine weevil (*Hylobius abietis* L.) is one of the most important pests in coniferous reforestation in Northern and Central Europe. Larvae develop in the stumps of recently felled trees, and the emerging adults feed on the bark of seedlings, and may kill them. Currently this pest is controlled by the application of chemical insecticides, but attention is focused on finding alternative methods of control. Two promising approaches are (1) the use of the fungus *Phlebiopsis gigantea* to accelerate the rate of decay of the stumps, and (2) application of entomopathogenic (insect-killing) nematodes. The aim of the present work is to test the compatibility of the fungus *P. gigantea* and the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis megidis*. Disks of spruce timber (*Picea sitchensis*) were inoculated with spores of *P. gigantea* and incubated for 2, 4, 6 or 8 weeks. Fungus-infested and control (fresh timber) disks were artificially infested with a single first instar pine weevil larva per disk. The insects in the timber were then exposed to nematodes in moist sand for 48 hours. Dissection of dead insect larvae indicated the number of nematodes infecting. Numbers of nematodes invading *H. abietis* in timber inoculated with *P. gigantea* were at least as high as in uninoculated timber, and in some treatments were significantly higher. From the results of these experiments we conclude that the use *of P. gigantea* and entomopathogenic nematodes are compatible for the control of pine weevil.

Keywords: *Hylobius abietis*, *Phlebiopsis gigantea*, *Steinernema carpocapsae*, *Heterorhabditis megidis*, forest protection, biological control, synergy, nematode behaviour

Introduction

The pine weevil (*Hylobius abietis* L.) is the greatest constraining factor on reforestation in Ireland and in much of central and northern Europe. Weevil larvae develop in the stumps of felled trees and the emerging adults feed on seedlings, killing them by gnawing on the bark. Current control methods rely heavily on chemical pesticides. In addition to the heavy economic costs of controlling the pest, there are potential environmental costs of continued application of chemical pesticides, which may severely restrict their use in the future. Alternatives to reliance on chemical control are being sought, and amongst the more promising is the use of biological control as a component in an integrated pest management programme. The more promising biocontrol agents include entomopathogenic nematodes and stump-degrading fungi.

Both adult and larval pine weevils are susceptible to entomopathogenic nematodes (Pye & Pye, 1985). Recent results of a research programme based in Scotland suggest that up to 70% of *H. abietis* larvae in spruce stumps can be killed with a one-off application of commercially available nematodes (*Steinernema carpocapsae*) (Brixey, 1997). Entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) can be mass produced in fermenters and are currrently used commercially for the control of several insect pests in Europe, the US and elsewhere.

A number of wood-rotting Basidiomycetes grow rapidly under the bark of stumps and roots, competing with weevil larvae for food. Treatment of stumps with the wood-colonising fungus *Phlebiopsis gigantea* is known to reduce the survival of pine weevil larvae (Skrzecz,

1996). This fungus is already commercialised for protection of stumps against root rot fungus infections (*Heterobasidion annosum)* but, despite its ready availability, its use against pine weevil has not been considered until recently.

Biological control agents frequently inflicts considerable mortality on pest populations, but may not on their own be sufficient to reduce the pest below economic threshold levels, or the level of control may be variable. A strategy that utilises two (or more) agents may result in more dependable control. Nothing is known of the interactions (such as synergy or interference) of the above agents.

The objective of this research was to evaluate the compatibility of two biocontrol agents: entomopathogenic nematodes *(Steinernema* and *Heterorhabditis*), and the wood-rotting fungus *P. gigantea*. In particular, we wished to ascertain whether the presence of *P. gigantea* affects the ability of entomopathogenic nematodes to find and invade pine weevil larvae.

Materials and methods

Cultures

Nematodes (*Steinernema carpocapsae* UK strain and *Heterorhabditis megidis* UK211) were cultured in last instar larvae of *Galleria mellonella*. After harvest, infective juveniles (IJs) were washed three times by sedimentation in tapwater. Pine weevil larvae were obtained from a laboratory culture fed on Sitka spruce. *Phlebiopsis gigantea* was cultured on malt agar under black light to encourage sporulation.

***Inoculation of timber disks with* Phlebiopsis gigantea.**

Young trees (basal diameter 7-9 cm) were harvested from a Sitka spruce (*Picea sitchensis*) plantation and brought to the laboratory where disks 3.5 cm high and 4-5 cm diam. were prepared from the trunks. Disks were washed in detergent solution and inoculated with 2 ml of a spore suspension (approx. 4 $x10^6$ spores in 0.1% Tween). Timber disks were placed individually in plastic drinking beakers ("Glacier", Rexam Plastic, Bristol, UK; 8 cm high, 5.2 cm diam. at base) with moist sterile paper in the bottom and incubated at 20°C. Inoculations were performed at 2-week intervals so that disks with fungus at different stages of growth (2, 4, 6 or 8 weeks) could be tested simultaneously.

H. abietis *bioassay*:

A hole (0.5 cm diam., 1 cm deep) was bored through the bark of fresh or fungus-colonised timber disks. A single first instar *H. abietis* larva was placed into the hole which was then sealed with spruce sawdust. For the fungus treatments, the sawdust used to plug the insect chambers had been inoculated with spores 2-3 weeks previously. The timber disks were then placed individually in plastic drinking beakers and covered with moist sand (heat sterilised sand moistened with 10% w/w tapwater). A suspension of nematode IJs (*S. carpocapsae*: 1750 IJs/ml; *H. megidis*: 2500 IJs/ml) was added to the sand surface and the beakers were cappped and incubated at 20°C. After two days, the insects were recovered from the timber and incubated at 20°C for a further 2 days. Dead insects were dissected and the number of nematodes recovered was used as a measure of infectivity.

Experimental design and statistical analysis:

S. carpocapsae and *H. megidis* were tested in separate experiments. In each experiment, there were 10 replicates per treatment arranged in randomised block design. The number of nematodes recovered per insect in each *P. gigantea* treatment was compared with the number per insect in fresh timber using Friedmann's test.

Results and discussion

Effect of P. gigantea *on the ability of* S. carpocapsae *and* H. megidis *to* infect H. abietis *and* G. mellonella *larvae*

The average number of *S. carpocapsae* invading pine weevil larvae in fresh timber was 7.4, while in none of the fungus treatments was the mean below 25 nematodes/insect. Differences between the fresh wood and fungus-colonised wood treatment were significant for the 4 week ($P < 0.05$) and 6-week ($P < 0.01$) *P. gigantea* cultures.

The average number of *H. megidis* invading pine weevil larvae in fresh timber was 47.2. Significantly more nematodes were recovered in insects from fungus-colonised timber (weeks 4 and 8, $P < 0.05$) than in those from fresh timber.

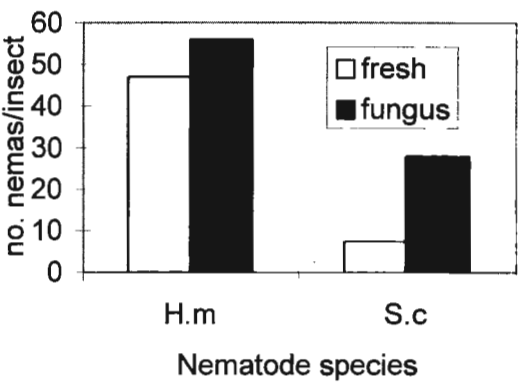

Figure 1. Mean number of nematodes recovered from *Hylobius abietis* larvae exposed to nematode infective juveniles in spruce disks in sand. The spruce disks were either of fresh timber or timber that had been colonised by *Phlebiopsis gigantea* for 2, 4, 6 or 8 weeks. H.m = *Heterorhabditis megidis* (Strain UK211); S.c = *Steinernema carpocapsae* (UK strain).

There is thus no evidence that colonisation of timber by *P. gigantea* for up to 2 months adversely affected host-finding or invasion of pine weevil larvae by either *S. carpocapsae* or *H. megidis*. Indeed, the reverse is true: where significant differences were detected between fungus-infected and fresh timber, the insects in the colonised timber had more nematodes than those in the fresh timber.

Possible explanations for the higher number of nematodes that was sometimes found in pine weevil larvae in timber colonised by *P. gigantea* are: (1) pine weevil larvae are rendered more susceptible to nematode attack in fungus-colonised timber; (2) fungus or fungus-infected timber has a stimulatory effect on the nematodes, or (3) fungus-colonised timber is more permeable to the nematodes.

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Preliminary study of pathogenicity of a local strain of *Steinernema abbasi* (Steinernematidae:Nematoda) to cruciferous pests

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A local strain of the entomopathogenic nematode *Steinernema abbasi* was collected in December 1998 by Professor Roger F. Hou and his colleagues from soil in eastern Taiwan. The life cycle of *S. abbasi* is comparable to known species of *Steinernema,* namely egg, four juvenile stages, and adult. The third-stage infective juveniles enter the hemocoele of the insect to deliver the symbiotic bacterium which cause death in a susceptible host. This species has not shown the nictating behaviour common to *S. carpocapsae* when applied to the leaf surface.

Bioassays were conducted in the laboratory to determine the pathogenicity of S. *abbasi* to Cabbage Leaf-webber *(Crocidolomia binotalis)* and Imported Cabbageworm *(Pieris rapae crucivora).* Treated and control larvae were placed in an incubator set at 25°C and a 12 L: 12 D photoperiod. Each treatment had 10 replications and each replication had 10 larvae. Third-stage juveniles of *S. abbasi* caused 100% mortality to first, second, third, and fourth instar larvae and 87.5% mortality to the fifth instars of *C. binotalis* at the concentration of 10 Us/larva. Third stage juveniles caused mortality levels of 100%, 100%, 100%, 94%, and 82%, respectively, to the first to fifth instar larvae of *P. rapae crucivora* at a concentration of 10 IJs/larva. From the above data it is evident that *S. abbasi* has the potential to control cruciferous pests, and further studies are needed.

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The influence of storage temperature and time on infectivity and reproduction of *Heterorhabditis megidis* (strain NLH-E87.3).

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Abstract: The effect of temperature, doses and storage period on the infectivity and reproduction capacity of *Heterorhabditis megidis* (strain NLH-E 87.3) infective juveniles (IJs) was studied in the laboratory. Infective juveniles were stored at 5, 10, 15 and 20°C during a period of 70 days (10 weeks). Every second week infectivity and reproduction capacity was examined with bioassays. Thirty and fifteen *Galleria mellonella* larvae were infested with the dose of one and 30 IJs, respectively. After 6 weeks of storage at 20°C the infectivity and reproduction of IJs diminished. Independently of the doses and storage period, the highest infectivity capacity was observed when IJs were stored at 10 and 15°C.

Key words: Entomopathogenic nematodes, dose-mortality, storage period, temperature, *Heterorhabditis megidis.*

Introduction

Nematodes of the genera *Steinernema* and *Heterorhabditis* are parasites of many insect pests, that carry mutualistically associated entomopathogenic bacteria *(Xenorhabdus* spp. and *Photorhabdus* spp. respectively) in their intestines (Akhurst & Boemare 1990). These entomopathogenic nematodes are used as biological control agents mainly to control soil-inhabiting insect pests in various crops (Klein 1990, Kaya & Gaugler 1993). The infective juvenile (IJ) stage actively seeks and invades a target host in the soil. After penetrating the insect, the infective juveniles (IJs) release into the host hemocoel the symbiotic bacteria carried in their intestine. The bacteria multiply, killing the host within 24-48 hours. The nematodes complete two to three life cycles in the host and the progeny IJs leave the cadaver in search of a new host. Entomopathogenic nematodes (family Steinernematidae and Heterorhabditidae) are being commercially mass produced and successfully introduced to many markets (Ehlers 1996, Jung 1996). However, the success of performance and commercialisation of these organisms depends on a series of abiotic and biotic factors. Although IJs of entomopathogenic nematodes have been recovered from the soil from a wide variety of climatic regions (Griffin *et al.*, 1991, Mracek & Webster 1993), the reproduction, development, dispersal and infectivity of entomopathogenic nematodes is known to be strongly affected by environmental temperature, not only in the field but also in mass reproduction systems (Griffin 1993, Steiner 1996, Jagdale & Gordon 1997 and 1998). Compared with other biopesticides, IJs age with time and this also affects the biological and physiological characteristics during their shelf life (Jung 1996), influencing thus the field efficacy. Shelf life and fitness of Heterorhabditis nematodes are still poorly understood.

The objective of this research was to study the influence of storage temperature, time and inoculum size on infectivity and reproduction of *Heterorhabditis megidis* (strain NLH-E 87.3) in *Galleria mellonella.*

Material and methods

The entomopathogenic nematode *Heterorhabditis megidis* (strain NLH-E87.3) (Smits *et al.*, 1993) was reared in last-instar *Galleria mellonella* larvae at 20°C. Groups of four wax moth larvae were put at the bottom of 50 ml plastics cylinders (50 ml, 36mm diameter) filled with moist silver sand (8% w/w, tap water). Four hundred infective juveniles (IJs) per pot in 1 ml tap water were added to the sand surface. The containers were closed and incubated in the dark at 20°C. After 96 hr, the host cadavers were removed from the sand and the red-colored infected wax moth larvae were placed on a layer of humid filter paper in a 5 cm Petri dish without lid. Dishes holding the cadavers were floated in water inside a large Petri dish (15 cm diameter) with lid and stored in the dark at 20°C (Modified White trap). Infective juveniles that emerged from the cadavers and moved from the small dish to the water, were collected daily for a period of 3 days. All the IJs collected were mixed and stored in 40 ml of tap water in four separate tissue culture flasks, at densities up to 4.500 infective juveniles per ml. Each flask was individually stored at temperatures of 5°C, 10°C, 15°C and 20°C during a period of 10 weeks.

Bioassays were carried out by placing a single last-instar *G. mellonella* larva at the bottom of a plastic cylinder (50 ml, 36mm diameter). The cylinders were filled with moist (8% w/w) sterilized silver sand. Two series of experiments were performed. In a first series with 15 replications, 30 infective juveniles in 1 ml tap water were added to the sand surface; the containers were closed with a lid and placed in the dark at 20°C. In a second series of experiments, with 30 replications, instead of 30 IJs one infective juvenile was put on the sand surface with a nematode fishing-rod (eyebrowhair glued to a bamboo stick). Only water was added to the controls. After 48 hours of exposure the *G. mellonella* larvae were removed from the sand and rinsed with tap water to remove any nematodes remaining on the outside. The larvae were transferred to moist filter paper and incubated separately at 20°C for 144 hours. Tests were conduced on the 1 st, 2nd, 4th, 6th, 8th and 10th week of emergence from the harvesting period. Before to be applied the IJs were rinsed in tap water and left at room temperature (18 - 20°C) during two hours. Each experiment was replicated three times.

Postexposure mortality was assessed for both experiments. To assess the penetration rate of the nematodes 11 of the larvae exposed to 30 IJs per larva were dissected and digested in a 0.8% pepsin solution and the invaded nematodes were counted (Mauleon *et al.*, 1993). To assess the production rate four infected host cadavers for each dose were transferred to individual modified White traps and all infective juveniles that emerged from single host over a period of 5 days were collected and counted. For the purpose of this study, we define emergence period as the time that nematodes leave the Petri dish that holds the host cadaver. The results were statistically analyzed by analyses of variance. Pairwise comparisons were made by least-significant-difference-test (LSD). (Genstat 5 committee, 1993).

Results

The effect of the two tested factors, storage temperature and time on the infectivity of the nematode strain *Heterorhabditis megidis* (NLH-E87.3) is shown in Figure 1 and 2.

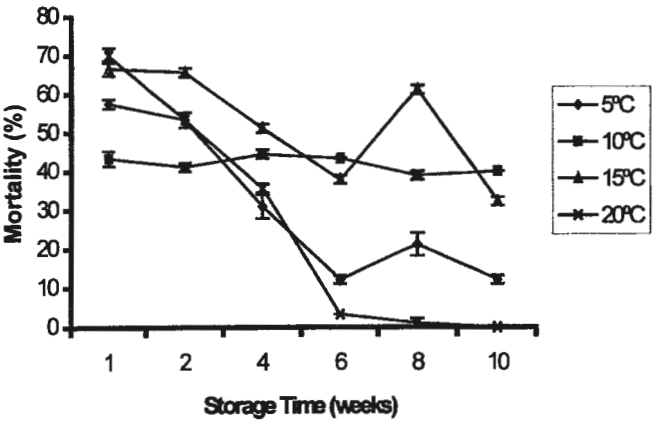

Figure 1. Mortality of *Galleria mellonella* larvae infested with one infective juvenile per larva of *Heterorhabditis megidis* (NLH-E 87.3) stored over time at different temperatures.

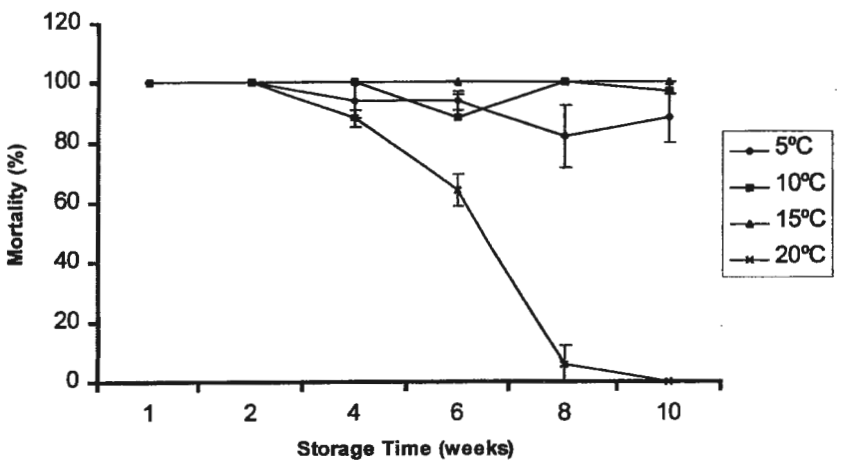

Figure 2. Mortality of *Galleria mellonella* larvae, infested with 30 infective juveniles per larva of *Heterorhabditis megidis* (NLH-E 87.3) stored over time at different temperatures.

Galleria mellonella larvae infested with one infective juvenile (IJs) per larva showed mortality over 30% on all temperatures until four weeks of storage. After this, at 6, 8 and 10 weeks, the same level of mortality was observed only at 10 and 15°C. The dose of 30 IJs per larva caused mortality over 80% independent of the storage period at 5, 10 and 15°C. No mortality, on both doses, was observed when larvae were treated with nematodes stored at 20°C during 10 weeks. Storage temperature and time (Figure 3), influenced the number of infective juveniles established in larvae. During the 10 weeks of storage the infective juveniles' penetration capacity was less affected when they were stored at 10 and 15°C than at 5 and 20°C.

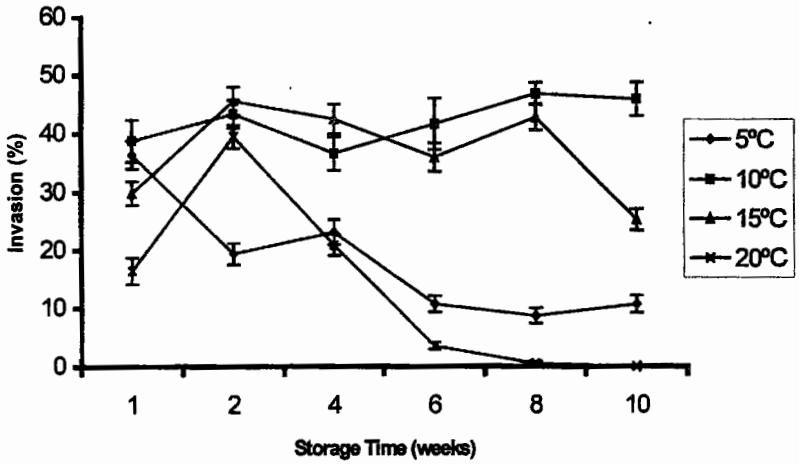

Figure 3. Percentage of nematodes invading *Galleria mellonella* larvae exposed to 30 infective juveniles per larva of *Heterorhabditis megidis* (NLH-E 87.3) stored over time at different temperatures.

The maximum capacity of invasion of nematodes stored at 5 and 20°C was seen after one and two weeks of storage, respectively. After this at both temperatures the number of infective juveniles that invaded decreased to very low levels at 5°C and to zero at 20°C.

Results of the infective juvenile production show that when the host larvae were infested with one infective juvenile (Table 1) the lowest production was given at storage time of one week on all storage temperatures except at 20°C. Infective juveniles stored at 10 and 15°C produced more juveniles than those stored at 5 and 20°C. No infection and consequently no production was observed at 20°C after storage time of 8 and 10 weeks.

Table 1. Production of infective juveniles in *Galleria mellonella* cadavers. Larvae were infested with one infective juvenile of *Heterorhabditis megidis* (NLH-E 87.3) stored over time at different temperatures.

	Storage Temperature (°C)			
Storage time	**5**	**10**	**15**	**20**
1 week	62 007 bE	17 259 dE	53 872 cE	100 572 aB
2 weeks	67 270 cD	102 950 abCD	106 967 aAB	84 858 bcC
4 weeks	96 950 cB	113 983 bB	118 813 aA	115 783 abA
6 weeks	87 551 cC	136 492 aA	101 005 bB	100 777 bB
8 weeks	105 792 aA	101 425 bD	102 068 abB	0 cD
10 weeks	68 048 cD	104 911 aC	81 006 bC	0 dD

Lowercase letters correspond to comparisons among storage temperatures. Uppercase letters correspond to comparisons among storage time. Data followed by different letters are significantly different from each other (P = 0.05).

Production of infective juveniles from larvae infested with 30 IJs (Figure 4) surprisingly decreased at all temperatures when the nematodes were stored for two weeks. No significant differences in production were observed when larvae were infested with infective juveniles stored during 8 and 10 weeks in the temperature of 5°C and between 4 and 6 weeks at 10° and 15°C. At 20°C no production was found after 8 weeks of storage because no IJs invaded the larvae (see figure 3).

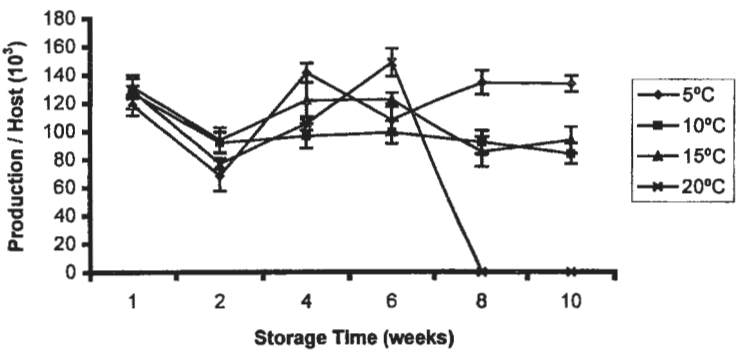

Figure 4. Number of infective juveniles emerged from individual cadavers of *Galleria mellonella* infested with 30 infective juveniles of *Heterorhabditis megidis* (NLH- E87.3) stored over time at different temperature.

Comparing the data from table 1 and figure 3 and 4 it is clear that the production of infective juveniles per invaded infective juvenile is much higher on larvae infested with one IJ, than in larvae infested with 30 IJs (Table 2).

Table 2. Production of infective juveniles per Infective juvenile invaded in *Galleria mellonella.* Single larvae were infested with 30 infective juveniles of *Heterorhabditis megidis* (NLH-E 87.3) stored over time at different temperatures.

	Storage Temperature (°C)			
Storage time	**5**	**10**	**15**	**20**
1 week	10 804	10 583	14 621	25 762
2 weeks	11 361	7 057	6 694	6 395
4 weeks	20 164	8 759	9 358	15 044
6 weeks	35 926	7 072	11 095	74 225
8 weeks	44 746	6 572	6 556	0
10 weeks	33 348	5 999	11 714	0

Discussion

Our results indicate that storage temperature, storage time and inoculum size significantly affected the infective capacity and number of infective juveniles that emerged from infested

Galleria mellonella larvae. Within the two first weeks of storage the percentage of nematodes infecting a host significantly decreased after storage at 5°C and increased at temperatures of 10, 15 and 20°C but this tendency changed with the passage of the time. Infective juveniles stored at 10 and 15°C maintained infectivity above 30% during 10 weeks and those stored at 5 and 20°C show a great decrease of infective capacity at both tested doses. Fan & Hominick (1991), Kaya & Gaugler (1993) and Griffin(1996) assumed that the number of nematodes infecting the hosts decreases after storage at low temperatures but is followed by an unexpected increase with passage of the time, indicating that the cold temperatures induces some nematodes to enter a non-infectious or "diapause" state. From our results there are indications of a similar phenomenon only during a period of 6 weeks with a clear difference in production but not in mortality or invasion rates. After this period until 10 weeks a decrease of infectivity occurred and infective juveniles became less infective. This response to low temperatures may have negative implications in their applications as inundative biological control agents and explain, in part, field-trial data in which a huge number of nematodes is required to obtain good results. A clear and progressive decline in infectivity of infective juveniles stored at 20°C may be due to the fact that nematodes' metabolism is temperature dependent. Warm temperatures increase the rate of lipid reserve used and decrease the time that the infective juveniles remain viable and pathogenic. Selvan *et al.,* (1993a) demonstrated that steinernematids retain a relatively high level of satured fatty acid contents, which could explain, in part, the higher infective capability as compared to Heterorhabiditids. As we stored the infective juveniles in water their active movements that resulted in expended energy could be another factor responsible for the differential infectivity when stored at 20°C longer than 6 weeks. Regardless of storage temperature and age of infective juveniles under presumably optimal conditions, the nematode strain that we tested showed that only a maximum of 47% of the nematodes infested the host at a dose of 30 infective juveniles per larva and a maximum mortality of 70% in larvae infested with one infective juvenile per larva.

The higher yield of *H. megidis* (NLH-E 87.3) per host larva treated with small dose (one IJ per larva) occurred 2-8 weeks of storage at almost all storage temperatures. However, during this period of time, storage temperatures of 10 and 15°C were those that showed the highest production. Zervos *et al.,* (1991) stated that several factors affect the in vivo production of infective juveniles, and these factors interact in unpredictable ways affecting the number of generations passed trough before emergence, the growth rate of the bacterial symbiont, or the biochemical composition of the cadavers. This may in part explain the low fecundity observed at first and last weeks of storage in larvae treated with either dose of infective juveniles.

When larvae were treated with the dose of 30 infective juveniles the cumulative production seems to be more related to the number of invaded infective juveniles than with storage time and temperature. From our results we can see that the lowest production occurred when a higher number of infective juveniles invaded the host. It is possiblc to conclude through data from penetration (n°. of infective juveniles per larva) that in larvae infected with 30 infective juveniles, each infective juvenile penetrated produced lower offspring than when larvae were infested with only one infective juvenile. We can notice also that at the storage temperatures of 10 and 15°C infective juvenile presented high levels of infectivity and mortality and consequently lower production. Our results enforces Selvan *et al.,* (1993b), studies that density of nematodes inside one larva has a important influence on nematodes fecundity.

In general it can be concluded that the nematode production varies with temperature and storage time of infective juveniles and that the optimal production is most likely to occur when a small, rather than a large, number of infective juveniles is inoculated at optimum

temperature. Clearly much further effort needs to be directed towards understanding the effects of storage temperature and time on infectivity, with a view both to possible manipulation of storage conditions for optimal conditioning of the nematodes and better results in field applications.

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The susceptibility of the second stage juveniles of *Meloidogyne javanica* to the bacterial symbiont from the entomopathogenic nematode, *Steinernema abbasi* (Steinernematidae: Nematoda)

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Summary: Two experiments, one in the laboratory and the other in a growth room were conducted to evaluate the influence of the bacterial symbiont, *Pseudomonas oryzihabitans* from the entomopathogenic nematode *Steinernema abbasi* on the root-knot nematode, *Meloidogyne javanica*. Second stage juveniles of *M. javanica* were disorientated in the presence of the bacterial symbiont and showed convulsive movements. Suspensions of the bacterial symbiont applied around the roots of tomato plants prevented the invasion of second stage juveniles of *M. javanica* into roots. The results indicated that further research on the efficacy of the bacterial symbiont might provide a new control strategy against root-knot nematodes.

Introduction

Inundative applications of entomopathogenic nematodes have been shown to be effective in reducing the invasion and development of *Meloidogyne* spp. (Bird and Bird, 1986; Gouge *et al.*, 1944) and *Globodera rostochiensis* (Perry *et al.*, 1998), possibly due to the competitive behaviour of the entomopathogenic Dauer juveniles around the root surface. A new species of *Steinernema, S. abbasi*, has recently been isolated in Oman (Elawad *et* al., 1997) which carries a bacterial symbiont *Pseudomonas* (*Flavimonas*) *oryzihabitans* (Elawad *et al.*, in press; Anzai *et al.*, 1997) belonging to a group of bacteria not hitherto associated with full progeny production in entomopathogenic nematodes. This bacterium is aerobic with one polar flagellum and has been shown to be effective against insect larvae and fungi in the absence of the nematode vector (Elawad, 1998). This paper reports on the results of experiments carried out to determine the influence of *P. oryzihabitans* on the behaviour and invasion of J_2s of *Meloidogyne javanica*.

Materials and methods

Steinernema abbasi was cultured in *Galleria mellonella* using techniques described by Woodring & Kaya (1988).

Isolation of bacteria

Dead infected larvae of *G. mellonella* were washed in distilled water and surface sterilised in 70% alcohol and left to dry in a laminar flow cabinet. Larvae were opened with sterile scissors and needles, care being taken not to damage the gut epithelium. A small drop from the oozing haemolymph was streaked onto nutrient agar. The Petri-dishes were sealed and incubated at 28°C in the dark for 24 h. Single colonies were selected and sub-cultured onto nutrient broth agar, the process being continued until uniform colonies were obtained. The pure colonies of the bacterium were multiplied in nutrient broth (30g/l) in an incubator at

28°C for 24 h and the bacterial suspension was centrifuged at 5000 rpm for 15 min in 250 ml tubes. A bacterial pellet was formed at the bottom of the tube, the supernatant broth solution was drained off, sterile distilled water was added to the pellet, mixed thoroughly to form a concentrated suspension of the bacterial symbiont. The concentrated suspension was diluted with sterile tap water to give the concentrations required for experiments. The concentration of the bacterial symbiont was determined by estimating the optical density of the suspension using a spectrophotometer adjusted to the 600-nm wavelength.

***Culture of* Meloidogyne javanica**

The root-knot nematode, *M. javanica* was cultured on tomato cv Tiny Tim in a glasshouse at 24-28°C. Galled roots with egg masses were washed free of soil, cut into pieces 2 cm long, placed in 0.26% sodium hypochlorite and triturated at 30 sec intervals at maximum speed in a two-speed blender. The suspension was poured through a series of sieves to separate the organic debris from eggs which were collected on a 20 µm-pore sieve and washed carefully with tap water ([technique modified from McClure *et al* (1973)]. The egg suspension was poured onto a cotton-wool filter (modified Baermann funnel), incubated at 28°C and the hatched second stage juveniles (J_2s) collected daily. Only freshly hatched J_2s, sterilised in streptomycin sulphate (0.1%) for 15 min (Mountain, 1955) were used for experiments.

***Effect of* P. oryzihabitans *on second stage juveniles of* M. javanica**

The concentrations of *P. oryzihabitans* used in this experiment were: 0, 10^4, 10^5. 10^6 and 10^7 cells ml^{-1}. The bacterial suspensions were mixed into nutrient agar without bromothymol blue as it cooled at 45°C and plated out on 9 cm Petri-dishes.

Thirty J_2s in a drop of sterile water (0.2 ml) were inoculated onto the plates and exposed for 30 min, 1 h, 3 h, 6 h, 12 h and 24 h, replication was 4-fold. The behaviour of the J_2s in the drop of water was observed. After exposure J_2s were transferred to sterile distilled water and were continually observed for a further 10 days, to see whether the J_2 recovered normal movement or were killed by exposure to the bacterial symbiont.

Effect of* P. oryzihabitans *on the penetration of second stage juveniles of* M. javanica *into tomato roots.

Forty tomato plants cv Tiny Tim were grown in 9 cm plastic pots in sterilised soil (3:1 loam/sand). At the two leaf stage 20 plants were each treated with 20 ml of a bacterial suspension containing 10^6 cells ml^{-1}, the untreated plants receiving 20 ml distilled water. At 1½, 3, 6, 12 and 24 days after application of the bacterial suspension each plant was inoculated with 200 sterilised J_2s of *M. javanica*. After 3 days exposure to the J_2s, plants were removed from the pots, thoroughly washed and repotted in sterilised soil. Each pot was placed in a small plastic tray to avoid cross-contamination and the tomato plants grown on in a growth room at 27 ± 2°C with a 16 h day/8 h night photoperiod.

The plants were harvested 21 days after the inoculation with *M. javanica* J_2s, the roots gently washed free of soil, stained in acid fuchsin, macerated in a blender and the number of different stages of *M. javanica* determined.

Results

At a concentration of 10^4 cells ml^{-1} some J_2s became disorientated exhibiting "convulsive" movements, the effect being more marked as the time of exposure increased (Table 1), none of the J_2s were paralysed. At 10^5 cells ml^{-1} disorientated movements were observed up to 3 h exposure, thereafter J_2s were paralysed, ie they were completely straightened out. At concentrations of 10^6 and 10^7 cells ml^{-1} all J_2s were paralysed (Table 1). When J_2s were placed in fresh distilled water after exposure there was some recovery to normal movement at all treatments except at 24 h exposure to concentrations of 10^6 and 10^7 cells ml^{-1} (Table 1).

Table 1. The effect of exposing J_2s of *M. javanica* to concentrations of the bacterial symbiont *P. oryzihabitans* for different times, estimated as the % responses: CV, % showing convulsive movements: P, % paralysed: NM, % which recovered normal movement after removal from contact with bacterial suspensions to fresh water.

Bacterial concentrations, cells ml^{-1}												
	10^4			10^5			10^6			10^7		
Exposure hours	CV	P	NM	CV	P	NM	CV	P	NM	CV	P	NM
½	8.0	0	100	18.5	0	100	0	100	73.9	0	100	61.0
1	11.0	0	100	32.6	0	100	0	100	64.0	0	100	34.7
3	38.5	0	100	57.5	0	100	0	100	43.5	0	100	12.0
6	57.2	0	91.5	0	100	67.4	0	100	44.7	0	100	8.4
12	65.0	0	81.8	0	100	47.0	0	100	21.8	0	100	5.0
24	76.0	0	68.0	0	100	6.0	0	100	0	0	100	0

Application of *P. oryzihabitans* around the roots of tomato plants significantly reduced the number of J_2s entering the roots (Table 2). The bacterium remained effective in soil even when the J_2s were inoculated 24 days after the bacterial treatment. There was clear evidence of interference in the development of the nematode because very few female *M. javanica* were produced in treated plants compared to untreated.

Table 2. The number of *Meloidogyne javanica* and the percentage of females in roots inoculated with J_2s at different times after the application of *P. oryzihabitans*

	The time in days when J_2s were inoculated after application of bacterial symbiont				
	1.5	3	6	12	24
Treated	0.5	2.25	1.75	2.5	4.5
% female *M. javanica*	0	11	14	60	94
Untreated	11.5	23.5	28.5	19.5	18.75
% female *M. javanica*	96	98	100	98	96

Discussion

The symbiotic bacterium, *Psuedomonas oryzihabitans*, from the entomopathogenic nematode, *S. abbasi*, disorientates and paralyses J_2s of *M. javanica*, prevents the J_2s invading roots and there is evidence of interference in the development of the nematode in roots. Symbiotic bacteria from other species of entomopathogenic nematode are known to produce antibiotics and toxins (Chen *et al.*, 1994; Akhurst, 1982) and the behavioural responses reported here indicate that *P. oryzihabitans* probably produced toxins which have antibiotic effects. The

behavioural responses of the J_2s of *M. javanica* to *P. oryzihabitans* have many similarities to the effects produced by the oxime carbamate, aldicarb, (Nelmes, 1970) who reported disorientation and convulsive movements of the J_2s of the potato cyst nematode, *Globodera rostochiensis*. Oxime carbamates also prevent the invasion of roots by J_2s of *Globodera rostochiensis* (Hague and Pain, 1973) and interfere with development of *Meloidogyne incognita* in roots (Wright *et al.*, 1980).

Inundative applications of entomopathogenic nematodes have been shown to be effective in reducing invasion and development of *Meloidogyne javanica* (Bird & Bird, 1986; Gouge *et al.*, 1994), possibly because entomopathogenic nematodes are attracted to carbon dioxide from roots and surround the root surface, thus preventing invasion by J_2s of *Meloidogyne*. A more plausible explanation is that, when very large numbers of entomopathogenic nematodes often in excess of 100,000 (Bird and Bird, 1986) are applied inundatively around roots, large numbers of these nematodes die, thus releasing symbiotic bacteria into soil around roots.

In summary it is proposed that the bacterial symbionts of entomopathogenic nematodes produce antibiotic toxins which are active against root-knot nematodes. Considerable research is required to investigate whether these toxic metabolites of *P. oryzihabitans* can be developed for commercial use. In particular more information is required on their stability in soil and water and whether temperature adversely affects their efficacy. Further research on the efficacy of the bacterial symbiont might provide a new control strategy against root-knot nematodes.

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A possible competition mechanism in insects parasitized with rhabditid nematodes making the insect carcass decay-resistant

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Abstract: No trace of antibacterial activity or an extremely low an antibiotic potency in *Galleria mellonella* throughout the life cycle of the rhabditid nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* cannot be the primary mechanism which keeps competing microflora from invading the insect carcass. The resistance of internal organs and host tissues to decay cannot be explained simply by antibiotic inhibition of bacterial contaminators. The lack of putrefaction could rather result from little or no competition for the multiplying bacterial symbionts in parasitized insects. A rapid colonization of the insect body by symbiotic bacteria creates unfavourable conditions for growth and multiplication of contaminating microflora. The phase II variant of *Xenorhabdus nematophilus* or *Photorhabdus luminescens* does not produce an antibiotic compound(s) active against proteolytic microbial invaders but it does tend to outgrow them in the host. The low antibiotic activity at an early stage of the parasitism could additionally contribute to the massive development of bacterial symbionts, notably *P. luminescens*, in the parasitized host.

Key words: *Steinernema carpocapsae*, *Heterorhabditis bacteriophora*, bacterial associates, antibiotic compounds, secondary invasion, competition mechanism

Introduction

Insect pathogenic rhabditid nematodes of the families *Steinernematidae* and *Heterorhabditidae* and their symbiotic bacteria represent a current focus for research because of their commercial use in insect pest suppression. *Photorhabdus luminescens*, formerly called *Xenorhabdus luminescens* (Boemare *et al.*, 1993), isolates are associated obligatory with its symbiotic nematode *Heterorhabditis*, whereas the bacteria *Xenorhabdus* spp. are facultative symbionts of nematodes in the genus *Steinernema* (Akhurst, 1983). The bacterial symbionts are carried monoxenically within the intestine of the infective dauerlarvae where the cells rest dormant. When the nematode has penetrated a host insect the bacteria are released and multiply in the insect's body cavity causing fatal septicaemias. The insect killed by the combined action of bacteria and nematodes retains its colouration and does not putrefy prior to emergence of new generation of infective juveniles.

The symbiotic bacteria *P. luminescens* and *Xenorhabdus* spp. exhibit phase variations: the phase I (primary form) variants produce agar-diffusible antibiotic compounds on bacteriologic media, whereas the phase II (secondary form) variants are unable to produce antimicrobials (Akhurst, 1982). It, therefore, has been widely accepted that the interactive nematode-bacterium complex prevents putrefaction by the production of inhibitory substances in the parasitized insect (Dutky, 1974; Poinar et. al. 1980; Akhurst, 1982). This suggestion is claimed only by interpolation to antibacterial compounds produced on artificial media by phase I variants. This investigation was consequently designed to evaluate the ability of *X. nematophilus* and *P. luminescens* to produce antibiotic compounds on an artificial medium and in the parasitized host, *Galleria mellonella*. Efforts were made to answer the question of

whether antibiotic compound(s) produced *in vitro* by the phase I variants depress really the secondary invasion of insect carcasses by bacterial (proteolytic) contaminators.

Material and Methods

Stock cultures and bacterial variants

Entomopathogenic nematodes and their bacterial associates used for experimentations are Polish indigenous nematodes, originally isolated from soil samples using the *Galleria* trap technique (Bedding and Akhurst, 1975). Nematodes were reared on 7th instar larvae of the greater wax moth *Galleria mellonella* L., according to the technique of Dutky *et al.*, (1964). Last-instar *G. mellonella* were obtained from the insectary cultures reared on honey drawn combs at 29°C in total darkness. Juveniles were maintained in a thin-layer of 0.004% formaldehyde solution at 6°C for no longer than 2 weeks.

Phase I variants were isolated by homogenizing surface-sterilized infective nematodes of *Steinernema carpocapsae* strain PLNc82 or *Heterorhabditis bacteriophora* strain PLHb81, but phase II cultures from larval plasma of *G. mellonella* parasitized with infective-stage nematodes (Akhurst, 1980). The isolates of phase I variants grew slowly on Tergitol-agar (Poinar and Thomas, 1978) as green, irregular debris-like colonies that were surrounded by decolourized zones after 7-12 days of incubation at 23°C. All isolates were routinely checked for purity by inoculation into larvae, determination of entomopathogenicity and symptoms of septicaemia. A characteristic red colouration and gummy consistency of insect carcasses, typical of *H. bacteriophora*/*P. luminescens* parasitism, existed only following infection with bacteria described herein as the phase I variant of *P. luminescens* (Jarosz and Skrzypek, 1992).

Developmental stages of the nematodes were determined by microscopical examination of nematodes dissected from *Galleria* larvae, starting from moribund insects.

Anti-microbial production in vitro

Using 1.5% nutrient agar as an artificial medium, the antibiotic-producing ability of bacterial symbionts was bioassayed by the double agar layer method of Gratia with the stab modification (Hamon, 1957). Three days were allowed for the slow-growing phase I colonies to develop, but only 36h were allowed for the faster-growing phase II variants. Bacterial colonies of both phase I and phase II strains had reached about 4.0 mm diam after this incubation time at 23°C. The colonies were then killed by exposing them to chloroform for at least 30 min. The bacterial colonies on the dish were covered with the log-phase broth cultures of each indicator bacterium in a soft (0.6%) nutrient agar. Growth inhibition zones were measured after 36h incubation of indicators at 28°C.

Assay for antibiotic potency in parasitized insect

The conventional agar diffusion assay procedure was used to evaluate the antibiotic potency in larval extracts prepared from parasitized insects. Larval extracts were prepared from *G. mellonella* parasitized with nematode/bacteria complex, and from larvae infected with the bacterial symbionts alone, the phase I or phase II variants. Ten individuals of each developmental stage of the nematode were blended thoroughly in 10 ml of distilled water, and then cold-sterilized by passage through a Schot G5 filter. By the same manner, there were obtained extracts from larvae challenged with phase I or phase II cultures.

Larval extracts from parasitized *Galleria* were bioassayed for anti-bacterial potency against a wide range of bacterial indicators. The diameter of growth inhibition zones produced around the well indicated antibacterial potency in the larval extract and to the sensitivity of the indicator bacterium tested.

The maximal inhibitory dilution (MID) determined against *Bacillus subtilis* ATCC 6633, a sensitive proteolytic test bacterium, by the 2-fold broth dilution was defined as the amounts of antibiotic contained in the greatest dilution of larval extract that completely inhibited the growth of the test bacterium at 32°C for an incubation period of at least 72h. A number of bacteriological tubes prepared with decreasing dilution of insect extracts in nutrient broth were inoculated with a 10^4 dilution of an overnight bacterial broth culture.

Results

Laboratory bioassays of the antibiotic-producing ability of bacterial symbionts unequivocally confirm the presence of a brood spectrum agar-diffusible antibiotic compound(s) produced *in vitro* on nutrient agar by the phase I variants of *X. nematophilus* and *P. luminescens*. Phase II isolates were inactive totally against a wide range of bacterial indicators tested (Table 1). The broad spectrum of antibacterial activity directed against gram negative and gram positive bacteria, with preferential activity against micrococci *Micrococcus luteus* and *Sarcina lutea* and against *Bacillus subtilis*, is similar for both bacterial symbionts, but *X. nematophilus* produced larger inhibition zones against more indicators. Only *Serratia marcescens*, a prodigiosin-producing strain of CCM 2222, was insensitive to antibiotics produced *in vitro* by both *X. nematophilus* and *P. luminescens*.

Table 1. Antibacterial activity produced *in vitro* on nutrient agar by bacterial associates of rhabditid nematodes bioassayed by the double agar layer technique of Gratia.

(Phase I strains: Xnp 51, Plp 73; Phase II strains: Xns 4, Pls 6)

	Xenorhabdus nematophilus		*Photorhabdus luminescens*	
	Phase I	Phase II	Phase I	Phase II
Bacterial indicators	Growth inhibition zone diameter (mm)			
Escherichia coli D31	27	0	18	0
Enterobacter cloacae β12	20	0	15	0
Serratia marcescens CCM 2222	0	0	0	0
Pseudomonas aeruginosa H3	39	0	24	0
Bacillus subtilis ATCC 6633	32	0	37	0
Bacillus cereus ATCC 8145	31	0	29	0
Bacillus brevis IP 5286	10	0	12	0
Sarcina lutea (lab strain)	38	0	26	0
Micrococcus luteus ATCC 4698	39	0	24	0

As can be seen in the *in vivo* antibacterial assays (Tables 2 and 3), no antibiotic activity or an extremely low antibiotic potency could be detected only in larval *G. mellonella* parasitized with infective nematodes or phase I variants of bacterial symbionts. In insects parasitized with *S. carpocapsae/X. nematophilus* complex or *X. nematophilus* alone, the antibacterial activity of a narrow spectrum directed preferably against aerobic spore-forming bacilli, but not against strongly proteolytic bacterium *Pseudomonas aeruginosa*, was demonstrated in trace levels. At a demonstrable titre the antibiotic activity could be detected in larvae parasitized with *H. bacteriophora* or with the phase I isolates of *P. luminescens*. No trace of antibacterial activity was seen in the case of phase II variants tested from either species.

Table 2. Antibiotic potency in moribund (M) larvae of *Galleria mellonella* infected intrahaemocoelicaly with phase I variants of bacterial associates alone, and in insect carcass 1 day after larval death (D)

(Compared by the conventional agar-diffusion assay procedure (well, diameter, 2.7 mm). Tr, trace amount of activity (diameter of growth inhibition zone less than 4.5 mm).)

	Xenorhabdus nematophilus		*Photorhabdus luminescens*	
	M	D	M	D
Bacterial indicators	Growth inhibition zone diameter (mm)			
Escherichia coli D31	0	0	Tr	5.5
Enterobacter cloacae β12	0	0	0	Tr
Serratia marcescens CCM 2222	0	0	0	0
Pseudomonas aeruginosa H3	0	0	0	0
Bacillus subtilis ATCC 6633	0	Tr	Tr	16
Bacillus cereus ATCC 8145	0	Tr	0	16
Bacillus brevis IP 5286	0	0	0	12
Sarcina lutea (lab strain)	0	Tr	7.5	12
Micrococcus luteus ATCC 4698	Tr	5.0	0	Tr

Against *B. subtilis* ATCC 6633, one of the most sensitive bacterial indicator in the *in vitro* assay, the crude larval extracts from animals parasitized with *S. carpocapsae* (or by its bacterial symbiont) showed trace activity only. Larval extracts from insects killed by *H. bacteriophora* exhibited higher, but still low antibiotic activity. The antibacterial potency found in *Galleria* parasitized with *H. bacteriophora* was 8 times higher (8 x MID) than that in *Galleria* killed with *S. carpocapsae*.

Briefly, antibacterial assays have shown a low antibiotic potency of a limited spectrum of antibacterial activity both in larval plasma from moribund insects and from insect carcasses throughout life cycle of the nematodes (Table 3), from insects blended in distilled water and in phosphate buffer (pH 6.8 and 7.6), in blended *Galleria* tissues centrifuged at 12000g and in those then sterilized by passage through a Schot G5 filter.

Discussion and Concluding Remarks

When an insect is killed by rhabditid nematode, *Steinernema carpocapsae* or *Heterorhabditis bacteriophora*, it subsequently is not attacked by secondary bacterial and fungal invaders. The internal organs and host tissues are resistant to decay. Only by interpolation to antibacterial compounds produced on artificial media by phase I variants it was claimed that bacterial symbionts depress the growth of proteolytic contaminators in insects parasitized with *Steinernema* or *Heterorhabditis* (Akhurst, 1982; Dutky, 1974; Poinar *et al.*, 1980). Some of these antibiotics have been isolated from liquid fermentation media and identified as indole derivatives (Paul *et al.*, 1981; Richardson *et al.*, 1988), trans-stilbenes (Paul *et al.*, 1981; Richardson *et al.*, 1988), xenorhabdins (McInerney *et al.*, 1991a), xenocoumacins (McInerney *et al.*, 1991b) or bacteriocins (Boemare *et al.*, 1992).

Table 3. Evaluation of antibiotic potency of larval extracts obtained during life cycle in *Galleria mellonella* of the insect parasitic rhabditid nematodes, *Steinernema carpocapsae* and Heterorhabditis bacteriophora

(0, No antibacterial activity; Tr, antibacterial activity in trace amounts (diameter of growth inhibition zone less than 4.5 mm; well diameter, 2,7 mm).)

Nematode life cycle	*Steinernema carpocapsae*				*Heterorhabditis bacteriophora*			
	Bacillus subtilis	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Bacillus subtilis*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia coli*
	Diameter of growth inhibition zone (mm)							
Fourth-stage juveniles (J4) dominate (moribund insect, fat body intact)	0	0	0	0	Tr	0	0	Tr
Fourth-stage juveniles (J4) dominate (disruption of tissues and larval organs)	6.0	Tr	0	0	15.5	16	0	4.5
Few first-stage juveniles (J1)	5.5	4.5	0	0	16	12	0	6.5
Second-stage juveniles (J2) dominate	5.0	0	0	0	14	10	0	Tr
Young adults of second generation	Tr	0	0	0	12	12	0	Tr
Second-stage juveniles (J2), mature females and males dominate	0	0	0	0	Tr	Tr	0	0
Few infective juveniles (IJ)	0	0	0	0	0	0	0	Tr
Infective juveniles (IJ) emerge abundantly	0	0	0	0	0	0	0	0

Bioassays for antibiotic activity by the double agar layer technique confirmed Akhurst's (1982) earlier observations that the phase I variants of *X. nematophilus* and *P. luminescens* produce *in vitro* agar-diffusible antibiotic compounds active against a wide range of bacterial species (Table 1), but involvement of antibacterial agents in inhibition of bacterial contaminators in insect carcass is negligible, if at all present during parasitism (Jarosz, 1996a). An extremely low or no antibiotic potency was demonstrated in *G. mellonella* larval extracts from each developmental stage of the nematodes (Table 3). Obviously, the lack of putrefaction cannot simply be explained by the trace activity of a narrow antibacterial spectrum detected mainly in the first stages of the parasitism. In addition, this low activity directed principally against some bacilli and micrococci declines sharply to a trace activity on day 5 when the second stage juveniles dominate in the life cycle of the nematodes. No antibiotic activity can be seen during hatching, development and emergence of infective first-stage juveniles. However, the dead *Galleria* still retained a natural and fresh body typical of insects killed by entomopathogenic rhabditid nematodes.

It is postulated that antibiotics produced by symbiotic bacteria associated with the *S. carpocapsae* and *H. bacteriophora* are not the primary mechanism which keeps competing microflora from invading the insect cadaver (Jarosz 1996b). This can also be supported by the fact that in a parasitized host or during the stationary period of *in vitro* culture, an antibiotically active phase I variants convert spontaneously to phase II symbionts that are unable to produce antibacterial compounds (Akhurst, 1982). This fact additionally arises the question of whether bacterial associates produce really antibiotic-like substances in parasitized insects that could prevent the putrefaction of the insect carcass.

Several competition mechanisms may be active in parasitized host, preserving the nutrient niche for completion of life-cycle of both rhabditid nematodes. On the basis of the *in vitro* and *in vivo* antibacterial assays, it seems be reasonable to conclude that a quite different competition mechanism, other than antibiotic production, operating in insects parasitized with rhabditid nematodes, makes the insect carcass decay-resistant. The lack of putrefaction could result from little or no competition for the multiplying bacterial associates in parasitized insects. A rapid colonization of the insect body by nematophilic bacteria creates unfavourable conditions for growth and multiplication of contaminating bacterial microflora. The phase II variant does not produce an antibiotic compound(s) active against proteolytic bacilli and other microbial invaders but it does tend to outgrow them in the host. The large number of bacterial symbiont in the dead insects results from the ability to outgrow many of the commensal bacteria rather than from the production of antimicrobials. By inhibiting bacterial contaminators, the low antibacterial activity could enable in an initial stage of the parasitism a rapid and massive colonization of an insect body by *X. nematophilus* or *P. luminescens*.

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Entomopathogenic nematodes from *Steinernematidae* and *Heterorhabditidae* (Nematoda: Rhabditida) in Bulgaria

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Summary: Seven species of entomopathogenic nematodes - 6 from Steinernema and 1 from Heterorhabditis were recovered from Bulgaria for a first time. Nematodes were isolated from soil samples taken mainly from natural meadow and forest ecosystems from different altitudinal and vegetation zones of Vitosha mountains, as well as from Zemen defile, Rila mountains and other areas in Bulgaria.

St. kraussei was the dominant species in the most of the natural ecosystems.

Introduction

One of the aims of the collective is to study the species composition, distribution and frequency of occurrence of entomopathogenic nematodes in Bulgaria (Shishiniova, Budurova, Gradinarov, 1997; 1998; 1998; 1999).

The investigations, started about 4 years ago involve results from 440 soil samples from 114 habitats.

Materials and methods

For isolation of nematodes from Steinernematidae and Heterorhabditidae the soil samples are collected by the marshrute method mainly from natural meadow and forest ecosystems from different altitude and vegetation belts in Vitosha mountain (near Sofia), where 348 samples from 60 biotopes are collected. Some samples and biotopes also from Zemen, Skakavitsa part of Rila mountain, Teteven part of Stara planina mountain, Botanic garden in Varna, region of Sofia and other parts of the country are studied. Totally 204 samples from meadow, 202 from forest and 34 from other sites (agroecosystems, riverside biotopes) are collected. Nematode extraction was done by the method of nematode baiting, using last instar *Galleria mellonella* larvae. The isolated nematodes were fixed in 4% formalin solution, than dehydrated in ethanol - glycerine solution and mounted in microscope slides. The species identification is done on the basis of used in taxonomy metric, morpho-anatomic characters and indexes. Infective juveniles and males from the first generation for *Steinernema* and second generation for *Heterorhabditis* were used.

Results and discussion

As a result of laboratory processing of the samples 7 species of entomopathogenic nematodes were found - 6 from *Steinernema* and 1 from *Heterorhabditis*. Species from genus *Steinernema* were wider distributed in the biotopes studied - they were found in 149 samples, while those from genus *Heterorhabditis* - only in 7.

Steinernema kraussei (Steiner, 1923)
In Bulgaria *St. kraussei* was isolated from different natural ecosystems in Vitosha mountain, Rila mountain, near village Lozen (Sofia region) at different altitude from 700 to 2250 m. It was found in 85 samples from 41 habitats. In the region of Vitosha mountain the species has highest frequency occurrence from subalpine meadow soil and spruce forests, followed by meadows in mid and low mountain belt, mixed oak - hornbeam and pine forests. From samples from Rila mountain (1500 - 1600m a.s.l.) the species was found in soils from spruce forests and meadows. Near v. Lozen (Sofia region) *St. kraussei* was isolated from soil near the river and pine forest.
Steinernema intermedium (Poinar, 1985)
In our material *St. intermedium* was found in 29 samples from 14 habitats in Vitosha mountain. Like *St. kraussei* it was found in different habitats - from meadows in low mountain belt to subalpine meadows with altitude about 2150 m. Most of the isolates were from beech, considerably less - from oak and pine forests. In subalpine belt in Vitosha *St. intermedium* was found in moist soil near the marshes. In spruce forest this species was not found. By transects sampling from Botanical garden in Varna, this species was found in riverside soil (70m a.s.l.).
Steinernema feltiae (Filipjev, 1934)
During our studies *St. feltiae* was found in 22 samples from 18 biotopes with altitude from 550 to 1850 m. In the region of Vitosha the species was isolated often from meadow soil from beech and lowmountain belt, its frequency of occurrence was lowest in subalpine zone. During the studies of Zemen defile along the river Struma 52 samples from 30 habitats were taken by the transect method. *St. feltiae* was found in 6 of them in all cases in sandy near river soil. The species was isolated also from meadow and near river soil samples from natural reserve "Boatin" (Teteven part of Stara planina). Except from natural ecosystems, *St. feltiae* was isolated from soils of corn agroecosystems near Sofia.
Steinernema affine (Bovien, 1937)
In our materials *St. affine* was isolated from 8 samples from 5 habitats of Vitosha mountain (1000 to 1300m above the sea level). Comparatively more often the species was found in meadow soils from the beech belt. It was rarely isolated from meadows in lowmountain belt and oak - hornbeam forest.
Steinernema carpocapsae (Weiser, 1955)
So far in the regions studied we isolated *St. carpocapsae* only from meadow soils in beech belt from Vitosha mountain (1250m a.s.l.) and from riverside soils near v. Polska Skakavitsa (550m a.s.l.) in Zemen defile.
Steinernema bicornutum (Tallosi, Peters & Ehlers, 1995)
The species is described by materials from Voivodina, Yugoslavia (Tallosi *et al.*, 1995). This work is the first confirmation of the species validity and for its presence in other regions of the Balkan peninsula. *St. bicornutum* was found in 3 samples from 1 habitat along the river Struma, near town of Zemen (550 m a.s.l.) in sandy meadow soil.
Heterorhabditis bacteriophora (Poinar, 1975)
In the regions we studied *Heterorhabditis* was found in 7 habitats with altitude from 70 to 1150 m. The species was isolated from sandy soils from riverside meadows near v. Lozen (Sofia region), Vitosha mountain (above v. Zheleznitsa) and near Zemen. We found the species from pine forest and mixed forest (with pine also) soil (Botanic garden, Varna, pine forest near Razhdavitsa - Zemen defile and agroecosystem near Svishtov), which is in contradiction with the published data about the preference of this species to sandy and moist riverside soils.

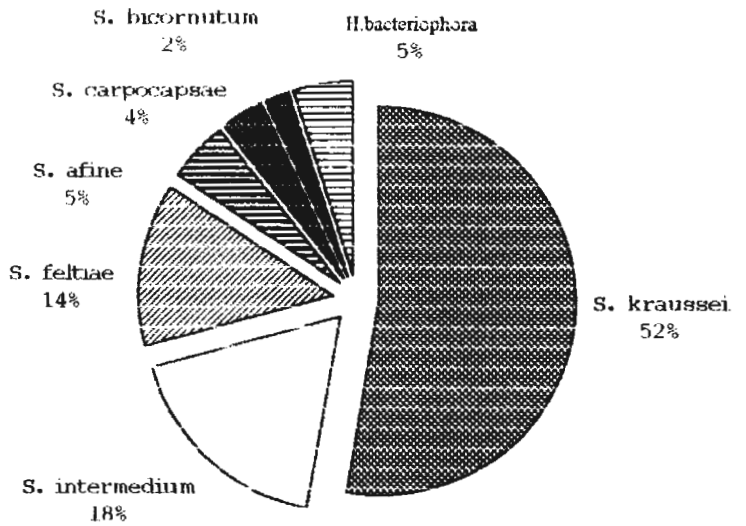

Fig.1. Frequency of occurrence of *Steinernema* and *Heterorhabditis* spp. in Bulgaria

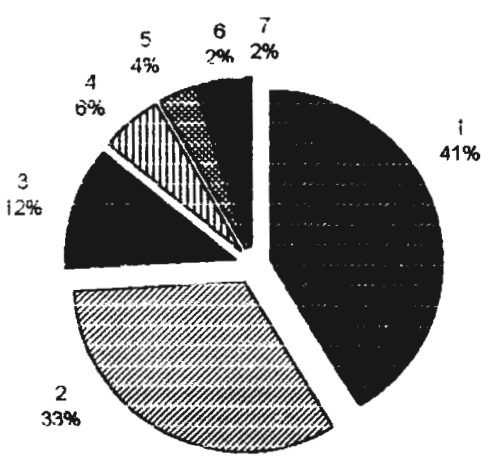

Fig.2. Occurence of S. kraussei

1 subalpine meadows 2. spruce forests 3. meadows up to 1600m 4. oak-hornbeam forests 5. pine forests 6. beech forests 7 riversite biotopes

Frequency of occurrence of so far 7 found species of entomopathogenic nematodes in Bulgaria are given on the Fig. 1. For the region and biotopes studied *St. kraussei* is strongly dominant and was found in 52% of the positive samples. Frequency of occurrence of the rest species is from 18 to 2%, ecading place have *St. intermedium* and *St. feltiae*. Ecological plasticity of *St. kraussei* (fig. 2) is combined with dominant status in almost all natural ecosystems. Among them subalpine meadows with 1800 - 2250m altitude and are preferred habitat for the species, where from the most isolates (41%) are taken. Next are spruce forests (33%) and meadows (up to 1600 m) - 12%. In the rest habitats, presented by different forest and riverside ecosystems frequency of occurrence of the species is lower - 2 - 5%. In the altitude of distribution of *St. kraussei* there are differences in our data and published by Steiner (1993) results from the Swiss Alps. According to his data maximum of the *St. kraussei* frequency of occurrence is at 1800m a.s.l., after which up to 2530 its frequency of occurrence abruptly diminishes. In our studies even at the 2250m (close to the maximum for Vitosha) the species has high frequency of occurrence.

Although initial and in restricted area of the country our studies show considerably high species diversity of entomopathogenic nematodes, most of the species known for Europe were found. The differences in frequency of occurrence of entomopathogenic nematodes in different habitats, probably are result of the influence of edaphic and climatic conditions, as well as the differences in the distribution of the attached to the concrete habitats insect - hosts.

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Nematode community structure in poplar (*Populus alba* L.) forest in eastern Croatia

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Abstract: Nematode community structure was analyzed in poplar forest in Eastern Croatia. Nematodes were analyzed in order to determine dominant feeding group. Sampling was done in September 1997, June 1998 and September 1998, in four replicates, on the depth of 20 cm, by using sonda with internal diameter Ø2 cm. After extraction and analyzing of nematodes greatest absolute abundance was determined in September 1998 (1056 nematodes/100 ccm of soil), and greatest number of genera in June 1998 (30). Trophic structure was dominant by plant feeders in June and September 1998 (44% and 47%, respectively), and by bacterial feeders (51%) in September 1997. The group of predaceous nematodes was less dominant in all three time of sampling (3%, 4% and 5%). The most common predators in all samples was *Mylonchulus* sp. Dominant plant parasitic nematodes was *Paratylenchus* sp. and *Tylenchidae*. Group of omnivores and fungal feeding nematodes also occurred - omnivores ranging from 4-12% in samples and fungal feeders from 4-9%.

Key words: poplar forest, nematodes, trophic groups

Introduction

Nematodes are suitable to reflect soil quality, and for years they have been used to reflect pollution (Zullini, 1976), impact of heavy metals (Sturharn 1986; Korthals, 1997), effect of acidification (Rues & Funke, 1992), effect of different tillage regimes (Ivezic *et al,*. 1998) etc. Nematode community structure was analyzed in poplar forest in Eastern Croatia in order to determine dominant feeding group, and proportion of the taxa in the samples in different time of sampling. The aim was also to determine which predators occurred in poplar forest.

Material and methods

The samples were taken in poplar forest (*Populus albus* L.) in Eastern Croatia, in the area of Osijek - Pampas (45°32"N; 18°44"E), in three different time:
1. September 1997,
2. June, 1998,
3. September 1998.

Samples were taken randomly, using sonda with internal diameter of Ø 2 cm, on the depth of 15 cm, and collected in plastic bags (cca. 1 kg per bag). Samples were taken in four replicates for every samplings period. Extraction of the nematodes (100 ccm of soil per repicate) was done at Laboratory for Entomology and Nematology at Faculty of Agriculture in Osijek, using Seinhorst Erlenmeyer method (Seinhorst, 1956). Identification was done to the genus level.

Chemical analyzes of the soil for 1997 and 1998 is presented in table 1.

Table 1. Chemical analyses of the soil in poplar forest.

Year	pH(H_20)	pH(KCl)	% humus	AL-P_20_5	AL- K_20
1997	7,80	6,75	2,62	32,27	15,08
1998	7,87	6,77	2,64	14,22	17,25

Results and discussion

Total number of nematodes was determined for September 1997 - 824 nematodes; June 1998 - 961 and for September 1998 - 1056 nematodes per 100 ccm of soil (average of four replicates). The number of genera was 26, 30 and 28 , respectively (Fig. 1).

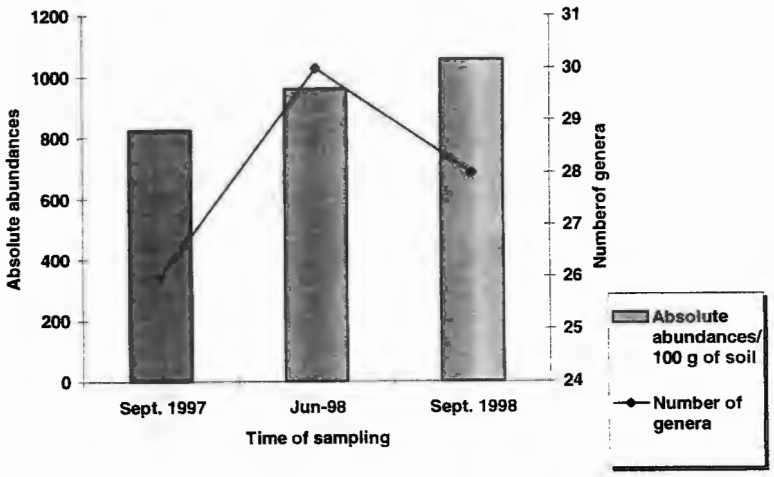

Figure 1. Absolute abundances of nematodes and number of genera which occurred in treatments.

Trophic structure were dominant by bacterial feeders in 1997 (fig. 2), while in 1988 in June and September, dominant taxa was nematodes which are plant parasitic (fig. 3 and 4).
The most common plant feeders in all treatments was *Pratylenchus* sp. and nematodes belonging to family Tylenchidae *(Tylenchus* a).
The group of predators was the less dominant in all treatments (3%, 4% and 5% respectively), with the most dominant predator in all samples - *Mylonchudus* spp.

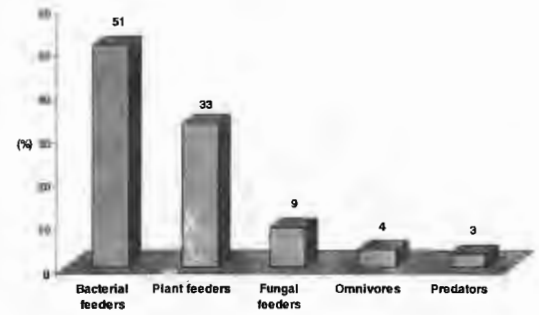

Figure 2. Trophic structure in September 1997.

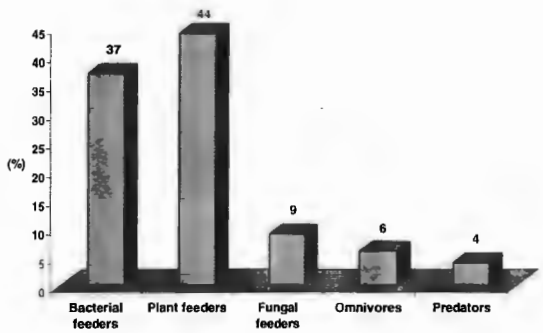

Figure 3. Trophic structure in June 1998.

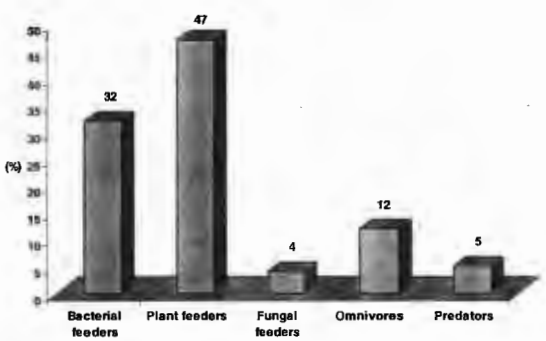

Figure 4. Thropic structure in September 1988.

The list of genera that occurred in poplar forest in Eastern Croatia are presented in table 2.

Genera			
Acrobeles	*Cylindrolaimus*	*Metateratocephalus*	*Prionchulus*
Acrobeloides	*Diploscapter*	*Monhystera*	*Prismatolaimus*
Acrolobus	*Dipterophora*	*Mylonchulus*	*Prodorylaimus*
Alaimus	*Discolaimus*	*Nygolaimus*	*Pungentus*
Aphelenchoides	*Ditylenchus*	*Panagrelus*	*Rhabditis*
Aphelenchus	*Drilocephalobus*	*Panagrobelus*	*Rhabdolaimus*
Aporcelaimellus	*Enchodelus*	*Panagrolaimus*	*Rotylenchus*
Cephalobus	*Eudorylaimus*	*Paramphidelus*	*Steinernema*
Cervidelus	*Gracilacus*	*Paratylenchus*	*Trichodorus*
Chiloplacus	*Helicotylenchus*	*Paraxonchium*	*Tylenchus* a
Chrisonemoides	*Heterocephalobus*	*Paraphanolaimus*	*Tylenchus* b
Chronogaster	*Heterodera*	*Pelloiditis*	*Tylencholaimellus*
Clarkus	*Labronema*	*Plectus*	*Tylolaimophorus*
Criconema	*Mesodorylaimus*	*Pratylenchus*	*Wilsonema*

Table 2. The list of genera occurred in poplar forest in Eastern Croatia.

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The potential use of entomopathogenic nematodes to control ticks

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Ticks and mainly tick-borne diseases remain a major factor in limiting the economic development of animal husbandry in many tropical and subtropical countries. Methods of controlling ticks are based almost only on topical applications of acaricides. Very little attention was paid as yet to biocontrol of ticks as compared to biocontrol of plant pests. Non insect arthropods were not regarded to be susceptible to entomopathogenic nematodes but Samish & Glazer found (1991) that entomopathogenic nematodes can kill ticks even though they do not go through their normal propagation cycle within tick cadavers.

The tick *Boophilus annulatus,* a vector of cattle babesiosis, was found to be far more susceptible to entomopathogenic nematodes than *Hyalomma excavatum, Rhipicephalus bursa* or *R. sanguineus.* Ticks seem to be resistant to nematodes while feeding on a host . Preimaginal tick stages were less susceptible to nematodes than adult ticks. Unfed females died most quickly, unfed males a bit slower and engorged females were the slowest to die. In Petri dish tests the LD50 and LT50 for engorged *B. annulatus* females was as low as 15 nematodes/dish and 0.8 days respectively . The virulence of nematodes to ticks varied highly according to nematode strain. In most cases the *Heterorhabditis* sp. IS-3 and IS-5 strains were the most virulent among 9 strains tested in petri dishes. In buckets containing sandy soil sprayed with 50 nematodes/cm 2 and engorged *B. anuulatus* females the LT_{50} of the ticks was less than 5 days. Adding manure to the soil or a manure extract to petri dishes reduced nematode virulence.

As ticks spend most of their life cycle in the upper humid layer of the ground and as many nematode strains share this same ecological niche their possible use for biocontrol of ticks seems to be promising.

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An image analysis of *Spodoptera littoralis* feeding behavior following ingestion of the insecticidal nematode *Steinernema riobrave* in an alginate gel carrier

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Abstract: The feeding behavior of *Spodoptera littoralis* larvae prefed with the insecticidal nematode *Steinernema riobrave,* was studied. The nematodes were embedded in the alginate gel that was addible-to-insects and offered to the larvae with leaves of castor-oil plants. The nematodes caused 100% mortality in the larvae after 48 h and leaf consumption was minimal. In the controls consisting of a leaf and an alginate gel without nematodes or the leaf without the gel, the larvae damaged the leaves and gained weight. The feeding rhythm of the larvae ingesting the nematodes in the alginate gel was evaluated with an image analysis system at a real time. 6th instar Larvae were starved overnight at 17°C, transferred singly to a 5 cm Petri dish and fed for 5 h on the nematode-gel product or on controls of gels without nematodes. Then, the gel products were removed and each of the larvae was exposed singly for 16 h to a cotton leaf to record defoliation by means of an image analysis system. The leaves were fixed in the dish above the larvae. In this way, the larvae were forced to feed on the leaf underside to avoid undesired imaging "noises". Image processing algorithms were developed to segment the leaf from the background of the arena. The imaging system included a black and white CCD video camera placed above an arena of 12 larvae in an illuminated chamber. The camera was connected to a frame grabber installed in a personal computer The defoliated leaf area was recorded as a function of time. The nematode-gel feeding as compared with the control, reduced the meal size and number of meals per larva by a half after 3-4 h and to a fourth after 20 h. The larvae fed on the nematode-gel preparation died after 48 h and nematode counts in the dead larvae were made after 24 h. The image analysis can be used to study the effects of the insecticidal nematode on the feeding behavior of the host during the time between nematode ingestion and mortality and to evaluate the effectiveness of the nematodes in biocontrol programs.

Keywords: Video image analysis, feeding rhythms, entomopathogenic nematodes, *Steinernema riobrave,* alginate gel carrier, *Spodoptera littoralis* larvae

Introduction

The effectiveness of entomopathogenic nematodes in pest control has been shown so far mostly against soil inhabiting pests (Kaya, 1990). Recently, a new hydrophilic carrier for these nematodes based on an alginate gel was developed (Navon *et al.,* 1998). In this carrier, the nematodes are embedded in a gel that was made edible to insects and showed promising activity against *Spodoptera littoralis* larvae. The purpose of developing this gel was to use it for managing defoliators on the plant.

To evaluate the potential of the nematodes in the gel as an on-foliar formulation, we studies the feeding behavior and rhythms of larvae prefed with the nematodes in this carrier. So far, insect feeding behavior was measured by nutritional indexing of feeding consumption and utilization (Waldbauer, 1968), mechanical and electronic monitoring of feeding rhythm

(Lance *et al.*, 1986) and mandible function (Navon *et al.*, 1992). Therefore, we developed a computerized video-image analysis system to describe the defoliation rhythms in larvae affected by *S. riobrave* infective juveniles.

Materials and methods

Automatic recording of feeding

The feeding behavior rhythm of the insect larvae was evaluated by recording the level of defoliation as a function of time. This recording was made by an image acquisition and analysis system. The system consisted of : 1 image acquisition and 2. image processing and analysis.

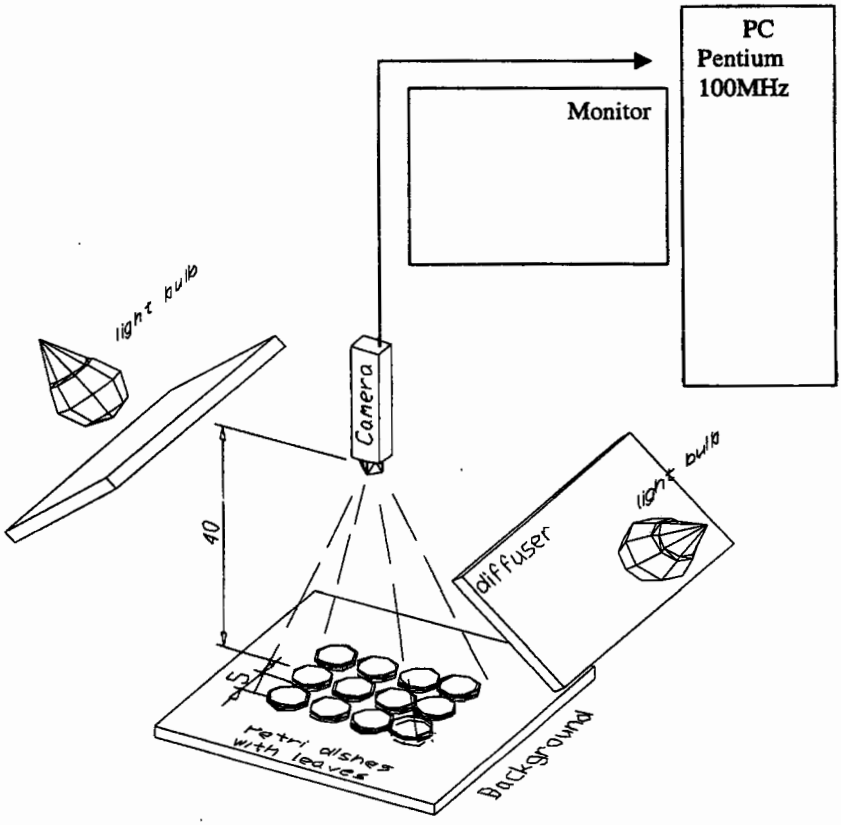

Fig. 1. Schematic description of a video-image system for measuring insect feeding behavior

1. Image acquisition

IA. illuminated cage with a feeding arena

The light was produced by two 150W halogen bulbs fixed at opposite sides of the cage, 20 cm from the cage side. The light was diffused through a plastic window. The illumination was constant throughout the experimental time. The cage arena sized 70 X 70 cm, had a black background on which twelve 5 cm diam. Petri dishes were placed. Each dish contained a leaf and a larva (Fig. 1).

1B. video camera

A black and white CCD camera (TM657, Pulnix Inc., CITY, USA) was used. The IR cutoff filter was removed from the camera in order to increase the camera sensitivity to wavelengths beyond the visible range (700-1000nm). The sensor of the camera was a 2/3" CCD with 768x584 pixels. The video camera exposure time was set to 1/50 sec. The camera was equipped with a 12.5-75mm f1.8-22 C-mount zoom lens with a macro feature. It was placed 0.4m above the arena. The lenses were operated in the macro feature so that all 12 Petri dishes, arranged in 3 rows of 4 dishes, were included in the camera view field (250x 185 mm). The lens aperture was set on f5.6 so that the contrast between the leaves and the black background was maximized, without causing saturation of the CCD sensor. Leaves showed high reflectance in the band of chlorophyll reflectance (740 nm), and therefore, by removing the IR cut-off filter from the camera, they appeared with high bright contrast against the black background.

1C. personal computer equipped with a frame grabber.

The images produced by the camera and grabbed with a frame grabber board (IVP150, Bargold Inc., Haifa, Israel) were installed in a personal computer with a Pentium 100MHz processor and Microsoft Windows 95 operating system. The frame grabber digitized the video signal with a resolution of 512x512 pixels. With a field view of 250x 185 m, the spatial resolution of the imaging system was 0.49 mm/pixel in X direction and 0.36 mm/pixel in Y direction, yielding an area of 0.176 mm^2 / pixel.

2. Image analysis and processing

The acquired images were divided to 12 sections. Each section included one Petri dish and was analyzed and calculated for each image individually. Image processing algorithms were developed to subtract the leaf area from the background, counting the number of leaf pixels in each section and storing the results on the disk. In this way, the difference between the detected leaf area at each image and the reference image of the unconsumed leaf, equaled to the leaf area consumed by the larvae at a real time. Feeding periods were in the order of minutes and inter-meal periods lasted tens of minutes. Therefore, sampling times with the camera was set on 30 seconds (Fig. 2)

Experimental work Nematode colony

The entomopathogenic nematodes *Steinemema riobrave* was reared on the last instar of the greater wax moth *(Galleria mellonella)* at 25° C according to the methods of Woodring and Kaya (1988). Nematodes were stored in distilled water at 10°C for 7-14 days and then allowed to acclimate at ambient temperature (23-25°C) for 24 h before use.

Insect colony

The insect colony of *Spodoptera littoralis* was maintained for the last 4 years on a premix diet based on the soybean-wheat germ diet "Manduca-premix-Heliothis Premix" (Stonefly Industries, Inc, Bryan, TX 77805, USA) . The colony was maintained in a rearing room at 25±2°C, 16:8 L:D photophase and 60-80% RH.

The nematode-alginate gel preparation

The edible-to-insects gel with the nematodes was prepared according to Navon *et al.*, (1998). In this preparation, the nematodes were embedded in the gel matrix. Yeast extract was used in the gel as a phagostimulant for the larvae. The nematodes concentration was 500 nematodes /ml gel. They were homogeneously dispersed before gel setting. The same procedure was used also for producing the gel without nematodes. Inside the gel the nematodes were immobilized by the colloid texture. Nematode counts in the gel after 5 hours at room temperature The mean count of live nematodes in the gel after preparation was 406± 42 SE / ml, lower than the theoretical count of 500/ml due to some mortality and migration of the IJ from the gel surface.

Defoliation bioassays with larvae fed entomopathogenic nematodes

Mature larvae weighing 290-345 mg were selected from the insect colony. Six larvae were placed singly in a 5 cm Petri dish with a 2 g cube of the alginate gel with the nematodes for 5 h. In the control, 6 larvae were fed with the gel without nematodes. Following the nematode-gel feeding the gel was removed and the larvae were offered leaves of cotton (Acala SJ-2) raised in Bet Dagan farm. The leaf was pressed to the dish bottom part with the lid so that the larvae were forced to defoliate the leaf from bellow. The 12 petri dish were put on the cage arena (Fig. 1), and the video recording was started immediately and was stopped after 20-24 h. The larvae died 48 h after the start of the nematode-gel feeding. Nematode count was made in the dead larvae by macerating the larval body with pincers in sterile water. The mean nematode count was 10.1± 1.7 SE per larva.

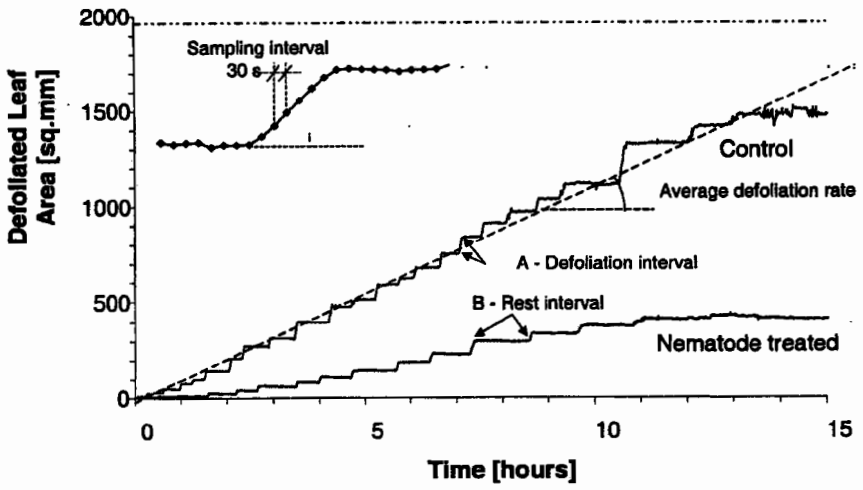

Fig. 2. Feeding behavior pattern of S. littoralis larvae prefed with the insecticidal nematode *S. riobrave* in a alginate gel carrier

Results and discussion

Figure 2 shows that that in the leaf area defoliated by the nematode-fed larvae after 10 h was about 250 mm^2 , about one fourth of the leaf area consumed by the control larvae. Also, the inter-meal periods in the nematode-fed larvae have been doubled in comparison with the control insects, and the meal sizes were smaller. The feeding rhythm of the larvae on the cotton leaf is shown in Figure 3.

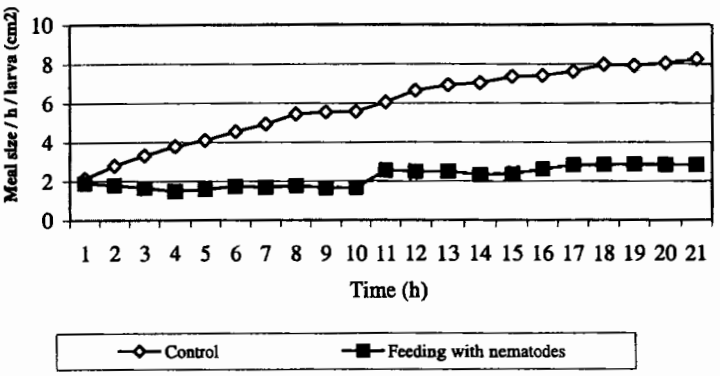

Fig. 3. Leaf feeding rhythm of *S. littoralis* larvae prefed with *S. riobrave* in an alginate gel carrier

The nematodes caused the larvae a reduction of 2-3 times in the meal size/ h between 0-21 h, as compared with the control larvae. This difference in meal size/ h suggests that the nematodes and their bacteriae substantially reduced larval capacity to feed on the leaf, and this effect was further increased until the larvae died as a result of the insecticidal effect of the nematode. The feeding reduction caused by the nematodes indicates that probably crop damage would be saved during the pre-mortal period of the larvae. One of the main aims in using biological pest management by means of entomopathogenic microbes and nematodes is that insect damage to the plant from the time of the microbe/nematode ingestion until its insect mortality would be minimal or nil. Larval intoxication with *Bacillus thuringiensis* has been characterized by a negative relative growth rate after the ingestion of the spore-crystal preparation (Navon *et al.*, 1992), and this effect in young larvae avoided crop damage. The present study showed that dietary *S. riobrave* in an edible alginate gel carrier substantially reduced leaf consumption in mature larvae by reducing meal size and frequencies. In future work, the mode of action of insecticidal nematodes and their bacteriae that lead to the pathological feeding effects , will be elucidated.

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PCR for identifying the entomopathogenic nematode *Steinernema carpocapsae* from overwintering codling moth larvae in Bulgaria.

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In an attempt to find natural enemies against the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), a serious pest of apples in the United States, the European Biological Control Laboratory (EBCL) has been conducting foreign explorations for natural enemies in different countries. Cardboard bands that had been placed around the trunk of apple trees in four locations in Bulgaria and containing overwintering stages of *C. pomonella* were brought back to the quarantine facility at EBCL. Several specimens were infected with nematodes. PCR analysis using Reid's *et al.* method (1997) revealed that banding patterns obtained were consistent with those of *Steinernema carpocapsae* Weiser. Subsequent taxonomic analysis based on morphological features confirmed the PCR results. *S. carpocapsae* has been previously reported infecting *C. pomonella* in Italy, Mexico, the United States, and the Czech Republic. This is the first report of *S. carpocapsae* infecting *C. pomonella* in Bulgaria. Tests are currently being conducted (by L. Lacey) to determine cold tolerance of this Bulgarian strain.

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The role and potential of secondary metabolities of the bacterial symbionts

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The remarkable diversity of organisms that coexist in the soil interact with each other in diverse ways. The chemistry of this interaction plays a major role in such areas as defence systems, food/host and mate location and symbiosis. The secondary metabolites produced by organisms are key communication conduits in this multitude of interactions. Several thousands of them have been identified many of which are derived from plants and bacteria, including some from the marine environment.

These secondary metabolites frequently are derived from a few basic compounds such as the polypeptides, phenylpropanoids and some amino acids. A remarkable number, as well as having evolved significant biological roles for the organisms that produced them, also have potentially useful pharmaceutical use. Some secondary metabolites from plants are used for medicinal purposes, such as morphine, quinine, digitoxin and taxol. Plants have been used as a method of treating diseases and medical ailments for thousands of years, and this knowledge has provided a useful lead when investigating the possible source of secondary metabolites as potential drugs. Bacteria, however, rarely provide such obvious leads to the potential value of their secondary products. However, the fact that entomopathogenic nematode-infected insect cadavers do not putrefy during the nematodes' "gourmet feast" has provided scientists with such a lead that has been followed up by several research groups.

The bacterial symbionts, *Xenorhabdus* spp. and *Photorhabdus* spp. of the nematodes *Steinernema* spp. and *Heterorhabditis* spp., respectively, produce an array of secondary metabolites that have antimycotic and antibiotic properties (Paul *et al.,* 1981; Boemare *et al.,* 1983; McInerney *et al.,* 1991). However, in nature these symbiotic bacteria are metabolically active only within an insect host.

Among these secondary metabolites the indole derivatives probably are produced via tryptophan, and nematophin is most likely derived from tryptophan and isoleucine. The stilbenes are presumably produced through the polyketide pathway and xenorxides are oxidized xenorhabdins. There are also some stilbenes and a great variety of anthraquinone derivatives produced, especially by *Photorhabdus* spp., under both *in vivo* and *in vitro* conditions. This is somewhat unusual as these substances are not common secondary metabolites of bacteria, though they occur frequently in plants and fungi.

Under what conditions are these secondary metabolites produced and are the products the same from *in vivo* and *in vitro* culture? We examined in particular the products of a strain of *P. luminescens,* the symbiont of *H. megidis.*

The isolation and purification of each metabolite from insect cadavers is complicated by the presence of a variety of impurities (Li *et al.,* 1998). Therefore, several chromatographic methods were used. The initial acetone extraction of substances from the macerated nematode - bacterium infected *Galleria mellonella* larvae was first re-extracted with methanol to remove the methanol-soluble impurities and then concentrated to a dark brown, oil-like mixture that was separated with flash silica gel chromatography using hexane and ether (Hu *et al.,* 1997).

An array of closely related pigments are produced by *P. luminescens* that vary in the positioning and/or number of hydroxyl and methoxyl groups around the central anthraquinone ring structure. The final identity and location of these groups was determined using MS, H-NMR analyses and demonstrated NOE effects.

There were two major groups of secondary metabolites, namely stilbene derivatives and anthraquinone derivatives, isolated from organic extracts of the bacteria in *G. mellonella* culture. All the identified metabolites produced in either 2- or 4-day tryptic soy broth culture were present also in the extract from nematode-bacterium infected *G. mellonella* larvae. However, there were two additional metabolites in the *in vivo* extracts that were not present in the extracts from the *in vitro* culture broths, namely the pigments 1-hydroxy-2, 6, 8-trimethoxy-9, 10- anthraquinone and 1, 4-dihydroxy -2,5-dimethoxy-9, 10-anthraquinone. As well, there was an unidentified red pigment (AQ) and an unidentified antibiotic (AT) that has not been previously reported. It appears, therefore, that from *in vivo* cultures of *P. luminescens* a total of seven distinct compounds have been identified by different researchers (Hu, *et al.,* 1998).

The precise reason for the difference in the metabolite composition between the *in vivo* and *in vitro* extracts is elusive though differences between the available nutrients, and in the prevailing environmental conditions, undoubtedly have a significant affect. The presence or absence of the nematode, *H. megidis,* was probably not a factor at the stage of the bacterial culture when the extracts were isolated. The relative composition of the metabolites was not different in experiments in which extracts were taken from bacteria infected *G. mellonella* cadavers that were or were not also infected also with the nematode symbiont.

Xenorhabdus and *Photorhabdus* produce several secondary metabolites that have antibiotic properties, but, generally, there is less diversity among the types of antibiotic substances produced by *Photorhabdus* spp. than by *Xenorhabdus* species. These antibiotics are most commonly active against Gram positive bacteria but there are some that are effective against Gram negative bacteria, even against species closely related to *Xenorhabdus.* There is evidence that some of these antibiotic compounds have significant potential value as pharmaceutical drugs (Webster *et al.,* 1998).

These antibiotic-type secondary metabolites occur in the infected *G. mellonella* larvae and in the bacterial culture broth within 24 h of inoculation, and the rapid increase in the quantities of these metabolites parallels closely the growth curve of the bacteria. By 96 h the levels have usually plateaued at a relatively high and the amount of active antibiotic compound per cadaver could be as high as 3-4 mg/g of wet insect tissue (Hu *et al.,* 1997). However, these secondary metabolites with antibiotic activity which are in the organic and aqueous components of the extract, show variation in concentration of some of the constituent metabolites over the 10-day incubation period of some bacterial species.

It is reasonable to speculate that a primary role has evolved for many of these secondary metabolites in the prevention or significant diminution of bacterial and fungal putrification of the insect after its death and before bacterial disintegration of the insect tissues has commenced. The integrity of the cuticle of the insect cadaver, as a major barrier to external saprophytes, appears to be an important component of this natural "fermentation chamber". Chitinase that is produced by the bacterial symbionts does not appear to be in sufficient concentration to break down the cuticle for at least 10 days, but may be a reason for the softness of the cuticle of the nematode-infected cadaver (Chen *et al.,* 1996). The cadaver is vulnerable to saprophytic bacteria and fungi that enter through the natural openings of the cadaver, and an array of opportunistic saprophytes that are released by breakdown of the insect's intestinal wall. It is reasonable to suppose, therefore, that there is significant competition for resources in the nutrient-rich cadaver from bacteria and fungi as well as from

several species of nematode that feed directly on the bacterial symbionts. Consequently, the collective evolutionary pressures of the alien microflora and fauna have resulted in the secondary metabolites of the symbionts acquiring antibiotic, antimycotic and nematicidal roles.

Once the cadaver bursts open to release the large numbers of infective juveniles and bacterial symbionts significant quantities of the antibiotics and nematicidal substances are released into the surrounding soil. These may, temporarily, diminish soil populations of bacteria and fungi and paralyse nematodes in the immediate vicinity. Although these organisms are not direct competitors with entomopathogenic nematodes in the soil such a changed level of the soil populations of these organisms may be significant in the rhizosphere of plants where the host insects of the entomopathogenic nematodes commonly occur. Such rapid, though temporary, influxes of these secondary metabolites from the bacterial symbionts could cause localized and rapid changes in rhizosphere microflora. It is in these regions of root growth where insect pests are attracted to the roots and where the entomopathogenic nematodes are attracted to their potential host insects.

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Insecticidal toxins from the bacterium *Photorhabdus luminescens:* gene cloning and toxin histopathology

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Photorhabdus luminescens (Enterobacteriaceae) inhabits the gut of entomopathogenic nematodes of the family Heterorhabditidae (1). Following invasion of an insect by the nematode, P. *luminescens* are released into the insect haemocoel. The bacteria and nematodes then replicate within the insect cadaver (2). At this stage the bacteria emit light causing the cadaver to glow. After several rounds of reproduction in which the nematodes feed off both the bacteria and the insect carcass, infective juveniles emerge to colonise new hosts.

P. luminescens can be readily cultured away from its host and the a few bacterial cells can kill a single insect (3). The work of others had suggested that insecticidal activity was associated with a range of different compounds including proteases, lipases and lipopolysaccharides (4-7). However, previous purification work had shown that insecticidal activity was associated with the high molecular weight fraction of the culture broth (8). Following further purification and a final high performance liquid chromatography (HPLC) step, four high molecular weight toxin complexes can be resolved from the orally toxic fraction: termed toxin complexes A, B, C and D (or Tca, Tcb, Tcc and Tcd). Individual toxin complexes migrate as single (or double) components on native gels, but can each be resolved into a number of different polypeptides by SDS-PAGE (9).

In order to further characterise the composition of each of the toxin complexes we raised both a polyclonal and a monoclonal antisera against the high molecular weight toxin fraction which contains all four toxin complexes. We then screened a *P. luminescens* genomic library with both antisera. The antisera recognised clones expressing components of four different *toxin complex (tc)* encoding loci: termed *tca, tcb, tcc* and *tcd.* Comparison of N-terminal protein sequences derived from purified polypeptides in the native broth with the predicted amino acid sequences of the *tc* loci, confirmed that *tca, tcb, tcc* and *tcd* encode the proteins *Tca, Tcb, Tcc* and *Tcd* respectively. The sequences of these genes have been reported elsewhere (9). The predicted amino acid sequences of the four *tc* loci have little, if any, similarity to other known protein toxins. However, short stretches of both *Tca* and *Tcc* share similarity with *Salmonella* plasmid virulence factors B and A respectively (termed spvB and spvA). These virulence factors are responsible for the ability of certain *Salmonella* strains to replicate in monocyte derived macrophages, and suggest a possible role for the *P. luminescens* homologs in overcoming insect haemocytes.

Despite our ability to reconstitute antigenicity, the toxin complexes are not exported from E. coli and the pattern of apparent protease cleavage seen in the P. *luminescens* broth is also not reproduced. Therefore in order to confirm the nature of these complexes as orally active toxins we carried out two approaches. Firstly, we purified sufficient quantities of *Tca* to perfrom LD_{50} determinations on neonate *Manduca sexta* exposed to toxin added topically to artificial diet. *Tca* is orally active in the ng/cm^2 which is equivalent to some *Bacillus thuringiensis* δ -endotoxins (10). Secondly we knocked out each of the *tc* loci in the same strain of *P. luminescens* (strain W 14) and then tested the effect of the mutant bacterial broths

in our oral bioassay. Deletion of either *tca* or *tcd* individually (as *tca-* or *tcd-* mutant strains) greatly reduced the oral toxicity of the broth to *M. sexta.* Whereas deletion of both *tca* and *tcd* together (in the *tca⁻ / tcd⁻* double mutant) eliminated oral toxicity altogether. These results suggest that both *Tca* and *Tcd* are involved in oral toxicity to Lepidoptera. However, we have been unable to purify sufficient quantities of *Tcd* to perform an LD50 determination.

In order to examine the effects of *Tca* on the lepidopteran gut and compare it to that previously documented for both the *B. thuringiensis* δ -endotoxins and Vegetative insecticidal proteins (Vips), and for cholesterol oxidase (11-15), we sectioned *M. sexta* neonates at intervals after oral ingestion of toxin. After several hours toxin treated midguts showed an accelerated rate of epithelial blebbing. This blebbing of the midgut epithelium into the lumen continues until the basement membrane is exposed and the epithelium is essentially destroyed. Both the columnar cells and the goblet cells appear to be attacked. Interestingly, a similar histopathology can be observed following injection of *Tca* directly into the insect haemocoel, which is presumably the normal route of delivery of the toxin by the bacterium (16).

In conclusion, we have purified four toxin complexes from the culture broth of *P. luminescens* and cloned the four toxin complex encoding loci. Genetic knockout of either *tca* or *tcd* reduces oral toxicity to *M. sexta* and knockout of both loci eliminates activity. Purified Tca shows effects specifically on the insect midgut, despite its putative normal delivery directly into the insect haemocoel. These *Photorhabdus* toxins (Pht's) may form useful alternatives to other orally active bacterial protein toxins such as those from *B. thuringiensis* (Bt).

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Cloning and expression of insecticidal toxin genes from *Xenorhabdus* species

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One of the most successfully exploited microorganisms used to control insect pest species is the bacterium *Bacillus thuringiensis.* The delta endotoxins produced during sporulation have variable activity against mainly Dipteran, Lepidopteran and Coleopteran insects. The recent use of *B. thuringiensis* on such a large scale has led to the development of insect populations resistant to some *B. thuringiensis* toxins and the use of transgenic plants with delta endotoxin genes may increase this. Therefore, a new drive to identify novel insecticidal toxins, particularly protein toxins that are capable of controlling insects has been initiated.

In an attempt to find new insecticidal toxins we have identified a number of insect active bacterial strains, originally isolated from insect parasitic nematodes (IPNs), belonging to the two genera *Photorhabdus* and *Xenorhabdus.* One isolate, *X. nematophilus* was shown to have activity against some pest species of Lepidoptera and mosquitoes. The insecticidal activity from this strain was shown to have a high molecular weight, to be heat sensitive and digested by certain proteases, therefore the toxin was believed to have a proteinaceous structure. To enable the entomocidal activity to be transferred to other microorganisms and plants for the control of insects, the structure of the protein toxin and an understanding of its expression is needed. A 40 Kb region of DNA from *X. nematophilus* PMF1296 that encoded proteins with insecticidal activity was cloned and sequenced. The identification of sequences important for toxicity was made by transposon mutagenesis. A single gene on the cosmid, *xnptA* was cloned and expressed in *E. coli* and shown to have insecticidal activity.

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Quality control of entomopathogenic nematodes

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Report on Workshop part II

Based on a intensive workshop at Merelbeke, Belgium 1998, the second part of discussions took place at the workshop here in Vienna. It is the aim of the working group two of COST Action 819 "Application and formulation" to structure this difficult task "Quality control and quality management of entomopathogenic nematodes". It is everybody's opinion, that it is essential to search for collaboration between scientists from different laboratories, producers of the industrial companies and persons from extension services. Everybody who is dealing with entomopathogenic nematodes is urgently forced to raise a monitoring system to check for the quality. Both organisms, such as nematodes and their symbiotic bacteria, that will be used for experimental purposes or as inoculum for production or as product for practical application should be of good quality.
The main question is; How to define a good quality nematode?

The quality control of a product based on entomopathogenic nematodes (EPN) is not a simple matter but it is the result of complex interactions among living organisms such as bacteria, nematodes, insects and human operators. Today we have no standard universal assays for evaluation of nematode quality or efficacy, although EPN's are listed among the important microbial control agents. Ideally, such assay should be simple, quick and sensitive. It should also reflect and correlate in a laboratory assay the behaviour under field condition in practical application.

In a basic part, four subgroups have been built to discuss on different aspects of quality control, were we have tried to find some consensus: Infectivity tests, viability and counting accuracy, evaluation of methods for prediction of pathogenity and shelf life, field and semi-field bioassays. Additionally there were several talk showing results and experiences in finding a specific assays to describe certain aspects of quality. Quality of insect parasitic nematodes includes the ability of the infective juveniles (IJs) to move and to locate, invade and kill the host insect. Also physical and biological factors like viability, visual food reserve, ensheathment, activity, waves/minutes, length of the infective juvenile (IJ), dryweight, triglycerid content, glycogen content, number of bacteria/IJ, penetration, migration and infectiousness were assessed and both related to one another and to efficacy, tested against black vine weevil larvae in climate room experiments. Of big interest was the collection of the protocols, that industrial companies and research groups have presented. Based on these discussions, guidelines and standards for quality control of EPN-products in semi-field trials have been developed. A proposition for a insect based quality assessment of entomopathogenic nematodes with standardised procedure has been presented and discussed.

This second meeting on structuring and developing commonly accepted and standardised guidelines for the quality control of EPN's was successful and appreciated by both sides, industry and research. It was agreed, that the results of the discussions, the protocols and definitions, as well the guidelines that have been developed by WG2 should be published in a handbook as proceedings for quality control of entomopathogenic nematodes.

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Estimation of errors in nematode counts from suspensions

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Abstract: With a series of nematode counts from suspensions could be shown, that in a series of counts the variance is equal to the mean. This facilitates the calculation of errors, because the statistics of the Poisson distribution can be applied to all counting. Approximate equations are given for calculating the confidence limits with 95% probability for every count. A consequence of the Poisson dependence is, that the same accuracy is gained with counting many samples or counting once a big sample. The sum of counted nematodes defines at the same time the mean and the variance, respectively the standard deviation as the square root of the variance. A summation of errors from pipetting and liquid handling can be neglected, as long as relative results between nematode solution are compared. Absolute estimation of nematode numbers have to incorporate inaccuracy of the used instruments. This could be done by including the tolerance value given by a calibration.

Keywords: nematodes, counting, Poisson distribution, confidence limits

Introduction

It is a general observation, that events occurring randomly in a continuum of time or space follow certain rules. If these events are counted from same units, they deviate around the average of counts. In most cases the probability of a certain count event can almost perfectly be predicted by the Poisson distribution (also Poisson series). That the probabilities predicted by the Poisson distribution are valid for samples of suspended nematodes too, will be demonstrated by analysing series of repeated counts.

Material and Methods

A suspension of nematodes was adjusted to three different concentrations (0.4, 1 and 2.5 ne/µl). From each concentration 30 samples were taken to reach an average count number around 10, 30, 80 and 200 nematodes. For 2.5 ne/µl the counting was repeated, so that 16 count series could be analysed regarding their expected value (mean) and variance (squared standard deviation).

Results

The mean and the variance were calculated for each series of counts and plotted against each other (Figure 1). Despite the different nematode concentrations in the solution, no effect on variance could be detected for equal count numbers. A linear trend function was fitted trough the values with the equation $VAR(ne) = a + E(ne)*b$. The parameter $a = -2.39$ and $b = 1.30$ were found with a high correlation coefficient $r^2 = 0.92$. As a was close to 0 and b close to 1, the variance can be estimated by the mean of the nematode counts.

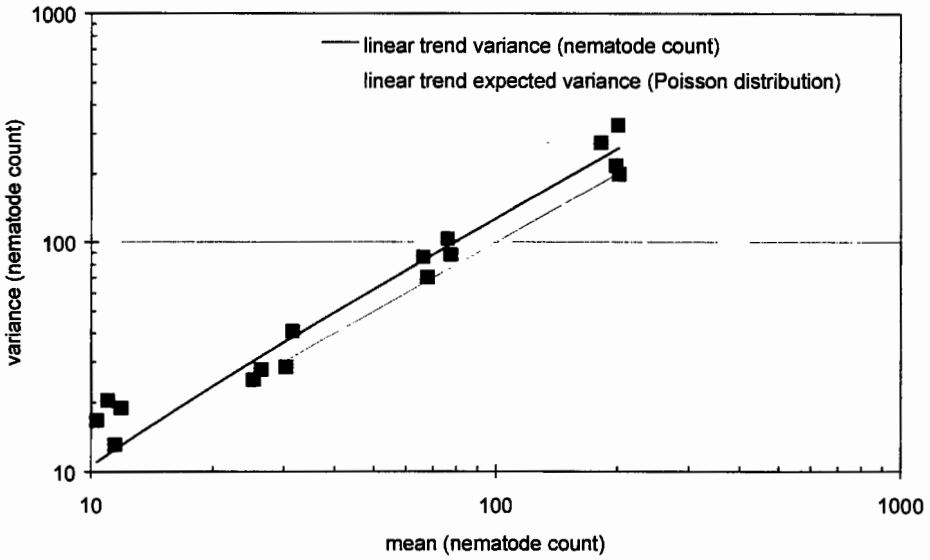

Figure 1. Variance and mean of 16 nematode count series plotted on a log/log scale. Each series consist of 30 counted samples taken from a solution with suspended nematodes. For each solution samples of 4 different volumes were compared to show the linear dependance of the variance from the mean according to the Poisson distribution.

The same dependence between variance and expected value exists in the Poisson distribution. Because the variance depends on the expected value, it has not to be calculated through the deviations of the single counts from the expected value. A known variance or standard deviation as the square root of the variance is a big advantage and should be considered for all estimation of errors.

Discussion

The characteristics of the Poisson distribution are shown in Equation 1:

$$1)\quad P(X=x) = \frac{\lambda^x}{x!}e^{-\lambda}$$

Expected value $E(x) = \lambda = \mu = \bar{x}$

Variance $VAR(x) = \lambda = \sigma^2 = sd^2$

The estimation of errors in nematode counts should base on confidence limits for the Poisson distribution. The confidence limits of the mean for 95% probability can approximately be calculated with the Equation 2 and 3 (Stahel, 1995). Tables with exact limits for small numbers can be found in some statistic books (Pearson & Hartley, 1966, Table 40).

$$2)\quad \lambda \approx x+2 \pm 2\sqrt{x+1} \quad \text{(for count numbers} < 31\text{)}$$

$$3)\quad \lambda \approx x \pm 2\sqrt{x} \quad \text{(for count numbers} > 30\text{)}$$

Note that x is the total number of counted nematodes and not the mean of the counts. As predicted by the Poisson distribution, x is alone responsible for the accuracy of the counting. You gain no additional accuracy from repetitions and optimal accuracy can be reached with the first counting. If you count small volumes in several steps, you end up with same accuracy as counting once the whole volume.

For most cases sufficient accuracy can be reached with a count of 200 nematodes. A counting procedure minimising the steps can be done by the following way. If the first count doesn't reach the desired end number, you continue counting with a second sample with the optimal volume calculated from the first counting. If you divide your count in two or more steps you can also check your single counts for additional errors. If the nematodes were not well mixed in the suspension, the confidence limits of the counts wouldn't overlap. You would also detect extreme volume errors or errors in counting by comparing the confidence limits for single counts. An alternative way for checking nematodes counts would be testing the "goodness-of-fit" for the Poisson expectations "by χ^2 (Elliott, 1977).

The nematode concentration of a solution is the proportion of the summations of all nematode counts and all volumes counted. Keep in mind that a calculation of the mean from several samples is not necessary and you are free to take different volume samples for each count. Suspect a calculation, if one count falls extremely out of the range predicted by the Poisson distribution. The best solution would be to discard this data and to repeat the counting procedure with 2 new samples.

For comparisons, relative confidence limits, defined as coefficient of confidence, are of practical use. Equation 4 and 5 are given for calculating different levels of accuracy or count numbers. For both reverse calculations numbers are given in Table1 and 2. The confidence limits predict the range of 95 % of all counts. 2.5 % at each end of the distribution fall out of this range.

4) Coefficient of confidence $CC\,(95\%) = \pm \frac{2}{\sqrt{\sum x_i}} 100\%$

5) Sum of countings for a given $CC\,(95\%)$ $x = \sum x_i = \left[\frac{200}{CC(95\%)}\right]^2$

Table 1. The coefficient of confidence (CC) for different count numbers (x) after Equation 4.

x	50	200	500	1000	2000
CC (95 %)	28 %	14 %	9 %	6 %	4.5 %

Table 2. Count numbers (x) for different coefficient of confidence (CC) after Equation 5.

CC (95 %)	20 %	15 %	10 %	7.5 %	5 %
x	100	178	400	711	1600

Additional errors can occur because of the inaccuracy of the volume defined in a graduated cylinder or the inaccuracy and low precision of volumes transferred in pipettes. Generally these errors lay between 0.5% and 2 %. Precision depends on the experience of a test person in handling with pipette in a repeated way. Accuracy is a question of materials and calibration. It's sometimes labelled as tolerance on the instruments.

Variance and average of an error follow different equations. The summation of errors like variances or confidence limits is calculated according Equation 6. The additional errors influence the confidence limits, but in general their small contribution can be ignored. Note that the squares in the equation decrease the contribution of small additional errors extremely.

$$6) \quad sd = \sqrt{(sd_1)^2 + (sd_2)^2 + \ldots}$$

Systematic errors due to the tolerance of volumetric instruments or inaccurate calibration of these instruments have an impact on the calculated result of a counting. The summation of errors like average numbers or means is counted according Equation 7. In general, all calculations accumulate systematic errors in the same direction. If you compare results generated by using the same instruments, the relative differences are not influenced by these errors.

$$7) \quad x = x_1 \pm x_2 \pm \ldots$$

If you want to guarantee a product specification, for example a fixed amount of nematodes in a package, these errors have to be considered. You can either add these errors to your calculated result or you can compensate these errors through better calibration. This can be done by calibrating your instruments (pipettes, graduated cylinders) by the help of an analytical balance.

Acknowledgements

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Insect Pathogens and Insect Parasitic Nematodes
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Insect based assays for entomopathogenic nematode infectiousness: definitions, guidelines, problems

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Abstract. The quality characters of entomopathogenic nematodes may be classified according to the steps involved in successfully killing an insect. Insect based infectiousness assays cover most of these steps and are therefore proposed as tools to predict field performance of nematode batches. Criteria for good assays are high resolution and good reproducibility which are strongly influenced by the choice of the test insect and the nematode dosage. Single dose assays are sufficient, if the nematode dose is chosen to give 50 % insect mortality using an average-quality batch of a certain nematode strain. No adverse affect on assay resolution was recorded when insects were treated in groups rather than isolated from each other. Based on these considerations a standard protocol for quality assessment of entomopathogenic nematodes is proposed. The widespread use of consistent assays and their relation to (semi) field trials will improve their role as predictive tools.

Introduction

For biological control agents 'Quality' is a broad term covering features like shelf-live, persistence, infectiousness and often also reproduction in the host insect. There is a large variety of analytical techniques and assays available to check these features in entomopathogenic nematodes. Most of these assays have been designed to improve the understanding of the factors affecting nematode quality. In the practice of using nematodes in the field, assays are needed which predict the performance of the nematodes in the field with a minimum of labor and cost involved. These requirements are in part covered by insect based infectivity assays.

Quality factors covered by insect based bioassays

The process of killing an insect with entompoathogenic nematodes involve host finding, host recognition, successful penetration, release of symbiotic bacteria, overcoming the insect immune response etc.. The most important steps in infection are summarised in Tab. 1 and classified according to commonly used terms in parasitology. The most common assays suggested for quality control evaluate host finding (e.g. Westerman & Stapel, 1992), nematode infectivity, i.e. the number of nematodes entering the host insect (e.g. Hominick & Reid, 1990) and overall pathogenicity, i.e. insect mortality. In most larger arenas where insects and nematodes are placed together, host finding over a limited distance is involved. Hence, assays evaluating insect mortality after placing nematodes and insects together may be termed infectiousness assays. By assessing just insect death after applying a single dose of nematodes, information on the sum of traits necessary to kill the insect is gathered. No information on the factor(s) limiting infectiousness can be deducted. These kind of assays are sufficient to predict the performance of a certain nematode batch when applied in the field. They are, however, not suitably for discovering the reasons for a limited infectiousness. For

the improvement of quality by altering production procedures, more specific assays are needed.

Tab 1: Events involved in successful control of insects by entomopathogenic nematodes and a proposed classification to commonly used terms in parasitology an pathology.

<table>
<tr><th>EVENTS</th><th colspan="4">TERMS</th></tr>
<tr><td>Host finding</td><td></td><td></td><td></td><td rowspan="9">Infectiousness</td></tr>
<tr><td>Host recognition</td><td rowspan="2">Infectivity</td><td rowspan="8">Establishment</td><td rowspan="8">Pathogenicity</td></tr>
<tr><td>Penetration</td></tr>
<tr><td>Release of bacteria</td><td rowspan="5">Virulence</td></tr>
<tr><td>Bacteria proliferation</td></tr>
<tr><td>Overcoming insects immune response</td></tr>
<tr><td>Nematode proliferation</td></tr>
<tr><td>Insect death</td><td></td><td></td></tr>
</table>

In infectiousness assays, usually only insect mortality is evaluated. For some applications like controlling white grubs in turf (Ehlers & Sulistyanto, 1996) the reproduction of nematodes in the insects is also crucial for field performance. It might therefore be a good idea to include this parameter in quality control assays.

Methods for assessing assays

Criteria for a good assay are high resolution and good reproducibility. The assay should be able to discriminate batches of a certain strain of nematodes. Therefore, the resolution of an assay should be tested with batches of the same nematode species, preferably also the same nematode strain, since in practice the quality of commercially available nematodes is to be assessed. Standard statistical techniques like Analysis of variance (ANOVA) can be employed to assess the resolution of assays. In a comparison of two assays using either G. *mellonella* or *T. molitor* as test insects, a larger F-statistic was found for *T. molitor* compared to G. *mellonella*. Hence, using *T. molitor* increases the resolution of this assay (Fig. 1) (the protocol for the assay can be found below).
The reproducibility of the assay using *T.* molitor was studied with batches of *H. bacteriophora* produced in bioreactors. The nematodes were harvested and a sample was sent to the company BIONEMA AB in Sweden. At the same time, the nematodes were tested for their infectiousness at the production site in Germany. The comparison of 10 batches gave a similar ranking in both laboratories. The SPEARMAN rank-correlation coefficient between the two measures was 0.57 ($p=0.08$). The agreement in ranking was good considering that the age of the nematodes tested varied. The time gap between testing in Germany and Sweden varied between 1 and 64 days. Insect mortality in Sweden seemed to be consistently higher (Fig. 2). Therefore, the results should always be related to a standard to eliminate the effect of

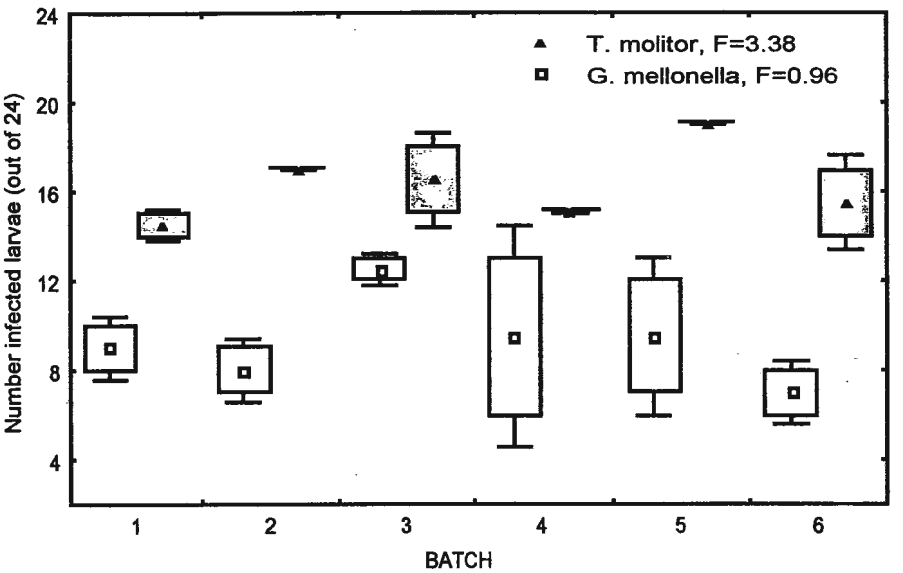

Fig. 1: Comparison of 6 batches of *Heterorhabditis bacteriophora* produced in bioreactors with a single dose infectiousness assay using *Tenebrio molitor* and *Galleria mellonella* as test insects. The dose used was 30 nematodes/larvae for *T. molitor* and 2 for *G. mellonella* and insects were isolated from each other. Two replicates of 24 larvae were evaluated Box=Std. error, Whisker=Std. deviation.

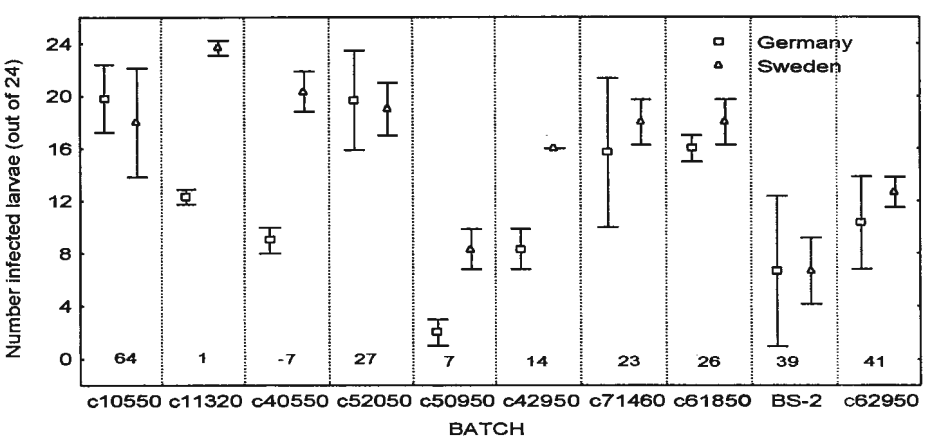

Fig. 2: Results of infectiousness assays performed in a German and a Swedish laboratory on 10 liquid produced *Heterorhabditis bacteriophora* batches (Means of 3 replicates ± standard deviation). The time gap (in days) between testing in Germany and Sweden is given below the figures. Whisker=Std. deviation.

Factors affecting assay performance

Assay performance can be improved by altering exposure time, dose applied, the host insect used, etc. To keep the assays simple, a single dose approach should be taken rather than a mufti-dose assessment. For optimal resolution the dose should be chosen to give around 50% mortality. As shown above, the choice of the test insect greatly influences assay resolution. Involved in this are also different doses, since G. *mellonella is* more susceptible and hence requires less nematodes (2 per insect) to suffer approx. 50% mortality. According to the interaction between infected insects and nematodes outside the host, described as secondary invasion (Hay & Fenlon, 1995) or aggregation (Westerman, 1996), assay performance would be influenced by testing isolated insects versus grouping them into one arena. If nematodes were attracted to infected insects, grouping should result in higher within batch variation and therefore decrease the resolution, whereas repellence of nematodes from infected insects (Shapiro & Glazer, 1996) should decrease within group variation and increase the resolution. In a comparison of grouped versus isolated T. *Molitor* exposed to different batches of H. *bacteriophora*, resolution was slightly enhanced in grouped-insect assays (Fig. 3). Since grouped insect assays are easier and quicker to perform, more insects may be used and the resolution increases even further. For H. *bacteriophora* and S. *feltiae*, a grouped insect assay is therefore proposed as a standard. It should be tested, however, whether other nematode species require isolated-insects assays for a good resolution.

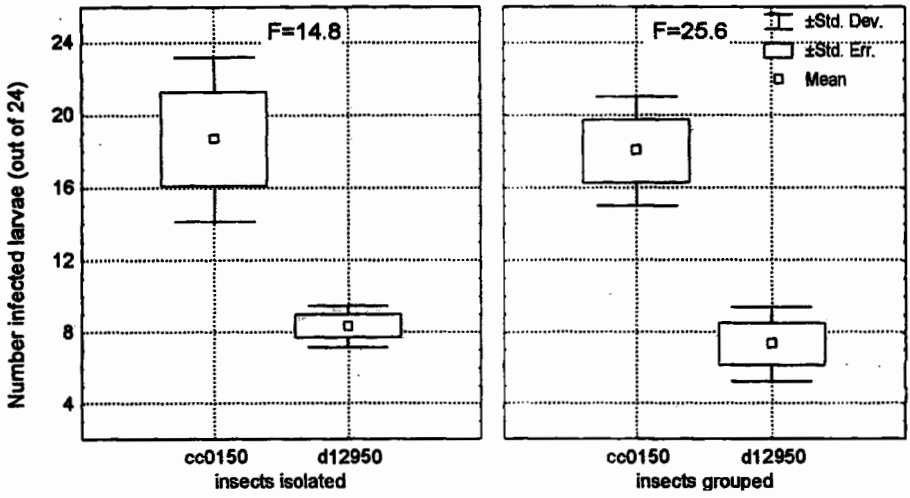

Fig. 3: Comparison of 2 batches of *Heterorhabditis bacteriophora* produced in bioreactors with a single dose infectiousness assay using *Tenebrio Molitor* isolated from each other versus grouped together. The dose used was 30 nematodes/larvae. Three replicates of 24 larvae were evaluated (see protocol below). Box=Std. error; Whisker=Std. deviation

A proposed standard protocol for the quick assessment of EPN infectiousness

Single dose infectivity test using larvae of the lesser mealworm, *Tenebrio molitor*.
- Insects grouped

Step 1: Preparing assay arena:
Prepare silver sand (200 to 400 µm core size) approx. 300 g for testing one batch of nematodes. Adjust to 10% moisture content by adding tap water to dry sand.
Step 2: Take 3 containers with approx. 10 x 10 cm area and fill with moist sand.
Step 3: Adjust nematode suspension to a density of about 30 DJs/100µl for *H. bacteriophora*. (Values for *S. feltiae* and *S. carpocapsae* should be about 20 an 10, respectively. Take at least 3 counts of the final suspension and divide 30 by the mean of these counts to get the adjustmentfactor for the volume containing 30 nematodes.
Step 4: Apply [100 x adjustmentfactor] µl to each well. Be sure to agitate the suspension by blowing air into it.
Step 5: Add the mealworms to the wells and close the lid of the container. Turn the plate around and knock it on the bench to ensure contact of nematodes and mealworms. Wrap plates in tin-foil and incubate at 25°C for 7 days.
Step 6: Count dead larvae. Correction for control mortality not necessary for *Heterorhabditis* spp. if luminescense is assessed. Otherwise include three containers without adding nematodes as an untreated control.

Variations:
- Insects may also be treated isolated from each other in 24-er multiwell plates (this assay has been employed in Fig. 1).
- More insects may be used per treatment to increase resolution.
- Nematode dosage should be adjusted to give approx. 50% mortality of an average quality nematode batch.

Problems remaining

The lack of a standard nematode or a standard nematode surrogate makes the comparison of results of assays done at different locations or at different times difficult. A large quantity of liquid nitrogen frozen nematodes might be a suitable standard since no alteration in quality would be expected during storage. However, the quality is probably very sensitive to inevitable variations in the thawing procedure. By including a chemical treatment, the vigour of the test insects could be tested, but it would not necessarily reflect their susceptibility to nematodes, unless it has a similar mode of action. These considerations demonstrate that there is no easy solution to this problem, if it can be solved at all.

The infection assays are done to check the potential of the nematodes for controlling a certain pest in the field. It should therefore be calibrated with field data. Field data, however, are subject to numerous factors that are hard to measure and often impossible to control (wheather, soil texture, biotic factors). All these factors contribute to the variation in field data and will make a calibration of infection assays to field data almost impossible. Standardized semi-field experiments should be chosen for the calibration of the quality control methods. Some guidelines for standardisation of semi-field trials against the black vine weevil are given by van TOL in this issue. Similar semi-field trials must be developed for other target pests.

The assays in use are sufficient for quality control of nematodes. They would be much more useful, however, if they were used in a consistent manner and if they were incorporated into standard semi-field experiments. The outcoming data could be processed to calibrate the assays and to make them better predictive tools.

Acknowledgement

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Influence of insecticides, carbosulfan and carbofuran on the mortality and pathogenity of entomopathogenic nematodes and fungus

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Summary: Carbosulfan and carbofuran are apply to control of *Melolontha* grubs in forest. Both insecticides could be possible candidates as a chemical factor in the IPM of grubs *Melolontha melolontha.* Susceptibility of entomopathogenic fungus *Beauveria brongniartii* and *B. bassiana* and nematodes *Steinernema glaseri, S. feltiae* and *Heterorhabditis megidis* to these insecticides has been studied. Fungus spore and infective juveniles of nematodes were treated with lower doses of insecticides, 3 mg/Kg soil (recommended dose - 30 mg/Kg). Both fungus species were susceptible on insecticides. Carbosulfan more restricted growth of fungus mycelium than carbofuran. Mortality of nematodes treated with carbofuran was higher compare to carbosulfan.

To examine the pathogenity of fungus and nematodes, infective juveniles of nematodes and fungus spores were kept in solution of insecticides within five days and after that they were treated to *Galleria mellonella* caterpillars. In principle both insecticides did not affect of fungus or nematodes pathogenicity. Our investigation shows that both tested insecticides could be potential candidates in IPM.

Introduction

Minimising of the application of insecticides requires some investigation of a new approach of a Melolonthine pests control. Studies show that biological control could be not enough efficient method in the case of *Melolontha* grubs (Zegler 1993, Vlug 1996). Bednarek *et al.*, (1999) studied the IPM concept for control of *Melolotha melolontha* grubs. They examined the possibility of increasing the effectiveness of entomopathogenic fungi and nematodes by the low concentration of an insecticide. The positive results show that it could be a new approach in the control of grubs infesting forest. Therefore, IPM methods are possible to apply when the chemical insecticide is not harmful for biological components of these methods. Therefore, tests were conducted to evaluate the effect of insecticides usually used under Polish circumstances on different species belong to the most promising genera: entomopathogenic fungi, *Beauveria* and nematodes: *Heterorhabditis* and *Steinernema.*

There were investigated two chemical insecticides belonging to carbaminian group: carbofuran (commercial name Furadan 5 GR, FMC, USA, including 5% of carbofuran) and carbosulfan as 1g/Kg (commercial name Marshal SuSCon 10 CG, including 10% of carbosulfan). Both toxins are systemic substances. Carbofuran affects grubs immediately in the soil, and carbosulfan belong to environmental friendly insecticide in a slow release formulation.

Methods

Growth of fungus mycelium was evaluated for two indexes: (1) average weight of the dry mycelium isolated from the liquid Sabouraud dextrose medium and (2) average diameter of the fungus colony growing on the solid Sabouraud dextrose agar medium (SDA), after 14 days of incubation. The diameter of fungus colonies were measured by means of Lilly and Barnets (1959) method. Fungus biomass is determinated as an average weight of dry mycelium. Mortality of the wax moth, *Galeria mellonella* caterpillars, infested with spores of fungus was investigated to evaluate of the fungus pathogenicity. There were 10 replications of 10 insects (1 petri dish) per treatment.

Petri dishes (9 cm diameter) were filled with solutions of carbofuran or carbosulfan (10 ml/dish), and the infective juveniles (IJs) of a nematode species were added and incubated in the temp. 19,5°C. There were 5 replications using 5000 IJs (per one Petri dish) per treatment. After seven days the number of death nematodes was counted under the stereomicroscope to evaluate the nematode mortality. Next, alive nematode juveniles were rinsed in sterile water. 500 IJs and 10 wax moth caterpillars were put in to the petri dish (9 cm diameter), filled with damp filter paper. The insects mortality was observed every day during 5 day a incubation at 26°C. There were 5 replications with 10 caterpillars (1 petri dish) per each treatment.

Conclusion

1. Results of our investigations showed that carbosulfan inhibited the colony growth of the both fungus species, but carbofuran stimulated growth of *B .brongniartii.* In the case of *B.bassiana* reaction of this fungus mycelium was stronger than of *B. brongniartii.* In contrast to fungi, insecticides did not affect IJs nematodes. The mortality of nematodes treated with insecticides did not differ significant as compared to the control.
2. There were no significant differences in the mortality of wax moth caterpillars treated with *B .bassiana* and *B. brongniartii* affected with insecticides during 21 days of incubation. When we compare the mortality dynamics during the period of 21 days, then we can note that in the case of *B. bassiana,* the percentage of mortality increased faster than for *B. brongniartii.*
3. The mortality of wax moth caterpillars treated with nematodes incubated with insecticides, was not differ depend to nematode species and insecticides. Also, we found that in the case of *S.feltiae* incubated with carbosulfan, the nematode pathogenity to *G. mellonella* caterpillars was higher as compared to another nematode species (the higher number of nematodes treated with the insecticide infested *G. mellonella* caterpillars). We can conclude that carbofuran could stimulate higher activity of IJs of *S. feltiae.*
4. .Our study proved that both recommended insecticides apply at lower concentration could be used in the IPM methods together with entomopathogenic fungi and nematodes to control of *Melolontha melolontha* grubs. It is possible that carbosulfan could stimulate the pathogenity process and give synergistic effect in pest control. But, this hypothesis need the investigation, yet.

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Interactions between Steinermatid nematodes and *Delia radicum* under field conditions

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Abstract: Soil samples from cabbage fields were surveyed for their content of Steinermatid nematodes and *Delia radicum* pupae. No clear correlation between nematode occurrence and pupal numbers appeared. In one field however, where nematode were found in restricted areas within the field, there was an indication that nematodes were more frequent where numbers of pupae were high. In another experiment *Steinernema feltiae* nematodes were introduced to a field by placing dead nematode-infected *Tenebrio molitor* larvae around cabbage plants in early summer prior to *D. radicum* egg-laying. In this field experiment nematodes significantly reduced the numbers of *D. radicum* pupae. In soil samples taken close to and away from plants it was found that soil samples taken close to plants caused higher mortality of bait larvae *(T. molitor)* than samples taken away from plants.

Key words: *Delia radicum, Steinernema,* population dynamics, cabbage, bait.

Introduction

Delia radicum is occasionally a serious pest insect of cruciferous plants due to its larvae feeding on the roots. No environmental acceptable control practices are so far available. On the other hand the larvae have several natural enemies including parasitoids, predators and insects pathogens harboring the soil. It is of increasing interest to investigate the potential of these pathogens to control *D. radicum* populations under field conditions. For instance Steinermatid nematodes are of interest since they are found in many soils and since some of these species actively seek and kill their host. In this work the presence of Steinermatid nematods were investigated in cabbage fields (experiment I). These fields were part of a crop rotation system and for that reason insects pathogens bound to the soil have a changing selection of hosts from one year to the other. To study the influence of cabbage plants, pairs of soil samples were taken. Each pair consisted of (1) soil from around a plant and (2) soil away from the plant. Since initial population size and fitness must be crucial to the regulatory role of entomopathogenic nematodes another experiment was carried out (experiment II). In this experiment *Steinernema feltiae* was introduced to a cabbage field. To simulate natural conditions this was done by infecting *Tenebrio molitor* larvae with the nematodes. The newly dead larvae were then buried around the root stem of the cabbage plants and effect on *D. radicum* survival was monitored.

Materials and methods

Experiment I

In 1997 a total of seven organic grown cabbage fields were sampled in the autumn after the end of the growing season. In each field 30 soil samples were collected at random. A soil sample consisted of the root of a plant and the surrounding soil (approx. 15 cm in depth and

diameter). In four fields additional samples were taken approximately 25 cm away from each of the plants resulting in 30 soil sample pairs. The two samples within a pair will be designated "At plant" and "Off plant" samples respectively. The samples were taken along a transect within the field. The distance between sampled plants were given by the use of random numbers and varied in general from two to nine metres. The samples were gently homogenized by hand and stored at 5°C until use. Three times during the winter, with an interval of approximately six weeks, subsamples of approximately 400 ml soil jars were baited in cylindrical jars. "At plant" and "Off plant" samples were baited parallel except for the first baiting were only "At plant" samples were baited. At each baiting five *T. molitor* larvae were used for one week at room temperature. On week days the jars were turned upside down to force the bait larvae to move through the soil. Dead larvae were placed individually on water traps to collect nematodes. Bait larvae mortality and infection by Steinermatid nematodes were recorded. Finally *Delia radicum* pupae from "At plant" samples were extracted by flotation and counted according to Finch *et al.*, (1978).

Experiment II

In June 1997 a number of *T. molitor* larvae were infected with *Steinernema feltiae* (DKI) in moist sand with a mean of 75 juveniles per larvae. After five days the dead larvae were taken to the field and buried around the stem of a cabbage plant just below soil surface. At control plants the soil was disturbed accordingly. Either 0,5 or 15 *T. molitor* larvae infected with *S. feltiae* were used in each replicate of 15 plants giving a total of 45 random plants within one row in an organic grown cabbage field. In August after cabbage plants had been cut all 45 plants were sampled similar to "At plant" samples in "Experiment I" and *D. radicum* pupae were extracted by flotation.

Results and discussion

Conditions "At plant" and "Off plant" (Experiment 1)

Results from the survey, where the mortality of bait larvae in pairs of soil samples were compared, are given in figure 1. It was found that the mortality was up to five times higher in "At plant" samples than in "Off plant" samples. One explanations to this difference could be that entomopathogenic nematodes are attracted to the plants due to higher levels of carbon dioxide or other substances produced by the plants or insects living in connection with the plants (Grewal *et al.*, 1993; Lei *et al.*, 1992; Royer *et al.*, 1996). Since the samples within a pair were taken only approximately 25 cm away from one another the distance from "Off plant" to "At plant" is within the distance that nematodes can travel. The difference in mortality could also be a reflection of insect distribution and activity in the period prior to soil sampling. In the case of *D. radicum* insects only occur "At plant" and thus the possibility of insect pathogen survival and propagation is higher.

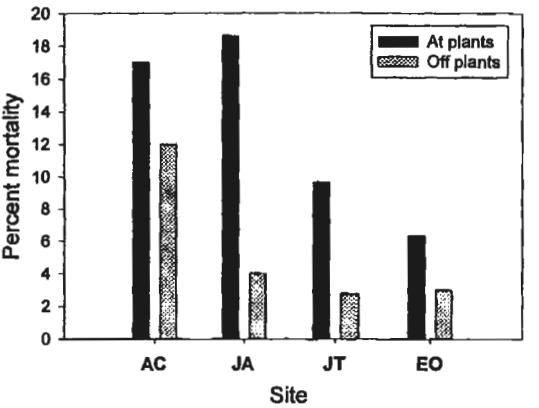

Figure 1. Mortality of bait larvae in soil samples from cabbage fields at four different sites. Each bar is the mean of second and third baiting (n=300).

Occurrence of Steinermatid nematodes (experiment I)

Nematodes were found in the two fields where the mortality of bait larvae was highest ("AC", "JA"). Figure 2 gives the positive baiting results from the comparison at site "JA" (data from "AC" not shown). It is remarkable that nematode occurrence was restricted to few plants and that nematodes in general were found repeatedly in the three baits. With one exception, only "At plant" samples were positive for nematodes. This difference between nematode occurrence "At plant" and "Off plant" correlates well with the observed difference in bait larvae mortality (figure 1). Both nematode occurrence and bait larvae mortality were distinct "At plant" .

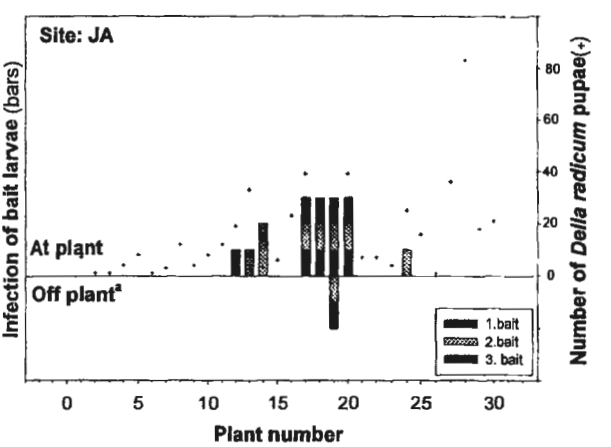

Figure 2. Infection of bait larvae by Steinermatid nematodes in relation to cabbage plants and number of *Delia radicum* pupae at site JA . [a]Off plant samples were not baited in the first bait

Interactions of nematodes and Delia radicum (Experiment I)
At site "JA" nematodes were restricted to few plants, and since this pattern did not seem to change by additional baiting, nematode occurrence was compared to numbers of *D. radicum* pupae (figure 2). At the plants positive for nematodes, the number of pupae was high in most cases. This could of course be a matter of coincidence but it could also reflect the dependency of the nematodes on insect activity for survival and population growth. To investigate such a connection more information on both insect and nematode population during the summer is needed. In the above case only the final situation in autumn is known and an important factor - the initial conditions in spring - are unknown.

Introduction of nematodes to cabbage plants (Experiment II)
In this experiment a high semi-natural population level was established by placing *T. molitor* larvae infected with nematodes in the field prior to *D. radicum* egg-laying. It was found that the number *of D. radicum* pupae were significantly lower at plants where either five or 15 infected *T. molitor* had been introduced compared to untreated plants (figure 3). This is in contrast to the observations in "Experiment I" and must be due to the raised numbers of nematodes being introduced early in the growing season and the ability of these nematodes to kill *D. radicum* larvae.

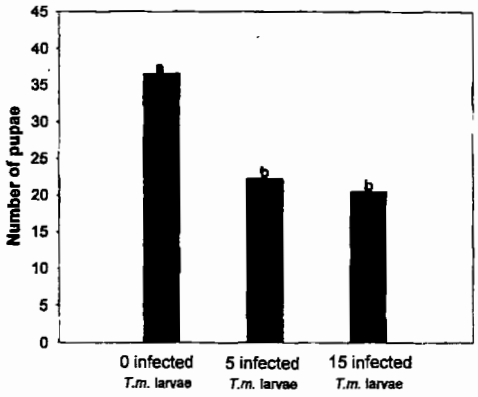

Figure *3.* The number *of Delia radicum* pupae found at plants inoculated with 0,5 or 15 dead *Tenebrio molitor* larvae infected with *Steinernema feltiae.*

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Do insect aggregations influence entomopathogenic nematodes occurrence?

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Abstract: Entomopathogenic nematodes belonging to the families Heterorhabditidae and Steinernematidae have been considered to be very efficient insect parasites to a broad host range. They are worldwide spread, recovered from most continents, including geographically isolated islands. They inhabit majority of terrestrial habitats, but prevail in those rich for soil-dwelling insects. Entomopathogenic nematodes significantly reduce insect populations, and moreover, have a great impact on the course of insect pest outbreaks.

Natural insect aggregations realize a huge concentration of suitable hosts for parasitism by entomopathogenic nematodes followed by the rapid nematode multiplication which facilitate their field recovering in dead insects or a laboratory isolation by using the *Galleria* trap method. Therefore, our main research objectives were aimed on the field recovery of entomopathogenic nematodes in some habitats characterized by the severe aggregations of these target insect species, the nematode frequency in these habitats, and the range of nematode species.

Localities inhabited by sawflies, *Cephaleia abietis, Pristrphora* spp. and *Pikonema spp.*, the winter moth, *Operophtera brumata,* including a complex of accompanying moths, bibionid flies, *Bibio marci,* and *Penthetria holosericea,* and one sciarid fly were tested for entomopathogenic nematodes occurrence. Their larvae form characteristic aggregations in the soil or nests beneath the litter with often tens to hundreds specimens spread on a small area of several quadrate meters and create thus an excellent xrucroniche for invasion by nematodes.

The only steinernematids were isolated, but their frequency was very high in all tested habitats. Nematodes were recovered in 61 (70.1%) of 86 sampled localities. Interestingly, all samples with nesting fly larvae were positive for nematodes, while the percentage of parasitism for sawfly and winter moth larvae attained 67% and 69%, respectively. At least, six steinernematid species were identified from this survey. The most frequent *S. kraussei* inhabited all habitats and was prevalent in sawfly localities while *S. feltiae* ocurred only in winter moth habitats. S. *affine* was common in the winter moth habitats. Other species, *S. intermedium, S. bicornutum* and *Steinernema* spp. were rare. Laboratory temperatures used for *Galleria* trap tests may significantly influence the number of nematode positive samples. Generally, in our experiments the steinernematids were mostly isolated at both experimental temperatures of 15°C and 22°C . However, if the *Galleria* traps would be tested at only 15°C or 22°C some nematode isolates would not be recovered. When looking for entomopathogenic nematodes in the field, the previous screening of for huge insect aggregations is recommended.

Key words: entomopathogenic nematodes, insect aggregations, sawflies, winter moth, bibionid flies, *Steinernema kraussei, S. feltiae, S. affinis, S. carpocapsae*

Introduction

Entomopathogenic nematodes belonging to the families Heterorhabditidae and Steinernematidae have been considered to be very efficient insect parasites to a broad host range (Weiser & Mráček, 1988; Gaugler & Kaya, 1990). They are spread worldwide, found in North America (Mráček & Webster, 1993), Europe (Hominick *et al.*, 1995), Australia

(Akhurst & Bedding, 1986), Asia (Mason *et al.*, 1996), and Africa (Waturu *et. al.*, 1997), and many islands (Hara *et al.*, 1991). They inhabit most of terrestrial habitats, but prefer those rich in soil-dwelling insects. Entomopathogenic nematodes significantly reduce insect populations in field experiments against different noxious insects, and moreover, have a great impact on the course of insect pest outbreaks (e.g. Georgis & Hague, 1981; Mráček, 1986; Akhurst *et al.*, 1992). Although, it has been proved that these nematodes are ubiquitous (Hominick & Briscoe, 1990a; Mráček *et. al.*, 1999), their field recovery is influenced by host range, suitability for penetration of different insect hosts by nematodes, possibility to meet each other in habitats, and nematode natural population density (Peters, 1996).

Among the many nematode suitable insects, different sawfly species, geometrid and noctuid moth complex with the most frequent winter moth, bibionid and sciarid flies to create aggregations of diapausing or hibernating larval populations in a relatively small area. Such aggregations occur because of a huge concentration of suitable hosts for paxasitation by the entomopathogenic nematodes followed by their rapid multiplication, facilitating their easy field recovery in dead insects or laboratory isolation using *Galleria* trap method (Mráček, 1980). There are numerous localities inhabited by these insects in the Czech Republic. These localities can be considered as examples of balanced, long-lasting nematode-insect associations (web-spinning sawfly) and unbalanced short-lasting nematode epizootics (e.g. nests of bibionid larvae) (Peters, 1996).

Despite the great advance in an elementary research and field applications by entomopathogenic nematodes, observations of the natural associations between these nematodes and their insect hosts are rare and have remained a potential field of studies. Such aggregations may provide important data on nematode distribution, their occurrence in habitats as well as on their impact on the oscillation of host abundance. Therefore, our main research objective was to focus on the field recovery of entomopathogenic nematodes in habitats characterized by severe aggregations of these target insect species, nematode frequency in these habitats, and the range of nematode species.

Material and Methods

Insect hosts and their localities

Web-spinning sawfly, *Cephaleia abietis, is* a serious pest of spruce habitats (60 to 80 years old monocultures) in Czech highlands and mountains. Sawfly larvae damage needles on spruce trees from 600 to 1100 m above see level. At the end of summer, the larvae fall from the spruce trees and enter the soil where they diapause for two or three years. They aggregate below the spruce canopies. The localities used for our studies ranged from moderate to severe (50 to 600 specimens/m^2 aggregated with diapausing sawfly larvae. 26 such localities (mostly in the Czech border mountains) and 4 additional localities with the other spruce sawflies *Pristiphora* spp. and *Pikonema* spp. were tested for entomopathogenic nematodes incidence.

Winter moth, *Operophtera brumata,* and a complex of accompanying moths (e.g. *Orthosia* spp., *Cosmia* spp., *Erannis defoliaria* etc.) are significant defoliators of various deciduous tree habitats in a Czech hill country. They feed on shoots, leaves and young fruits. In late May and beginning June they enter the soil below the tree canopy to pupate. The localities tested in our studies belonged to moderately and severely defoliated apple trees on roadsides and hedgerows, each containing approximately 20 to 100 inhabiting winter moth and accompanying moth larvae. In our study 50 such localities, mostly in South Bohemia, were tested.

Larval bibionid flies, *Bibio marci, Penthetria holosericea,* and Sciaridae, feed on soil organic compounds and plant roots. During autumn, insect larvae form characteristic nests

beneath the litter with hundreds of specimens spread in a small area of several quadrate decimetres, thus creating an excellent microniche for invasion by nematodes. Seven nests, five with *B. marci*, one with *P. holosericea*, and one species from the family Sciaridae were collected in the vicinity of České Budějovice and tested for the incidence of entomopathogenic nematodes.

Field and laboratory experiments

Soil samples in chosen outbreak spruce forest and apple tree habitats were taken using an iron core (volume 200 cm^3), 5 cores per habitat (to get the constant volume of experimental soil) and transported to the laboratory in plastic bags. Nests of fly larvae were found by digging beneath the canopies of roadside apple trees and small oak forest stands. The soil samples from these nests together with insect larvae were collected by a small iron shovel. Field collections were performed from spring 1996 to autumn 1998, throughout the season in sawfly localities, mostly in May and June in winter moth localities, and during October and November for the nesting fly larvae.

For the laboratory tests, each soil sample was mixed, divided into two parts, and assayed using *Galleria* traps (Bedding & Akhurst, 1975; Mráček, 1980) that were kept in incubators at two experimental temperatures, 15 and 22°C, respectively. The *Galleria mellonella* larvae that were used as baits were set in a small, iron mesh pocket and placed at the bottom and centre of each petri dish. Mortality of *G. mellonella* was assessed after 5 days. Dead *Galleria* larvae were divided in two batches, one was dissected to obtain adult nematodes of the second generation and the second batch was used for culturing on a water trap for obtaining infective juveniles (IJs). Adults and infective juveniles (IJs) were heat-killed in Ringer's solution and fixed overnight in TAF fixative, transformed to permanent mounts by Seinhorst's (1966) method, and then identified to species under a light microscope using morphological characters.

Results and discussion

Only steinernematids were isolated during our survey and their frequency was very high in all tested habitats inhabited by aggregated insects. No heterorhabditids were found. Nematodes were recovered from 61 (70.1%) of 87 sampled localities. Interestingly, all samples with nesting fly larvae were positive for nematodes, however, the only seven sites were sampled, while the percentage of parasitism in *Galleria* traps from localities with sawfly and winter moth larvae attained 65.4% and 68%, respectively (Table 1).

Table 1. Nematode sampling in niches with insect aggregations

Insect host	Number of samples	Nematode positive	%
Cephaleia spp.	26	17	65.4
Operophtera brumata	50	34	68.0
Nesting Diptera	7	7	100.0
Pikonema spp. & *Pristiphora* spp.	4	3	75.0

At least, six steinernematid species were identified from this survey (Table 2). *S. kraussei* was found in all habitats (niches), however, with one exception the only inhabiting species in sawfly localities. *S. feltiae* occurred frequently in apple tree habitats and surprisingly never

has been recovered in nest niches or spruce habitats. *S. affine* was common. *S. intermedium* and unidentified species were rare. Surprisingly, S. *carpocapsae* was not found in the apple tree habitats, even though we sampled three times its type locality (Weiser, 1955) in the vicinity Holovousy village. However, *S. feltiae* was recovered in this locality. Unidentified species need more morphological studies. In six samples two nematode species were found, occurred (*S. feltiae* and *S. affine* 3x, *S. feltiae* and *S. kraussei* 2x, *S. kraussei* and *Steinernema* spp. 1x).

Table 2. *Steinernema* species in three habitats and their target insect species

NP - number of EPN positive samples
S. spp - unidentified species
IN - infection by EPNs, but only found nematode females or samples overgrew by saprophytic nematodes
* six samples contained two species

	habitat / host		
Nematode	**Apple tree / winter moth**	**Spruce /sawflies**	**Nest /fly larvae**
S. kraussei	6	7	1
S. feltiae	15	0	0
S. affine	7	0	0
S. intermedium	1	2	1
S. bicornutum	2	0	0
Steinernema spp.	2	0	1
IN	7	11	4
NP	34 (40*)	20	7

Laboratory temperatures used for *Galleries* trap tests may significantly influence the number of nematode positive samples. Generally, in our experiments the steinernematids were mostly isolated at both experimental temperatures of 15 and 22°C (70%). However, if the *Galleria* traps would be tested at only 15 or 22°C, respectively, some nematode isolates would not be recovered. E.g., if only temperature of 22 °C would be tested all nematodes (23.3%) isolated at only 15°C were missing (Table 3). Generally, our results are not surprising and may support the well-known relationship in a parasite-host system where not only entomopathogenic nematodes and target insect species playa substantional role (Mráček & Spitzer, 1983). However, more data on insects and nematodes associations are needed to solve questions why nematodes settle in one locality and not other, or why they are sometimes isolated once and not again in the same sampling site. When looking for entomopathogenic nematodes in the field, the previous screening of for huge insect aggregations is recommended.

Table 3. Results of the *Galleria* traps at two experimental temperatures (*)

Host	Total number of samples	Number of positive samples	Positive samples					
			at only 15°C		at only 22°C		15°C a 22°C	
			N	%	N	%	N	%
Cephaleia spp.	22	14	1	7.1	0	0	13	93
Operopthera brumata	11	6	1	16.7	1	17	4	67
Diptera larvae	7	7	2	28.6	1	14	4	57
Pikonema spp.& *Pristiphora* spp	4	3	3	100	0	0	0	0

(*) not all soil samples were tested for the temperature preference

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3. Entomopathogenic fungi

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Entomophthoralean fungi infecting the green spruce aphid *(Elatobium abietinum)* in the North-western part of Europe

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Abstract: During a survey performed between 1997 and 1998 in the North-western part of Europe (Denmark, France, Northern Ireland and Iceland) the following entomophthoralean fungi were identified from *Elatobium abietinum: Entomophthora planchoniana, Conidiobolus obscurus, C. osmodes, Pandora neoaphidis* and *Neozygites fresenii.* Despite very intensive sampling only two species of entomophthoralean fungi were documented from Iceland: *E. planchoniana* North-west of Reykjavik and *N. fresenii* South-east of Reykjavik. Possibly these two fungi were imported to Iceland at different times and to different places and have so far not been spread throughout Iceland. Even though *P. neoaphidis* was never documented in Icelandic *E. abietinum* it was possible to infect Icelandic *E. abietinum* with a Danish isolate of this fungus.

Key words: *Elatobium abietinum,* Entomophthorales, *Neozygites fresenii,* prevalence.

Introduction

The green spruce aphid *Elatobium abietinum is* an economiccally important pest insect in Sitka spruce plantations in the North-western part of Europe. Feeding by *E. abietinum* on needles may in some years cause widespread defoliation and reduction in tree growth in spruce forests (Bejer-Pedersen, 1962; Straw *et al.,* 1998). *E. abietinum* was introduced from Central Europe to United Kingdom, France and Denmark more than 150 years ago but was not observed in Iceland until 1959. In oceanic regions of Europe *E. abietinum* develops anholocyclic and outbreaks are usually seen in spring and early summer except in Iceland where outbreaks occur in fall or early winter (Carter & Halldorsson, 1998).

Austara *et al.,* (1997) reported six different species of entomophthoralean fungi attacking *E. abietinum* in the North-western part of Europe (Iceland, United Kingdom, Norway and Denmark). Only *Neozygites fresenii* was documented in all countries, and for Norway and Iceland it was the only documented species. No prevalence studies has so far been done, however for other aphid-crop systems (eg. *Sitobion avenge* feeding on cereals) fungal prevalences up to 50% have been measured (Feng *et al.,* 1991; Steenberg & Eilenberg, 1995), indicating that at least in some periods these fungi are responsible for a reduction of aphid populations.

The spatial distribution of infected aphids may be affected by behavioural changes caused by the infecting, a behavioural change has for example been reported for root aphids infected with *Pandora neoaphidis* (Harper, 1958). It is important to understand these behavioural aspects since they may favour or disfavour the possibility of dispersing conidia or resting spores into the environment and the following successful infection of a susceptible host.

Materials and methods

Sampling localities

In Denmark samples of *E. abietinum* were taken in 1997 and 1998 from six Sitka spruce plantations all located in coastal areas. In France samples were taken in spring 1998 from two localities in Normandy and six localities in Brittany. In Northern Ireland all samples were taken in 1998 on three localities. In Iceland thirteen localities were selected for sampling of *E. abietinum* in 1997 and 1998.

Species composition of naturally occurring insect-pathogenic fungi

Shoots were cut from Sitka spruce and brought to the laboratory or immediately sent by mail to The Royal Veterinary and Agricultural University, Copenhagen. Living aphids were carefully transferred to small plastic cups (30 ml) which contained 3% water agar in the bottom. Twigs of Sitka spruce were provided as food. In each cup two to five aphids were incubated at 15-20°C and the mortality was recorded daily for at least one week. Dead aphids were removed and examined for entomopathogenic fungi. Sporulating cadavers of aphids were placed over glass slides to allow discharge of primary conidia. Further, non-sporulating cadavers were checked for the presence of resting spores from Entomophthorales. Entomopathogenic fungi were identified under microscope using morphology of the conidia and resting spores as the main characters. More than 400 *E. abietinum* from France, 1200 from Northern Ireland, 4000 from Iceland and 500 from Denmark were incubated during this study.

Spatial distribution of* Neozygites fresenii *within a single tree

To get more detailed information about the spatial distribution of both *E. abietinum* and entomophthoralean fungi additional samples were collected from Iceland. The first set of samples were taken October 28, 1997 and the second set of samples were taken December 1, 1997. All samples were divided into five subsamples according to the year of development of the needles (93, 94, 95, 96 and 97). Samples were collected from five and four trees for the two sampling dates respectively. For each subsample at least three branches at different height and direction were cut. For each subsample density of aphids (nymphs and adults), density of sporulating cadavers and prevalence of entomophthoralean fungi were recorded. A total of 871 and 677 aphids at each sampling were incubated and checked for symptoms of entomopthoralean fungi.

Statistical methods

Standard methods (PROC CORR and PROC GENMOD) for analysis of density data and binary data were conducted in SAS (v. 6.12).

Results

Species composition of naturally occurring insect-pathogenic fungi

In Denmark the following entomophthoralean fungi were identified: *Entomophthora planchoniana, Conidiobolus obscurus, C. osmodes, P. neoaphidis* and *N. fresenii.* Prevalences never exceeded 5%. *C. osmodes* was recorded for the first time on *E. abietinum.*

In France *E. planchoniana* and P. *neoaphidis* were documented. *E. planchoniana* was the most abundant species however the prevalence never reached epizootic levels.

From Northern Ireland *E. planchoniana* and *N. fresenii* were identified. *N. fresenii* was the most abundant species accounting for more than 90% of the cadavers sporulating with conidia and 100% of the cadavers filled with resting spores. The infection level was probably high since the ratio between living aphids and fresh sporulating cadavers was as high as 1:3

when they were received at The Royal Veterinary and Agricultural University two days after sampling.

From Iceland only *N. fresenii* and *E. planchoniana* were found *E. planchoniana* was the only species North-west of Reykjavik and *N. fresenii* was the only species found South-east of Reykjavik. A similar East-West distribution pattern has also been recorded for *E. abietinum* populations in Iceland (Sigurdsson *et al.*, in prep.). An explanation of this result may be that these two fungi were imported to Iceland at different times spread separately throughout Iceland. It seems that, at least in autumn, *N. fresenii is* highly prevalent with values of prevalence ranging between 15% and 45% depending on locality. Even though *P. neoaphidis* never was documented in Icelandic *E. abietinum* it was under laboratory conditions possible to infect Icelandic *E. abietinum* with a Danish isolate of this fungus (originally isolated from *Brevicoryne brassicae).*

Spatial distribution of* Neozygites fresenii *within a single tree

In figure 1 a summary of the detailed study carried out in Iceland at the end of October, 1997 are shown. The study was carried out at Skaftafell and Kirkjubæjarklaustur, where only one species of entomophthoralean fungus, *N. fresenii,* occurred. Compared with many other systems of aphids and entomophthoralean fungi this a very simple system with good opportunities to study the distribution and possible behavioural change of aphids infected with *N. fresenii.*

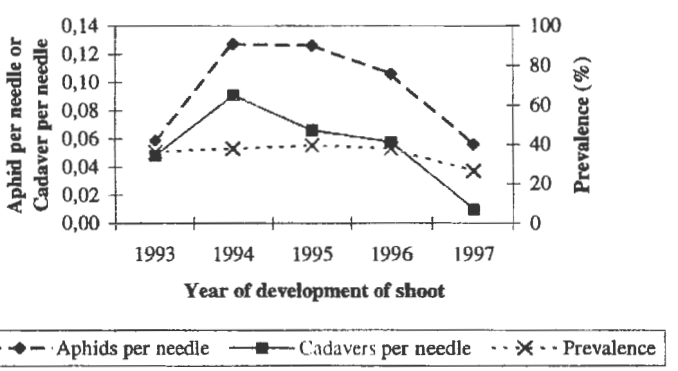

Figure 1: Density of *Elatobium abietinum,* density of cadavers sporulating with *Neozygites fresenii* and prevalence of *N. fresenii* (conidial and resting spore infection pooled) are plotted as a function of year of development of needles. Samples are collected October 28, 1997 from five Sitka spruces at two localities in Iceland (Skaftafell and Kirkjubæjarklaustur).

The highest density of both aphids and cadavers is present on needles developed between 1994 and 1996. Data analysis (PROG CORR, SAS v. 6.12) showed that densities of aphids and cadavers were significantly correlated (1‰). On figure 1 it appears visually that the prevalences are evenly distributed, however the curve is the average of five trees with big variation in prevalences and logistic regression (PROG GENMOD, SAS v. 6.12) showed that prevalence was significantly correlated with density of cadavers (1‰).

Discussion

Five entomophthoralean fungi could be documented on *E. abietinum*. *C. osmodes* has never been described before on *E. abietinum*. However, the species occurs on other aphid species in Europe (Ba=azy, 1993; Keller, 1987). Prevalence up to 45% was observed during this study which documented that *N. fresenii* in some cases is an important mortality factor for *E. abietinum*.

In Icelandic *E. abietinum* the correlation between the densities of aphids and cadavers was significant (1‰) indicating that no obvious change in the behaviour was seen for infected aphids. Prevalences were statistically correlated with the density of cadavers. However, the correlation coefficient between the density of cadavers and the prevalence varied between the two investigated dates (only data from one date are shown). This is probably due to the fact that the prevalence is influenced by climatic factors such as temperature and humidity.

Acknowledgements

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Insect Pathogens and Insect Parasitic Nematodes
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Advantages and disadvantages of different sampling methods for obtaining prevalence data of fungal infections in root flies

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Abstract: Brassiceye® traps baited with ethylisothiocyanate were modified and used to collect adults of *Delia radicum* and *D. floralis* from the field to observe the infection level of *Entomophthora muscae* and *Strongwellsea castrans*. This study confirms that both *E. muscae* and *S. castrans* contain the basic properties to establish epidemics and act as important mortality factors in the field. Our results also suggest that *E. muscae* dominates under warm conditions and that *S. castrans* might be more dominating under cold conditions. The study also indicates that modified Brassiceye® traps are effective and very selective for *D. radicum* and *D. floralis*. Advantages and disadvantages of using different capture methods for fly population monitoring, pathogen sampling, and autodissemination are presented.

Keywords: *Delia radicum, Delia floralis, Entomophthora muscae, Strongwellsea castrans,* traps, pathogen sampling, prevalence.

Introduction

Delia radicum (L.), the cabbage root fly, and *D. floralis* (Fallen), the turnip root fly, are considered to be economically important pests in Brassica vegetables, root crops and canola in the north temperate region (Rygg, 1962; Coaker & Finch, 1971; Finch, 1993; Broatch & Vernon, 1997). Both *Entomophthora muscae* (Cohn) Fresenius and *Strongwellsea castrans* Batko & Wiser have been recognized as pathogens on adult *D. radicum* and *D. floralis* and high infection levels are found for both fungal species (Smith, 1927; Nair & McEwen, 1973; Nair & McEwen, 1975; Lamb & Foster, 1986; Eilenberg, 1991; Eilenberg *et al.*, 1994; Eilenberg *et al.*, 1995). Few studies, however, have been conducted on the seasonal trends and the interactions of S. *castrans* and *E. muscae* in a *D. radicum/D. floralis* field population. The aim of this study was therefore to find the natural infection level of *E. muscae* and *S. castrans* on adult *D. radicum* and *D. floralis,* and to assess the interaction of these insect pathogenic fungi and their importance as naturally occurring mortality factors. The final goal for this investigation was to obtain knowledge that advances the understanding of these insect pathogens as biological control agents. This paper presents the study in brief while more detailed work will be published elsewhere.

Materials and methods

Ten marigold-yellow Brassiceye® tube traps baited with the attractant ethylisothiocyanate were modified to catch live flies in sufficient numbers for calculation of infection levels. Traps were placed along the interface of a hedgerow and a white cabbage (*Brassica oleracea*

L. capitata L.) field in the township of Ski in eastern Norway (59° 43' N:10° 50' E). The field was bordered on two sides by a hedge consisting of a mixture of birch, aspen, sallow, Norway spruce, raspberries and grass. Ten traps were placed with the bottom of the trap at about 25 cm above the ground and spaced about 10 meters apart. They were, with some exceptions, emptied and cleaned with ethanol three times a week. Live flies were transferred individually to 25 ml plastic cups containing 5-10 ml 2% water-agar as described by Eilenberg & Philipsen (1988) and incubated at 17.5° C, 70% RH and 24 h light. Mortality was recorded daily except during the weekends. Flies were observed until they died and were then identified, sexed and species of Entomophthorales were identified. To compare the infection level recorded using the trap method with the sweep-net method described by Eilenberg & Philipsen (1988), the edge of the border vegetation was also sampled by sweep-net four times during 1997. Weather data were obtained from The Norwegian Crop Research Institute (1998).

Results and discussion

E. muscae was an important mortality factor and caused epizootics in the *D. radicum/D. floralis* field population both in 1996 and 1997. The highest *E. muscae* infection level was observed in 1997 when the highest temperatures were recorded (Fig. 1), and both in 1996 and 1997 a significant (P=0.008, 1996, P=0.004, 1997) correlation was found between the *E. muscae* infection level and temperature. The *E. muscae* infection level was significant (P=0.03) and negatively correlated to precipitation in 1997. No correlation with precipitation was found in 1996, nor any correlation with macroclimatic RH in any of the years. *S. castrans* also acted as an important mortality factor in the field, and the highest *S. castrans* infection level was observed in 1996 when the temperatures were lower than in 1997 (Fig. 1). No correlation, however, between the *S. castrans* infection level and any of the climatic factors were found in any of the years. Our results therefore suggest that *E. muscae* dominates under warm conditions and that *S. castrans* might dominate under colder conditions. This indicates that these two fungi might complement each other at different times of the season. Laboratory studies are, however, needed to confirm this. We found no strong indication that any difference in infection level of *E. muscae* or *S. castrans* on *D. radicum* or *D. floralis* exists. Nor did we find strong evidence for differences in infection rate between sexes. The *E. muscae* and *S. castrans* infection level when using the sweep-net method was not significantly different from the trap infection level for the same period.

The modified Brassiceye® traps were highly effective and very selective for *D. radicum* and *D. floralis*. Of flies identified, 98.4% in 1996 and 93.7% in 1997 were either *D. radicum* or *D. floralis*. More than 94% of flies caught in 1996 and more than 80% in 1997 were females. Several different methods have been used to monitor *D. radicum* and *D. floralis* fly populations. Similarly different methods have been used to sample these flies for prevalence of *E. muscae* and *S. castrans* infections. Advantages and disadvantages of using these different methods for fly population monitoring, pathogen sampling, and autodissemination are presented in table 1. For all methods used, there is a danger that the fly population might be biased for infected/not infected flies. This aspect, however, is not discussed in the table. In IPM systems for Brassica vegetables it is important to conserve and enhance *E. muscae* and *S. castrans*, and autodissemination of these two natural enemies might be possible using the modified Brassiceye® traps developed in this project. Further development of the trap and studies on the type of inoculum are being done.

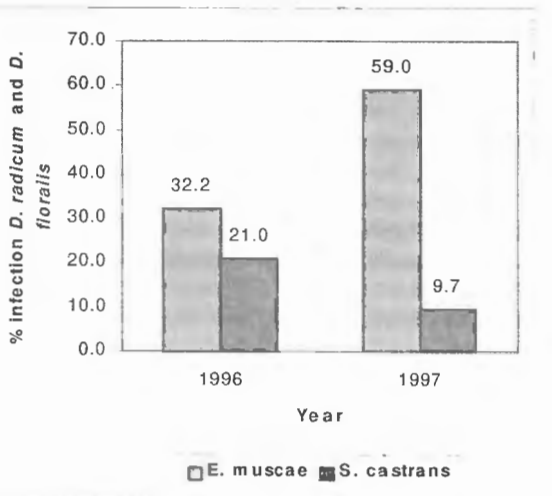

Figure 1. The *E. muscae* and *S. castrans* infection level in a *D. radicum/ D. floralis* field population in 1996 and 1997

Table 1. Advantages and disadvantages in using different methods for assessment of the prevalence of insect pathogenic fungi in populations of adult *Delia radicum/ D. floralis*

Sampling method	**Advantages**	**Disadvantages**
Modified Brassiceye® traps (this study)	Catch high numbers of live flies over time with low labor input. Highly specific, easy handling and easy identification. Flies can be incubated and diagnosed both for *S. castrans* conidia and resting spore infection, *E. muscae* infections, and other fungal infections. Catch mostly females, which are the target both for monitoring, direct control, and autodissemination.	Must be emptied quite often to avoid cross contamination. It is more difficult to distiguish *D. radicum* from *D. floralis* when female individuals are used.
Water traps (Finch & Skinner, 1982a; Finch &	Catch high numbers of flies over time with low labor input especially if allylisothiocyanate baited.	Not highly specific. Quite difficult and time consuming to identify flies.

Skinner, 1982b; Finch, 1995; Broatch & Vernon, 1997; Eilenberg & Michelsen, 1999)	Flies can be diagnosed for *S. castrans* conidial infection. Rarely saturated by the numbers of insects caught .	Flies can not be diagnosed for *S. castrans* resting spore infection, *E. muscae* infection or other fungal infections.
Sticky traps (Finch, 1989; Finch & Collier, 1989; Broatch & Vernon, 1997; Eilenberg & Michelsen, 1999)	Catch moderate numbers of flies over time with low labor input.	Not highly specific. Flies sticks to traps and are very difficult and time consuming to identify. Flies with an early *E. muscae* infection might die and dry out before the fungus can develop/ be identified. Ventral side of abdomen sticks to the trap and *S. castrans* infections might be difficult to identify. Become saturated by the numbers of insects caught. Flies might be diagnosed for fungal infection which have developed external symptoms
Sweep-net (Eilenberg & Philipsen, 1988; Eilenberg & Michelsen, 1999)	Flies are caught live and incubated. They can be diagnosed for both *S. castrans* conidia and resting spore infection, *E. muscae* infections, and other fungal infections.	Time consuming to catch enough flies. Difficult and time consuming to identify.

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A molecular method to discriminate between *Entomophthora* species using frozen or alcohol preserved cadavers

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Abstract: Entomophthoralean specific primers were designed for the ITS II and the first part of the LSU rDNA, which enabled the use of DNA extracted from sporulation cadavers, thereby allowing analyses of strains not present *in vitro*. The ITS II region for the studied isolates was very long compared with many other fungi, ranging from 1200 by to 2000 bp. Both the ITS II and the first part of the LSU rDNA showed high variation within the genus *Entomophthora using* PCR-RFLP. Preliminary analyses of the RFLP separate the *E. muscae*-complex into three clusters, supporting the validity of *E. schizophorae and E. syrphi.*

Key words: Entomophthorales, *Entomophthora muscae*, *Entomophthora syrphi*, *Entomophthora schizophorae*, *E. muscae*-complex, PCR-RFLP, genetic variation, *in vivo*, *in vitro*, ITS, LSU, 25S rDNA.

Introduction

The insect pathogenic genus *Entomophthora* sensu stricto includes approximately fifteen species and have been recorded from several orders: Hemiptera, Thysanoptera, Neuroptera, Coleoptera, Hymenoptera and Diptera. The well known species *Entomophthora muscae* is pathogenic to higher Diptera, and is now recognised as a complex of species; the *E. muscae*-complex. Four types are found within the complex, separated by conidial size, nuclear number, nuclear size, and host range (Keller, 1984).

Proliferation and spread of obligate insect pathogenic fungi are dependent on the presence of host insect, therefore knowledge of their host range is important. It is generally believed that species within *Entomophthora* have a narrow host range, but in the laboratory transmission of *Entomophthora* spp. between host families and even orders has been successful (Eilenberg *et al.*,1987). Molecular characters could elucidate the taxonomic significance of morphological and biological features within the genus *Entomophthora* including the *E. muscae*-complex

In vitro isolation of *Entomophthora* spp. is difficult and is reflected by the low number of isolates available in culture collections, like the "Collections of Entomopathogenic Fungal Cultures, ARSEF-USDA". A molecular technique allowing use of *in vivo* material was therefore desirable. Within Entomophthorales the PCR based RAPD (Hajek *et al.*, 1996; Hogdes *et al.*,1995; Rohel *et al.*, 1997) has been used to differentiate between isolates or species, but to use that technique the isolates have to be grown *in vitro.*

Specific PCR followed by cutting the products with restriction enzymes PCR-RFLP (Restriction Fragment Length Polymorphism) was used in this study. Variation in the ITS regions has been found within certain fungal genera (Bridge & Arora, 1998), and within glomalean genera the first part of the LSU rDNA has also been found to vary (Van Tuinen *et al.*, 1998). Entomophthoralean specific primers were designed for those two regions thereby avoiding problems with amplification of non-target DNA.

Material and Methods

Isolates

7 species from *Entomophthora* were represented in the 26 isolates, most from the *E. muscae*-complex: *E. schizophorae* (5), *E. syrphi* (4) and *E. muscae* (12), but E. *thripidum* (1), *E. chromaphidis* (1), *E. planchoniana* (2) and *E. culicis* (1) were also used. The material was either *in vitro* isolates grown in liquid media (GLEN + 5% FBS) or it was *in vivo* as sporulation cadavers preserved in alcohol or freshly frozen. The isolates were from different host orders and different locations.

DNA extraction and Amplification

All the material was freeze-dried before the total DNA was extracted by a method of Bulat *et al.* (1998).

Two PCR-amplifications were carried out for each of the isolates used, amplifying the ITS II (Internal Transcribed Spacer II) and the first part of the LSU rDNA (hereafter termed LSU). For each of the two regions a new designed Entomophthoralean specific primer together with a fungal universal primer were used (Fig. 1). The two new primers were designed using the sequence of the ribosomal repeat from *Entomophaga aulicae* with the genebank accession no: U35394.

For the ITS II the primers were 5.8s-5': TCATCGATGAAGAACGTAGT (this study) and ITS 4: TCCTCCGCTTCTTGATAGC (White *et al.*, 1990) and for the LSU the primers were nu-LSU-0018-5': GTAGTTATTCAAATCAAGCAAG (this study) and nu-LSU-0805-3': CATAGTTCACCATCTTTCGG (Gargas, Unpub.)

The PCR conditions were initial denaturation for 5 min at 96°C, followed by 35 cycles with denaturation for 1 min at 96°C, annealing for 1 min at 55 °C, extension 1 min at 72°C and a final extension 10 min at 71°C.

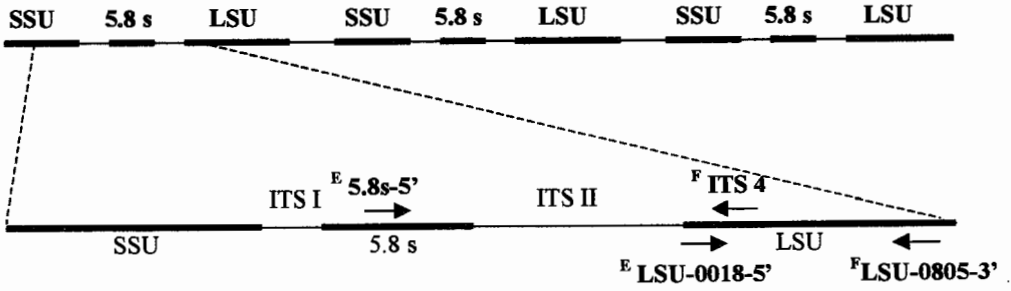

Figure 1. The nuclear ribosomal RNA is coded by three genes: SSU (Small Subunit), 5.8s and LSU (Large Subunit). These three genes, also known as the ribosomal repeat, are sitting in gene clusters, with many copies next to each other. The repeat is transcribed as one unit and the ITS (Internal Transcribed Spacers) are spliced out later. The arrows represent the primers' position and direction. [E]Entomophthoralean specific primer (this study), [F]Fungal universal primer.

RFLP

Each amplification was cut with eight different restriction endonucleases: Alu 1, Hae III, Dde 1, Rsa 1, Cfo 1, Taq 1, Sau 3A, Dra 1 (Boeheringer Mannheim or New England Biolabs)

using 5 µl PCR-product, 1 µl of the recommended buffer, 2 unit enzymes and adding milliQ-H_20 up to 10 µl. The reactions were incubated over night at 37°C, then separated on a 1.5% agarose gel and the fragments were visualised with EtBr.

Cluster analysis

For each enzyme the different fragments' lengths were used as a character and all the fragment lengths (characters) were scored as present or absent for each of the isolates. This gave two data matrix, one for the LSU and one for the ITS II, which were analysed separately. With the program NT-SYS the similarity between the isolates were calculated using the Jaccard coefficient, and from those dendrograms were made using the UPGMA.

Results and Discussion

The new primers for the ITS II and the LSU gave only PCR amplifications when an entomophthoralean fungi was present on the insect, while no specific amplifications were seen when uninfected insects were used (Fig 2. Lane 1-6). This shows that the primer was able only to amplify fungal DNA and did not amplify DNA from insect or other microorganisms associated with it. The specificity of the two new primers thus made it possible to amplify the ITS4 and the LSU from *in vivo* material.

The RFLP-patterns of an *in vitro* culture and of the same insect, from which the *in vitro* culture originally was isolated, were identical (Fig 2. Lane 7-10), showing a high reproducibility between *in vivo* and *in vitro* material.

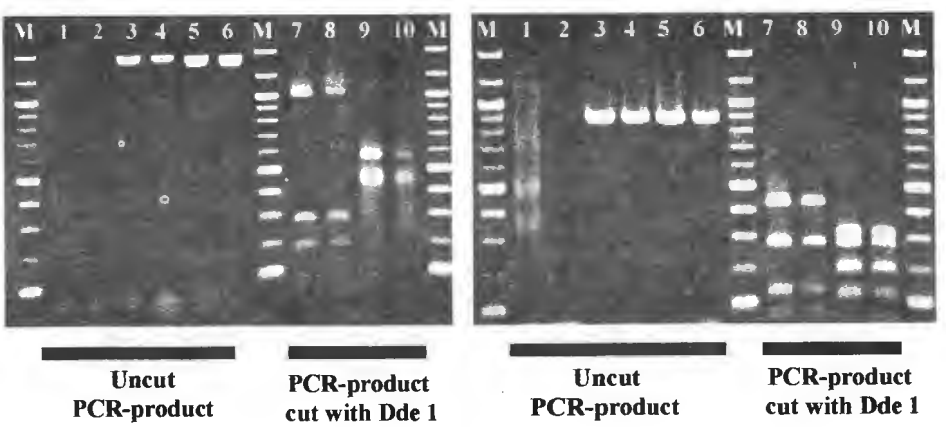

Figure 2: Agarose gel electrophoresis of ITS II-PCR (left) and LSU-PCR (right) and examples of restriction fragment length polymorphism between *E. muscae* and *E. syrphi.* Lane 1: Uninfected *Musca domestica,* lane 2: Uninfected *Delia radicum,* lane 3: *E. muscae in vitro,* lane 4: *E. muscae in vivo* , lane 5: *E. syrphi in vitro,* lane 6: *E. syrphi in vivo,* lane 7: *E. muscae in vitro,* lane 8. *E. muscae in vivo , lane* 9: *E. syrphi in vitro,* lane 10: *E. syrphi in vivo* .The *E. muscae in vitro* (lane: 3 and 7) were originally isolated from the sporulating insect used as the *E. muscae in vivo* (lane: 4 and 8), likewise with the *E. syrphi.*

The fungal specificity of the primers is not yet fully solved. But preliminary studies suggest that the IST II primers are less specific than the LSU primers. Thus the ITS II have

been amplified from fungi belonging to the genera *Entomophthora, Eryniopsis, Entomophaga, Erynia, Zoophthora,* and *Conidiobolus,* and even weak amplifications was detected when using a hyphomycete *Metarhizium anisopliae,* whereas amplifications of the LSU have only succeeded with fungi belonging to genera within the family Entomophthoraceae.

With the described methods we were able to extract DNA and get good PCR amplifications from both alcohol preserved and freshly frozen cadavers, but there was a tendency that the PCR failed when the material had been stored several years in 70% alcohol. This might be due to degeneration of the DNA, but with another extraction method it might be possible to get some quality DNA.

The length of the LSU was the expected 900 by for all the tested *Entomophthora* isolates, but more surprisingly was the length of the ITS II, which range from 1200 bp to 2000 bp. Such a large ITS size polymorphism is not frequent within fungi, but have previously been reported within another entomophthoralean genus; *Erynia* (Rohel et *al.,* 1997).

Preliminary analyses of the RFLP from the ITS II and the LSU gave similar topologies for the two regions, but the distances between the isolates was higher for the ITS II than for the LSU (data not shown). The ITS II is not, compared to the LSU, a part of a functional structure, and thus more mutations will accumulate over time. The isolates from the *E. muscae*-complex grouped into three clusters in our preliminary studies, which supports the validity of *E. schizophorae* and *E. syrphi* as separate species.

This method can provide a new set of characters useful together with morphological and biological characters for identification and validation of species within *Entomophthora,* and could also be a helpful tool in ecological studies.

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Natural occurrence of entomophthoralean fungi on collembolans

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Abstract: A total of 8223 collembolans representing 14 species were incubated. Infection with *Neozygites* or *Neozygites*-like species was recorded in two of the sub classes within Collembola. However the prevalence of *Neozygites* spp. was low, in Symphypleona it was up to 10% in *Sphaeridia pumilis*, 6% in *Sminthurus viridis* and below 1% in *Deuterosminthurus sulphurus*, from Arthropleona only very few specimens of *Orchesella cincta* and *O. villosa* sporulated with *Neozygites* sp.

Key words: Collembola, Entomophthorales, *Neozygites*

Introduction

Very few studies have focused on the natural occurrence of insect pathogens associated with Collembola. At a quantitative level, however several groups of pathogens have been documented to infect collembolans including bacteria, fungi, nematodes, protozoan and virus (Drechsler, 1944; Christiansen, 1964; Purrini, 1983). No well documented observations of infection with obligate entomopathogenic fungi have been reported, until *Neozygites sminthuri* was recently described from *Sminthurus viridis* (Steenberg *et al.*, 1996; Keller & Steenberg, 1997). During 1998 collembolans were collected and incubated to assess the diversity, host range and prevalence of collembolan pathogenic fungi from the order Entomophthorales.

Material and methods

Locality
Collembolans were collected in hedgerows and organically grown clovergrass fields around at the experimental farm of the Royal Veterinary and Agricultural University. The farm is situated at Taastrup, 18 km West of Copenhagen.

Sampling
Collembolans were collected alive by sweep netting in two clovergrass fields every two weeks in July, August and September, pit-fall trapping in three hedgerows (30 traps in each) twice a week during August and September, and extraction from soil cores using a high gradient funnel extractor from May to December.

Incubation
The collembolans were incubated alive for five days in 30 ml. plastic vials with 5 ml of 1.5% water agar on the bottom, a clover leaflet was added as food for the phytophagous species.

Pathogens
Sporulating cadavers were transferred to microscope slides and incubated for one day in water saturated atmosphere. The conidia were stained with lactophenol-cottonblue and examined under light microscope.

Results

A total of 8223 collembolans representing 14 species (Table 1) were incubated. Infection with *Neozygites* spp. were recorded from five different collembolan species representing two of the subclasses within Collembola (Table 1). The highest prevalence were found in the subclass Symphypleona, however the prevalence of *Neozygites* spp. was generally low: up to 10% in *Sphaeridia pumilis*, 6% in *Sminthurus viridis* and below 1% in *Deuterosminthurus sulphurus*. In Arthropleona only very few specimens of *Orchesella cincta* and *O. villosa* were observed to sporulated with *Neozygites sminthuri*-like fungi. Additionally, sporulation of the facultative pathogen *Conidiobolus coronatus* was recorded from several hosts.

Table 1. Total number of collembolans incubated, and number of cadavers sporulating with entomophthoralean fungi

Collembolan species	**Total number incubated**	**Number of sporulating cadavers**		
		Neozygites sminthuri	***Neozygites* spp.**	***Conidiobolus coronatus***
Arthropleona				
Hypogastrura assimilis	195	0	0	0
Onychiurus sp.	52	0	0	0
Mesaphorura sp.	547	0	0	0
Proisotoma minuta	745	0	0	0
Isotoma anglicana	812	0	0	3
Orchesella cinta	225	1	0	1
Orchesella villosa	1088	2	0	4
Lepidocyrtus cyaneus	338	0	0	0
Symphypleona				
Sphaeridia pumilis	375	0	26	0
Sminthurinus elegans	40	0	0	0
Dicyrtoma sp.	110	0	0	11
Deuterosminthurus sulphurus	1384	4	9	0
Sminthurus viridis	2090	37	0	34
Neelipleona				
Megalothorax minimus	222	0	0	0
Total	8223	44	35	53

The morphology of the primary conidia of *Neozygites* spp. varied, splitting into separate groups. One group consisting of *Neozygites sminthuri* (Keller & Steenberg, 1997), while the other potentially represents undescribed species.

Discussion

This study shows that collembolans are infected by a range of *Neozygites*-like fungi, the identity of them is not yet clarified. Infection was mainly found in species belonging to Symphypleona, however sporulation of *Neozygites sminthuri*-like fungi were also recorded for the first time from the subclass Arthropleona. The prevalence of entomophthoralean fungi found in this study was usually low, but further work is needed to assess the prevalence over time. As collembolans often have a life span of about a year (Hale, 1967), even a low prevalence might ad up to play an important role in the population dynamics of collembolans especially species belonging to the subclass Symphypleona.

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On mycoses of phytophagous mites (acari)

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Introduction

Little is known about fungi parasitic on, or otherwise associated with mites, but interest in them is growing, particularly because of their potential as agents for biological control of many pests and disease vectors among these arthropods. Since mites (especially eriophyids) are so small as to be almost invisible to the unaided eye - pathogenic fungi are extremly difficult to observe macroscopically during collection in the field, but only after plant material has been examined carefully in the laboratory.

The most frequently encountered fungal pathogens of mites belong to the genera *Neozygites* of the order *Entomophthorales* and *Hirsutella* of the group *Hyphomycetales* group. These two fungal genera differ not only taxonomically but they also have different biology and pathogenic abilities.

The fungi of the genus *Neozygites* belong to obligatory pathogens and are specific in relation to the host. Till now only one case of *Neozygites* growth on artificial medium was successful (Grundschober *et al.*, 1998). The species of the genus *Hirsutella* seem to be more common pathogens on mites than on insects. They are of special interest because most of their strains can be grown on artificial media and multiplicated by this means for application against pest mites, as e.g. *H. thompsonii* in form of the mycoacaricide called Mycar (McCoy 1996)

Recently a research project has been undertaken by the Agricultural University, Chair of Plant Protection, in Siedlce, Poland within its main aims to determine fungous disease frequency, species composition and significance of these infective agents for regulation of phytophagous mites population.

Material and methods

Research was carried out in 1995-1998 in the vicinity of Siedlce on cultivated and wildgrowing plants from May to November. Field samples were studied for the presence of dead mites which were transferred to microscope slides with lactophenol-aniline blue or acetoorcein and investigated for presence of fungi. Dead mite specimens with mycose symptoms were collected and the strains were isolated on SDA media. Attempts to grow the fungi from *Neozygites* genus on SDA enriched with egg yolk and powdered milk were unsuccessful. For quantitive estimations, mean percentage mortality of mites due to fungi was determined in samples of collected plants containing 50 leaves.

Results

Epizootic occurrence of the entomophthoralen species *Neozygites floridana* was observed on *Tetranychus urticae.* Infected individuals of *T. urticae* were observed from the third week of May, in particular on strawberry and dead nettle to the end of October on many different plants (Tab.I). In mid-September, a mean dencity of 10 mites per leaf of kidney bean was observed and of these 85% were infected by *N. floridana.* Few specimens of *Bryobia* sp. feeding on *Potentilla argentea* infected by this fungus were also found.

New entomopathogenic fungus of the genus *Neozygites* has been found on the eriophyoid mites - *Abacarus hystrix* and *Vasates mckenzie* and the formal description of the pathogen is in press. It is the first case of an entomophthorosis eriophyoid mites. The fungus differs from the other *Neozygites* species affecting mites by small, globose primary conidia, short-ovoid, smoky capilioconidia of delicately rough surface and very short capiliophores-usually not longer than the spore length. This pathogen infected single mite individuals in autumn (from September till first week of November) on *Lolium perenne* and *Agrotis stolonifera* and caused 2-3% of host mortality.

Of entomopathogenic hyphomycetes few cases of *Verticillium lecanii* were found on mites while the greatest bilolgical diversity appeared among the anamorphs ascribed to the genus *Hirsutella. Two* strains of *Hirsutella nodulosa* with strongly rough of mycelium were isolated from the species of Tarsonemidae, Ceamasidae and *Oribatida* species on *Potentilla argentea.* The same fungous species was noted on the strawberry mite *Stenotarsonemus fragariae* on productive strawberry plantations near Skierniewice. Mortality during plant growth season was rather low and increased by the end of October to the level of 13%.

The species *Hirsutella* cf *thompsonii* and *Hirsutella kirchneri* were isolated from *Abacarus hystrix* and *Vasates mckenzie* on *Lolium perenne* and *Agrostis stolonifera* and *Festuca rubra.* The first specimens of these mites infected with *Hirsutella* cf. *thompsonii* appeared just in the second week of September and were noted till the end of October. Mites infected by *H. kirchneri* were observed from the end of June to first week of November.

Aforementioned *Hirsutella* species caused at this time in average about 45-55% mortality of the mites.

Another strain of *H.* cf. *thompsonii synnematosa* was also isolated from pear leaf blister mite *Eriophyes piri,* which diferrs from these isolated from *Abacarus hystrix* by numerous, stout, spirally interwoven white synnemata in old cultures. Its features do not fit, however, the variety *synnematosa* separated by Samson *et al.,* (1980). Probably the differentiation within this species is considerably greater than discussed by these authors and more precise methods for its diversity assessment should be applied in further studies, as e.g. RAPD (Mozes-Koch *et al.,* 1995).

All fungi isolated from mites in this research except *Neozygites floridana* are new for Poland. Researches show that the biodivercity of fungous pathogens of phytophagous mite is considerably smaller than in groups of macroentomofauna as far limited only to the genera *Hirsutella, Neozygites* and occasionally *Conidiobolus* and *Verticillium* species. On the other hand high variability and insufficiently precise taxonomic critera of *Hirsutella* and *Verticillium* species cause serious difficulties in correct and sure determination of strains.

Tab.l. List of entomopathogenic fungi infecting phytophagous mites in Poland

Fungus	Host	Host plant	Term of mycosis
Neozygites floridana	*Tetranychus urticae*	*Phaseolus vulgaris*	20.07 - 20.10
		Cucumis sativus	20.07 - 20.10
		Fragaria grandiflora	20.07 - 20.10
		Zea mays	10.08 -15.09
		Ribes nigrum	10.08 - 20.09
		Lamium album	30.05 - 20.10
	Bryobia spp.	*Potentilla argentea*	5.06 - 25.08
Conidiobolus spp.	*Bryobia* spp.	*Potentilla argentea*	5.06 - 25.08
Verticillium lecanii	Tarsonemidae	*Potentilla argentea*	15.09
Neozygites abacaridis n.spp.	*Abacarus hystrix*	*Lolium perenne*	20.09 - 3.11
	Vasates mckenzie	*Agrostis stolonifera*	
Hirsutella nodulosa	Tarsonemidae		
	Coemasidae	*Potentilla argentea*	15.08 - 20.09
	Oribatida		
	Steneotarsonemus fragariae	*Fragaria grandiflora*	17.06 - 25.10
Hirsutella cf. *thompsonii*	*Abacarus hystrix*	*Lolium perenne*	12.09 - 29.10
	Vasates mckenzie	*Agrostis stolonifera*	
Hirsutella cf. *thompsonii* var. *synnematosa*	*Eriophyes piri*	*Pyrus communis*	21.07 -10.09
Hirsutella kirchneri	*Abacarus hystrix*	*Lolium perenne*	
	Vasates mckenzie	*Agrostis stolonifera*	24.06 - 3.11
		Festuca rubra	

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The pathogenicity of entomopathogenic fungi to *Boophilus annulatus* ticks

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Introduction

The increasing resistance of ticks to pesticides coupled with their high cost and the growing demand for safer animal products and for a cleaner environment are leading animal growers towards a search for alternative methods for the control of ticks. But as yet there exist no commercial bio-control agents, to suppress a tick populations.

One of the most important cattle ticks in wide areas of the world is the one host tick *Boophilus annulatus* (Hoogstraal 1956). Their females feed on cattle and when engorged they drop off and lay eggs on the upper layers of the ground where they remain until the eggs hatch.

Fungi are reported in literature to be major pathogens of ticks because of their wide dispersal, their wide spectrum of hosts and their ability to enter via the cuticle (Samish & Rehacek 1999).

Representatives from only six genera out of the 57 major entomopathogenic fungi (Eumycota, Deuteromycotina) are known to attack (Tanada & Kaaya 1993). However their effect upon the cattle tick *B. annulatus* was not reported as yet.

Materials and methods

Ticks

Boophilus annulatus ticks were collected in 1984 from cattle in Israel and fed every 2 months on Friesian calves. Off-host stages were incubated in the dark at 26°C, 80% RH. The engorged female ticks were tested for fungi susceptibility within 24 hours of repletion.

Fungi

The virulence of 13 strains of enthomopathologic fungi (Deuteromycetes, species: *Beauveria bassiana, Metarhizium anisopliae, M. flavoviride, Paecilomyces fumosoroseus* and *Verticillium lecanii)* to *B. annulatus* eggs, larvae and engorged female ticks were recorded. The spores were harvested by washing the plates with aqueous solution of 0.005% Triton X100 and the spore suspension was filtered through several layers of cheesecloth. The concentration of the spores was determined by a haemocytometer and adjusted to 1x 10^7 spore/ml.

The conidia were harvested after the fungi have been cultivated for 2 weeks at 25°C on malt extract agar.

Bioassay

Engorged female ticks were surface sterilized by dipping into solution of soap following by water, saline solution, then for 5 min in 0.25% Roccal solution and for 3 min in 70% alcohol and rinsed twice in water. The females were incubated in sterile containers in order to obtain fungi free eggs. The eggs or unfed tick larvae were transferred into petri dishes (50 mm diam.) with filter paper, which has been previously moistened with a 0.5 ml suspension of the

fungal conidia and incubated at 25°C for 7 -15 days. Virulence was determined by recording the direct infection of eggs or unfed larvae during 7 days, as well as by the number of larvae hatching from fungi infested eggs after 15 days. Each test consisted of at least 4 dishes with 25-40 eggs or larvae per dish.

Engorged females ticks were immersed into conidia suspensions (1x 10^7 conidia/ml) for 3-5 seconds, transferred to petri dishes with moist filter paper and incubated for 14 days at 25°C. Mortality was recorded daily and the egg mass produced by the females weighed after 14 days of incubation. Each test consisted of at least 4 dishes with 5 female ticks per dish.

Results

Susceptibility tick eggs to fund.

Nine isolates belonging to five species of fungi were tested. The most virulent strains *Beauveria bassiana* and *Metarhizium anisopliae* caused 70-98% mortality of treated eggs. The first signs of infection appeared where the egg darkened 2-3 days post treatment. Microscopic examination indicated that these fungi penetrated through the egg wall and developed within the eggs. Sporulation of *B. bassiana* and *M. anisopliae* on the egg surface began 5-7 days after infection. Larvae hatched from 2-10% of eggs treated with *B. bassiana* and from 10-30% of the eggs treated with M. *anisopliae* in comparison to 85-90% from untreated control eggs.

Strains of *P. fumosoroseus* colonized on the surface of the eggs, but did not penetrate. The growth of *P. fumosoroseus* on the eggs surface didn't prevent larvae hatching.

Susceptibility to fungi of unfed tick larvae

M. anisopliae and *M.flavoviride* strains killed unfed larvae from 1-3 days after the treatment. The infected larvae darkened and became immobile, fungal hyphae inside larvae were observed 48 hours post infestation and sporulation on the larval surface 72 hours after treatment. Within 6 days 80-100% of the larvae died. at a concentration of 10^7 spore per ml and within 3-4 days at a concentration of 10^8 spore per ml. Unfed larvae were resistant to infection by *B. bassiana, P. fumosoroseus* and *V.lecanii.*

Susceptibility to fungi of engorged female ticks

Most of the tested fungi strains killed *B. annulatus* females within 3-14 days after treatment. Mortality of infected females on day 7 ranged between 10 to 85%, while untreated control ticks mortality was only between 0 to 15%. The *M. anisopliae* strains were most virulent and caused 85-100% mortality within 5-10 days at the concentration of 1 x 10^7 spore per ml and within 3-6 days at the concentration 1 x 10^8 spore per ml. The first signs of infection appeared as hemorrhages below the cuticle and as external exudate 3-5 days post treatment. The less virulent strains *B. bassiana* and *P. fumosoroseus* caused the mummification of the female ticks.

With many of the tested fungi the female ticks stopped laying eggs already several days before their death. *M. anisopliae* strains were most efficient in reducing egg production. At a concentration of 1 x 10^5 conidia per ml several *M. anisopliae* strains reduced egg production by 2/3. With higher concentrations no eggs were produced.

Female ticks treated with either of the 3 *M. anisopliae* strains and incubated at 20°, 25° or 30°C died at a similar rate. Strains of other fungus genera were more efficient at 30°C than at 20° or 25°C.

Discussion

The most commonly investigated entomopathogenic fungi are *M. anisopliae* and *B. bassiana* (Deuteromycotina), because of their wide geographic spread an host range. *M. anisopliae* seems superior to *B. bassiana,* their strains cause 0 to 96% tick mortality (Castineiras *et al.,* 1987, Mwangi *et al.,* 1995, Barci 1997). All experiments reported here demonstrate that out of the 4 fungi genera tested against *B. annulatus*, *M. anisopliae* is the most virulent. Dipping *Boophilus microplus* eggs in 1 x 10^8 conidia/ml of either fungi *M. anisopliae* or *B. bassiana* or engorged females in 2.7x10^8 *M. anisopliae* caused 96-100% mortality (Gorshkova 1966, Castineiras *et al.,* 1987, Bittencourt *et al.,* 1994, Barci 1997, Zhioua *et al.,* 1997). It seems that *B. annulatus* ticks is either even more susceptible than *B. microplus* or the fungi strains used in our experiments were more virulent to ticks. Fungi from the genera *Beauveria* and *Metarhizium* are used increasingly in commercial amounts against insects. Only few field experiments to kill ticks with fungi were performed as yet (Correlia *et al.,* 1998). Their value as commercial tick control agents has still to be proven.

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Fungi associated with the coffee berry borer, *Hypothenemus hampei*

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The coffee berry borer, *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae; CBB), is a serious pest of coffee throughout the world. Endemic to Central Africa, it has now spread to most coffee growing regions. The female bores a hole into the berry where she deposits her eggs; larvae feed on the endosperm, lowering the quality of the berry. For the past two years, the European Biological Control Laboratory (EBCL) has been conducting foreign explorations for natural enemies of the CBB, mainly in Africa. So far, Togo, Ivory Coast, Benin, and Uganda have been visited with additional journeys planned to India, South Africa and Zambia.

In the field, coffee berries showing the characteristic hole bored by the female were individually collected and placed in plastic jars. Back at EBCL's quarantine facility, the berries were placed in sealed cardboard canisters with an opening at one end containing a translucent plastic tube, to which emerging insects are attracted. Tubes were examined daily and insects other than CBB were mounted for later identification, while CBB's were collected and placed with their dorsum touching the surface of water agar in sealed Petri dishes, in order to promote growth of any fungal spores carried by the insect.

The following fungi have been isolated from CBB's: *Aspergillus flavus, A. niger, A. ochraceus, Fusarium* spp., *Penicillium chrysogenum, P. brevicompactum* and *Verticillium* spp., in addition to the fungal entomopathogens *Paecilomyces farinosus, P. lilacinus,* and *Beauveria bassiana.*

The CBB-isolated *P. farinosus* and *B. bassiana* were used in experiments testing six different C:N ratios for liquid medium production. The CBB-*P. farinosus* strain outperformed all other fungal entomopathogens, including some commercially available strains. Experiments are currently underway to test dessication tolerance of the CBB - P. *farinosus* strain.

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Laboratory evaluation of *Beauveria bassiana* (Bals.) Vuill. and *Beauveria brongniartii* (Sacc.) Petch against the four eyed spruce bark beetle, *Polygraphus poligraphus* (L.)(Coleoptera, Scolytidae).

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Abstract: Sensitivity of *Polygraphus poligraphus* against *Beauveria bassiana* and *Beauveria brongniartii* was tested in laboratory experiments. *B. bassiana* was significantly more effective than *B. brongniartii* in nearly all cases if beetles had limited contact to fungus treated bark pieces and after direct inoculation of beetles (at 20°C). Even short contact periods caused high infection rates. Incubation of beetles at three different temperatures (10-13°, 20-23° or 30-33°C) caused highest infection rates in most cases after inoculation with *B. bassiana* spore powder or spore suspension, mean life span of beetles was longest at 10-13°C. Mean time to death was short in *P. poligraphus* after inoculation with different spore powder concentrations especially with the highest dose ($5x10^6$ conidia/g).

Key words: *Beauveria bassiana, Beauveria brongniartii, Polygraphus poligraphus*, Scolytidae; efficacy, temperature, dose; laboratory tests.

Introduction

The insect pathogenic fungus *Beauveria bassiana* can be found in distinct bark beetle species (Balazy, 1966; Mills, 1983; Wegensteiner *et al.*, 1996). Laboratory experiments provided high fungus infection rates in most cases testing *B. bassiana* against *Ips typographus* (Neuzilova, 1956; Matha & Weiser, 1985; Wegensteiner, 1992; 1996), *Pityogenes chalcographus* (Wulf, 1983) and against *Trypodendron lineatum* (Prazak, 1991; 1997). *B. brongniartii* is known from soil living insects especially from beetles of the family Scarabaeidae (Müller-Kögler, 1965; Burge, 1988; ref. in Samson *et al.*, 1988; ref. in Krieg & Franz, 1989).

The idea for testing *B. bassiana* and *B. brongniartii* against *P. poligraphus* is of basic and of practical interest. Permanent bark beetle outbreaks in distinct European regions and the lack of efficient control measures are some of the main problems in forestry since decades. There exist several reports that bark beetles hibernate in the bark of trees as well as in soil. Mass occurrence of *Polygraphus poligraphus,* normally known as a "secondary" pest species (attacking only dying trees), in several Austrian localities were the reason to check the sensitivity of this species to these two *Beauveria* species.

One of the expected goals of these experiments was to gain information on the sensitivity of this bark beetle species for potential use of entomopathogenic fungi in control measures. Furthermore, it was of interest if it might be possible to provide conidia for inoculative or augmentative release of entomopathogenic fungi in bark beetles' habitat with special focus on more aggressive ("primary") bark beetle species (e.g. *Ips typographus)* using *P. poligraphus* as an alternative host to keep entomopathogenic fungi in a local bark beetle population.

Material and Methods

Adult *Polygraphus poligraphus* freshly emerged from log sections (from a standing infested tree from a locality near Flatz, Lower Austria) were used for three different infection experiments. The conidiospores had been produced in the Czech Republic (*B. bassiana*-strain 90311/48 and *B. brongniartii* strain 256/29288) by the method of Samsinakova et al. (1981).

In a first experiment four groups of beetles were exposed to spruce-bark pieces (size: 4x5cm), treated with *B. bassiana* or *B. brongniartii* spore powder ($5x10^7$ conidia/cm^2), alternatively for 1 minute, 10 minutes, 60 minutes, 24 hours. One group was inoculated by dipping the beetles into *B. bassiana* or *B. brongniartii* spore powder ($3x10^{10}$ conidia/g). All variants were incubated at 20°C (± 1°C). One group remained totally untreated as a control.

In a second experiment three times two groups of beetles were inoculated with *B. bassiana,* either spore powder ($3x10^{10}$ conidia/g) or spore suspension ($1x10^7$ conidia/ml), and were incubated at three different temperatures, 10-13°, 20-23° or 30-33°C. One untreated group was used as a control at each temperature.

In a third experiment four groups of beetles were inoculated by dipping them into four different *B. bassiana* spore powder concentrations alternatively ($5x10^6$, $5x10^5$, $5x10^4$ and $5x10^3$ conidia/g); they were incubated at 23°C (± 1°C). One group remained untreated as a control group.

The beetles of each variant were incubated together with some spruce bark pieces, without light at 93.5% to 91.0% relative humidity using saturated potassium nitrate. Mortality was recorded daily, removing dead beetles, till the death of the last beetle. Dead beetles were incubated in moist chambers (at room temperature) to propagate conidia production. The daily recorded mortality data were used for homogeneity analysis according to Kolmogoroff and Smirnoff. Differences in numbers of infected beetles were calculated using Chi^2 test.

Results and discussion

Beetle mortality after inoculation with B. bassiana or B. brongniartii spore powder

Beetle mortality corresponded in the *B. bassiana* inoculated groups with time of beetles' contact to spore powder, short contact to treated bark pieces yielded significantly slowest mortality in the beetles of the 1min. and 10min. group in comparison with beetles from all other groups ($p< 0.001$). No differences were found between the 60min. and the 24hrs. group ($p> 0.05$), powder inoculated beetles died significantly faster in comparison with all variants after limited contact to treated bark ($p< 0.05$ to $p< 0.001$). Infection rates were significantly lowest in the beetles of the 60 min. group in comparison with all other *B. bassiana* groups respectively with the *B. bassiana* powder inoculated variant ($p< 0.001$) (tab. 1).

No differences were found in mortality data within the *B. brongniartii* variant between the 1min., 10min. and the powder inoculated groups ($p> 0.05$), and between the 60min., 24hrs. and the powder inoculated groups ($p> 0.05$). Significant differences in beetle mortality were found only between in the short time contact groups (1min. and 10min.; MLS >5days) and the 60min. and 24hrs. groups (MLS <5days) ($p< 0.001$). No significant differences in infection rates were found between all the different *B. brongniartii* groups ($p> 0.05$) (tab. 1).

No statistical differences were found in mean time to death between the two *Beauveria* variants with one exception only (between the two 1min. groups; $p> 0.05$). In all other cases *B. bassiana* caused more rapid mortality than *B. brongniartii* ($p< 0.001$). No differences in infection rates could be found only after 60min. contact with spore powder treated bark pieces ($p> 0.05$), in all other cases the infection rates in the *B. bassiana* groups were significantly

higher ($p < 0.001$) (tab. 1). Beetles in the control group lived significantly longer than in all inoculated variants ($p < 0.001$) (tab. 1).

Table 1: Mean life span (MLS) in days and mortality by mycosis (% inf.) in *P. poligraphus* (n) after beetle inoculation by limited exposure to *B. bassiana*- and *B. brongniartii* spore powder treated bark pieces (contact periods: 1 minute (1 min), 10 minutes (10 min), 60 minutes (60 min), 24 hours (24 hrs) and in powder inoculated beetles (pow.) and in an untreated control (contr.; % spontaneous infection) at 20°C (± 1°C).

	Beauveria bassiana					*Beauveria brongniartii*					contr.
	1 min	10 min	60 min	24 hrs	pow.	1 min	10 min	60 min	24 hrs	pow.	
MLS	5.1	4.4	4.0	4.0	3.8	5.5	5.3	4.6	4.5	5.0	22.3
% inf.	97.0	97.0	72.0	100.0	98.9	81.3	81.4	70.1	69.6	76.5	10.9
n	100	100	100	91	94	91	102	107	102	98	46

It was shown that *B. brongniartii* can cause infections in *P. poligraphus* but that this species is less effective than *B. bassiana*. Even a brief contact to treated bark pieces for 1 min. and 10 min. caused high infection rates. Wegensteiner (1992) was also able to observe highest *B. bassiana*-infection rates in *I. typographus* as well as very short mean life spans even after brief periods of beetle contact with treated bark pieces (1 min. to 24 hrs.).

Beetle mortality after inoculation with B. bassiana spore powder or spore suspension at three different temperatures

Beetles died significantly earlier after spore powder- than after spore suspension inoculation ($p < 0.001$) in the 10-13°C variant. No differences could be found between these two variants at 20-23°C and 30-33°C ($p > 0.05$). Comparing the mortality data within the spore powder inoculated groups no differences could be found between beetles in the 20-23° and 30-33°C group ($p > 0.05$) but both higher temperatures caused significantly faster mortality than in both 10-13°C groups ($p < 0.001$). Similar results were found in the variants after inoculation with spore suspension. Beetles in the control groups lived significantly longer in all cases ($p < 0.001$) (tab. 2).

No differences were found in infection rates of the two variants at 10-13°C ($p > 0.05$), a significantly higher infection rate was found in the spore powder inoculated beetles at 20-23°C ($p < 0.01$). Spore suspension inoculation caused a significantly higher infection rate at 30-33°C ($p < 0.001$). No statistical difference in infection rates was found between 10-13° and 20-23°C spore powder inoculated groups ($p > 0.05$), the low infection rate in the 30-33°C spore powder group was significantly different from the infection rates in beetles at both lower temperatures ($p < 0.001$). A significant difference was found within the spore suspension variant only between the 20-23° and 30-33°C groups ($p < 0.05$). Infection rate was relatively high in the 10-13°C control group (tab. 2).

Table 2: Mean life span (MLS) in days and mortality by mycosis (% inf.) in *P. poligraphus* (n) after beetle inoculation with *B. bassiana*-spore powder (s.p.) or spore suspension (s.s.) and in an untreated control (contr.; % spontaneous infection) at three temperatures (ranging from: 10-13°, 20-23° and 30-33°C).

	10 - 13°C			20 - 23°C			30 - 33°C		
	s.p.	s.s.	contr.	s.p.	s.s.	contr.	s.p.	s.s.	contr.
MLS	7.2	12.2	9.6	4.5	4.7	7.2	4.8	4.7	8.4
% inf	100.0	95.8	20.6	99.3	93.4	8.7	70.9	100.0	3.8
n	66	71	86	167	158	69	79	78	79

Range of temperature during the experiments (3°C) is most probably too wide to allow exact conclusions about effects of temperature on efficacy of *B. bassiana*. The higher inoculation dose using spore powder was shown to be important at 10-13° only. 20-23°C seem to be best for efficacy of *B. bassiana*. Temperatures above 30°C may have negative consequences on efficacy of spores extenuated partly by the presence of sufficient contact water 30-33°C, as found in the suspension variant. Similar results are reported from *I. typographus* testing *B. bassiana* at different temperatures (Wegensteiner 1992; 1996). Biotic factors, especially temperature and humidity are known to play a key role in the efficacy of insect pathogenic fungi (ref. in Müller-Kögler, 1965; ref. in Feng *et al.*, 1994). Temperatures above 30°C are known to affect *B. bassiana* as well as bark beetles adversely. The range from 10° to 30°C is generally known as being tolerable for both *I. typographus* (Postner, 1974) and *B. bassiana* (ref. in Feng *et al.*, 1994).

Mortality of beetles after inoculation with different spore powder concentrations

Beetle mortality was significantly faster in the group inoculated with the highest spore powder dose ($p < 0.001$) only, no differences were found between mortality data of all other groups ($p > 0.05$). Beetles in the control group lived significantly longer in all cases ($p < 0.001$). Infection rates were significantly highest in the group with the highest inoculation dose ($5x10^6$; $p < 0.001$), no differences were found between all other groups ($p > 0.05$) (tab. 3).

Table 3: Mean life span (MLS) in days and mortality by mycosis (% inf.) in *P. poligraphus* (n) after beetle inoculation with four different *B. bassiana*-spore powder concentrations and in an untreated control (% spontaneous infection) at 23°C (± 1°C).

conidia/g	5×10^6	5×10^5	5×10^4	5×10^3	control
MLS	3.2	4.7	4.9	4.9	22.3
% inf.	99.4	92.2	87.0	92.9	10.9
n	179	141	154	155	46

Even low spore concentrations caused high infection rates in contrast to results with *I. typographus* (Wegensteiner, 1996). Inoculation dose may play an important role, but all doses tested affected infection rates of 87% and more. The reason might be that tested doses were too high or/and virulence of this *B. bassiana* strain was too high that individual distinctions in vitality of *P. poligraphus* played a negligible role.

Especially the *B. bassiana* spore preparation was shown to have a good virulence on *P. poligraphus*. The high *B. bassiana*-infection rates in all experimental groups emphasise especially this entomopathogenic fungus species. The discrepancy between laboratory and field tests known also from other experiments with distinct entomopathogenic fungus species (ref. in Bathon, 1991; Tanada & Kaya, 1993) forces to continue these investigations under field conditions. It is expected that mortality will be delayed and infection rates will be lower under field conditions than in the laboratory experiments. This fact is of eminent importance from the practical point of view even for selecting application strategies in field. Augmentation of entomopathogenic fungi in spruce bark beetle habitats seem to be theoretically possible "using" *P. poligraphus* as an alternative bark beetle host. However, special attention must be paid on application strategies because of unspecific effect of *B. bassiana* against distinct, beneficial and indifferent insect species.

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Preliminary investigations on the use of *Beauveria bassiana* (Bals.) Vuill. and other control methods against the bark beetle *Ips typographus* L. (Col., Scolytidae) in the field

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Abstract: The bark beetle, *Ips typographus* (Coleoptera, Scolytidae), is considered to be one of the most important insect pests of spruce in Europe. Effective and environmentally safe, longterm biological control methods are still lacking. Our work describes investigations to assess the suitability of the entomopathogenic fungus *Beauveria bassdana* (Hyphomycetes, Moniliales) for its integration into an autodissemination technique by which *I. typographus is* attracted to a pheromone lure and dosed subsequently with conidia in a specially designed trap. Contaminated bark beetles leave the trap and carry the pathogen into the pest population. With respect to a combination of *B. bassiana,* also other control methods, such as the mineral dust silicic acid, the plant extract Neem Azal T/S® and the insecticides Karate WG Forst® and Fastac® were tested. In lab experiments, all tested reisolates of *B. bassiana* showed a high virulence against *I. typographus.* However, the mortality and mycosis of the beetles varied depending on the bioassay method. Therefore, a suitable, standardised method was developed. The best reisolates causing the highest mortality within one week were Bba Z, originally isolated from *I. typographus,* and the strain from the industrial product Boverol® (Bba 138). The mineral dust and the insecticide Karate WG Forst® induced a 100% beetle mortality within three days at 25°C, the plant extract Neem Azal T/S® suppressed the beetle fertility. Based on these laboratory investigations, preliminary field experiments on the treatment of nuptial chambers and on the combination of pheromone traps with *B. bassiana* were conducted. In the nuptial chamber test, the pathogen induced a high beetle mortality within one week and a mycosis rate of 91%. In first field tests with pheromone traps and *B. bassiana* a high mortality up to 100% within six days and a mycosis rate of 70-96% were noted. In one of the experiments, traps were located on flight cages with spruce trunks in order to study the behaviour of the infected beetles. Beetles contaminated with Boverol® died before reaching the spruce trunks or formed only reduced brood systems.

Key words: *Ips typographus, Beauveria bassiana,* alternative control methods, nuptial chamber, pheromone trap, flight cages, biological control, autodissemination method

Introduction

The bark beetle *Ips typographus is* considered to be one of the most important insect pests of spruce in Europe. During an outbreak it is able to induce an extensive destruction of spruce stands. In the national park 'Bavarian Forest' the beetle killed more than 30% of the high plateau spruce forest in the last years. Experts are still talking about a progradation.The entomopathogenic fungus *Beauveria bassiana is* a well known, naturally occurring and widely distributed antagonist of *I. typographus* (Wulf 1979; Bathon 1991; Wegensteiner

1992; Nierhaus-Wunderwald 1993). Although there are a lot of laboratory investigations, the use of this fungus to control the beetles in the field is still not verified.

Our work describes preliminary investigations on the use of *B. bassiana* and other control methods against the bark beetle *I. typographus* in the field. The basic principle is the so called „trap and release or autodissemination-method" (Figure 1), by which the bark beetles will be dosed with fungal conidia in a specially designed pheromone trap and then may leave the trap (Pelletal. 1993; Zimmermann 1994). Our aim is to infect the beetle with the pathogen as a vector, so that it can infiltrate the pest population and cause a so called "snowball effect" (Vaupel & Zimmermann 1996; Kreutz & Vaupel 1999).

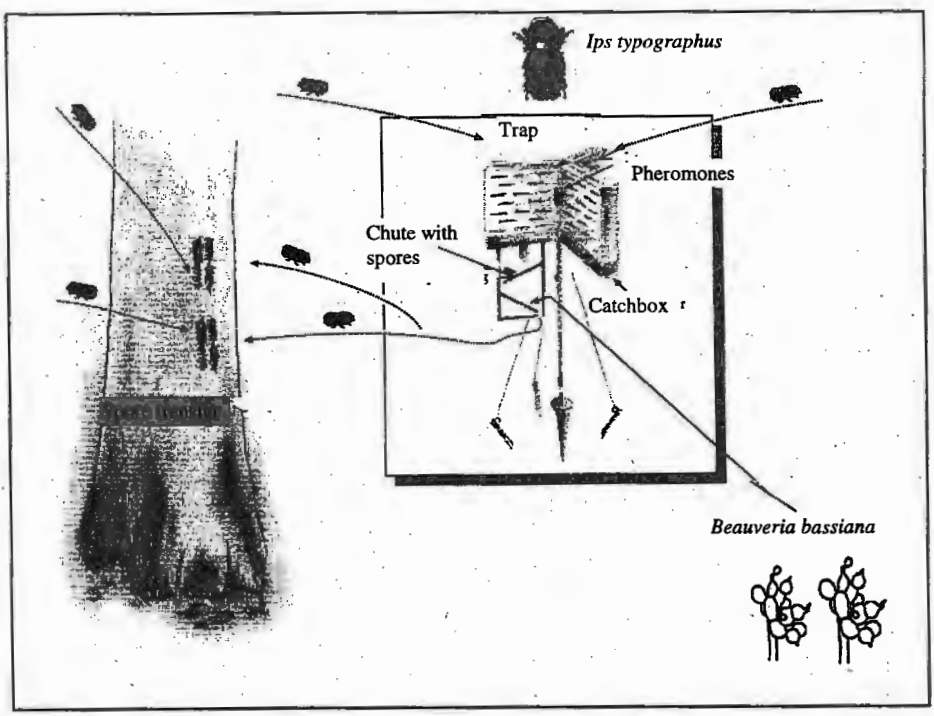

Figure 1. Trap and Release-Method -Infection of *Ips typographus* in a pheromone trap and transfer of the pathogen into the pest population

Material and methods

Laboratory experiments

Various bioassay methods were developped using different substrates, spore application methods, temperatures, rel. humidities and spore concentrations. Finally, the standard test was: 20 bark beetles from a rearing (10 beetles per petridish), three repetitions, 25°C and 70% R.H., darkness and a duration of 14 days. Four strains of *B. bassiana* and two of *Metarhizium anisopliae* were tested (Table 1). The *B. bassiana* reisolates are the strains Z and 136 from *I. typographus* and two isolates from the industrial products Boverol® (Fytovita) and Conidia® (AgrEvo).

With respect to a combination of *B. bassiana,* also other control methods such as the mineral dust silicic acid, the insecticide Karate WG Forst® and the plant extract Neem Azal T/S® were tested. The aim is an artificial weakening of the bark beetles in order to increase the effect of the pathogen.

Table 1. Reisolates of *Beauveria bassiana* and *Metarhizium anisopliae* tested in the laboratory (Bba = *Beauveria bassiana,* Ma = *Metarhizium anisopliae)*

Strain	Source	Host insect	Geographic origin
Bba 134	AgrEvo Conidia®	-	Columbia
Bba 136	Zimmermann	*Ips typographus*	Germany
Bba 138	Fytovita, Co Ltd Boverol®	-	Czech. Repub.
Bba Z	Kreutz	*Ips typographus*	Germany
Ma 43	Müller-Kögler	*Carpocapsa pomonella*	Austria
Ma Z	Rupprecht	*Ips typographus*	Germany

Field experiments

In summer 1998, first field experiments were carried out in the forest district `Reinhardswald' in a one hundred year old spruce stand in the north of Kassel (Germany). Based on the laboratory results, these experiments were concentrated on the treatment of nuptial chambers with *B. bassiana,* on a pheromone trap test with a box and a pheromone trap test in flight cages.

Treatment of nuptial chambers

First, nuptial chambers in laying spruce trunks were treated in order to control the effect of the fungus and some stressors and a combination of both in the field. In 1-m trunk pieces, 15 bore holes were treated with silicic acid, Boverol®, a Boverol®/Silicic acid-mixture, the insecticide Fastac® (0,001 %) and a Boverol®/Fastac®-mixture. Untreated nuptial chambers were used for control. After five days the bark pieces were punched out and the brood systems were controlled for dead and mycosed beetles.

Pheromone trap test with a box

In order to test the mortality due to the fungus in the field, a commercial pheromone trap was combined with spores of *B. bassiana* attached on special chutes below the trap (Figure 1). The attracted beetles fell through the pheromone trap, they were contaminated with the pathogen, and then collected in a white, wooden box. In this experiment, conidia produced on maltextract-peptone-agar and freeze dried and air dried blastospores produced in a liquid culture of *B. bassiana* Z were used. In order to control the mortality and mycosis rate, the box was emptied daily and the captured beetles were put in petridishes on filterpaper. Dead beetles were transferred in a moist chamber to control the mycosis.

Pheromone trap test in flight cages

A pheromone trap with chutes was fixed on flight cages with a size of 2 x 3 x 2m. These were installed in a spruce stand to observe the behaviour of the contaminated beetles. One chute was combined with Boverol® and the other was used as a control. Every cage contained four lm spruce trunks as brood substrate for the beetles leaving the trap. After two weeks the trunks were brought in a greenhouse at 25°C. Six weeks later, bark pieces of 20 x 50 cm were punched out to check the formation of brood systems and to count the number of dead and mycosed beetles.

Results and discussion

Laboratory experiments

All tested strains of *B. bassiana* had a high virulence against *I. typographus*. Mortality and mycosis varied depending on the bioassay method. The best method was: Beetles three days on a diet, dipped in 1 x 10^7 spores/ml and then transferred on bark at 25°C and 70% R.H.. In the virulence tests, the strain Bba Z and the isolate from Boverol® (Bba 138) had the highest virulence. Silicic acid and Karate WG Forst® resulted in 100% beetle mortality in three days, and Neem-Azal T/S® suppressed the beetle fertility within four weeks.

Field experiments

Treatment of nuptial chambers

With 38 maternal, 105 larval galleries and 80 pupal chambers, the most and biggest brood systems were found in the control (Table 2). The insecticide Fastac® showed a strong toxic effect because no brood systems could be found. Silicic acid also showed a good effect. Although the beetles treated with Boverol® formed well developed brood systems, the contamination resulted in a high mycosis rate of 91%. In the combination of silicic acid with Boverol® the effect is comparable to the insecticide.

Table 2. Effect of Boverol®, Silicic acid and Fastac® alone and in combination on *Ips typographus* after treatment of the nuptial chamber in spruce trunks in the field

Treatment	Samples	Beetles (dead)	Mycosis (%)	Brood system			
				Nupt. chamb.	Mat. gall.	Larv. gall.	Pup. chamb.
Control	15	18 (10)	-	16	38	105	80
Silicic acid	15	12 /10)	-	8	14	13	4
Boverol®	15	27 (22)	91	14	31	42	20
Boverol®/ Silicic acid	15	9 (5)	80	12	27	2	-
Fastac®	12	17 (16)	-	10	22	-	-
Bov®/Fast®	14	19 (19)	5	12	20	-	-

Pheromone trap test with a box

After six days, 100% of the conidia-contaminated beetles, 88% of those treated with freeze dried blastospores and 76% of those treated with air dried blastospores were dead (Figure 2). The control mortality was about 28%. The highest number of mycosed *I. typographus* (96%) was observed after conidia treatment (Figure 3). The rate of mycosed beetles treated with freeze-dried blastospores was 88% and with air-dried ones 70%.

Pheromone trap test in flight cages

The results on the combination of Boverol® with the pheromone trap are presented in Table 3. In the eight punched bark pieces we only found four bore holes, while in the control 24 were counted. Therefore, after contamination with Boverol® only few beetles were able to form brood systems. The effect of Boverol® is also shown by the size of the maternal galleries with an average length of 3,7 cm in the contaminated beetles and 13,75 cm in the control.

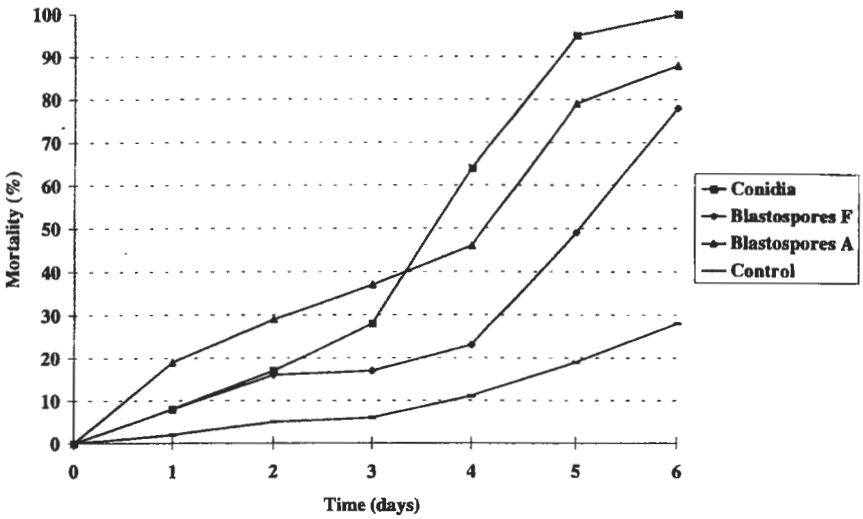

Figure 2. Effect of blastospores and conidia of *Beauveria bassiana* Z on *Ips typographus* in a pheromone trap (F = freeze dried, A = air dried)

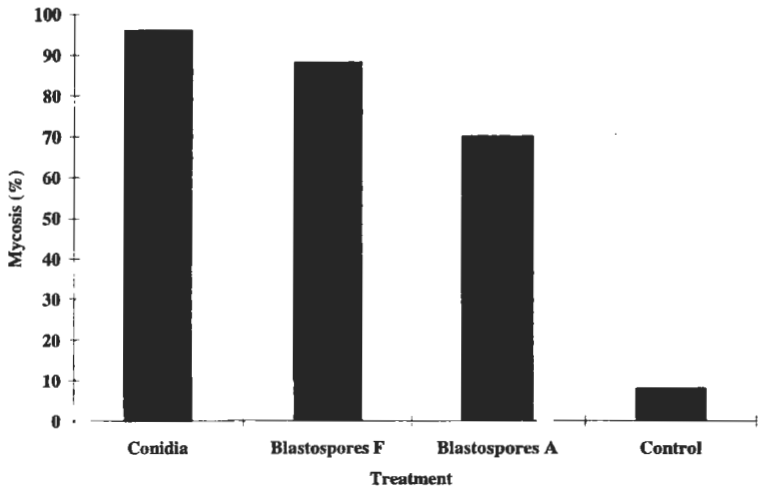

Figure 3. Number of mycosed *Ips typographus* after treatment with *Beauveria bassiana* Z in a pheromone trap (F = freeze dried, A = air dried)

Table 3. Number of beetles and brood systems in spruce trunks after contamination of *Ips typographus* with Boverol® in flight cages in the field.

Treatment	Bore holes	Number of beetles (dead)				Brood system	
		Larvae	Pupae	Juveniles	Adults	Mat. gall. (n/cm)	Pup. chamb. (n)
Boverol® (4 trunks)	4	1 (1)	22 (10)	24 (15)	5 (4)	5/3,7	68
Control (4 trunks)	24	43 (3)	348 (71)	114 (15)	40 (8)	67/13,75	617

The laboratory experiments showed that *B. bassiana,* silicic acid and the insecticide Karate WG Forst® caused a high mortality of *I. typographus.* After combination of conidia or blastospores of *B. bassiana* with a pheromone trap, the same results were obtained. First experiments with pheromone traps in flight cages had shown that the beetles which were contaminated with Boverol® died before reaching the spruce trunks or formed only reduced brood systems. Therefore, our laboratory results were confirmed in the field experiments. In the next step, we are investigating if a transfer of spores occur from contaminated to healthy beetles, e.g. during the copulation, and if we can optimize the autodissemination effect by combining the pathogen with silicic acid, Neem Azal T/S® or a low dosed insecticide. If there is no or only a limited pathogen transfer, the killing of beetles after leaving the trap may be interesting and important for economical reasons, because regular evacuations of the traps are cost and labour intensive. Further field trials in this summer are planned.

Acknowledgement

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Characterization of *Beauveria brongniartii* by the BIOLOG™-system

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Abstract: The monitoring of virulence is a central problem in the commercialization of entomopathogenic fungi as biological control agents. Negative physiological changes such as the attenuation of virulence of those fungi make it difficult to standardize the products and cause in consequence expenses in production and sales costs. Currently the control of the efficacy of biological control agents to their host organisms can only be evaluated by time consuming bioassays. Therefore, the goal of our research is to test simple and quick screening techniques such as the microtiter plate system BIOLOG™ to accelerate the identification of virulent entomopathogens.

The BIOLOG™ system has already been used successfully for characterizing microorganisms. With the help of the two BIOLOG™ test systems SF-N and SF-P the carbon utilization patterns of six *Beauveria brongniartii* strains (highly and less virulent) have been studied. Furthermore, the BIOLOG™ system has been evaluated if it is suitable to indicate virulence and non-virulence of the strains. The feasibility of this method as a prospective tool for the screening for virulent entomopathogenic fungi is discussed.

Keywords: *Beauveria brongniartii,* strain-identification, enzyme-profile, virulence, BIOLOG

Introduction

Entomopathogenic fungi become more and more important as effective biological control agents. Applications of hyphomycetes demonstrated their promising economic potential as ecologically beneficial alternatives to pesticides (Samson *et al.,* 1988). However, in order to take advantage of entomopathogenic fungi as registrated mycoinsecticides, basic knowledge on their physiology and on the mode of infection is still needed.

The monitoring of virulence is a central problem in the registration process and the commercialization of entomopathogenic fungi as biological control agents. Negative physiological changes such as the loss of virulence make it difficult to standardize the products and cause in consequence expenses in production and sales costs. Currently the control of the efficacy of biological control agents in the field as well as the quality control of the final product can only be evaluated by time consuming bioassays. Furthermore, in the screening for virulent strains the identification of entomopathogenic fungi is still based on classical taxonomic characteristics such as conidium morphology and conidiogenensis (Fargues *et al.,* 1981). This makes it sometimes - e. g. in the case of *Beauveria* - difficult to assign isolates unequivocally to one species. Therefore, the goal of our research is to test simple and quick screening techniques such as the microtiter plate system BIOLOG™ to accelerate the identification of virulent entomopathogens. Furthermore, the results of this study should contribute to the development of cheap media for mass production.

The BIOLOG™ system has already been used successfully in different fields for characterizing bacteria, yeast and fungi (Seifert *et al.,* 1997). It consists of a 96 well microtiter plate with 95 different carbon sources and one reference well without any carbon source. Different types of microtiter plates are available. The SF-N and SF-P MicroPlates™ offer the

possibility to characterize the biochemical profile of filamentous fungi towards 129 different carbon sources. The utilization of a specific carbon source is detected by reading turbidity in each well (Kiil & Sasa, 1997).

With the help of the two BIOLOG™ microplates SF-N and SF-P the carbon utilization patterns of six more or less virulent *Beauveria brongniartii* strains have been measured. Furthermore, the carbon utilization patterns have been evaluated if they are suitable to indicate virulence and non-virulence of the strains.

Materials and Methods

Strains

In this study *B. brohgniartii* strains IMBST 95041, IMBST 95031, IMBST 97011, IMBST16694 (isolated from soil samples of cockchafer infested pastures in Kramsach/Austria) and the Swiss strains W 4574 and CH I (both obtained from FAL-Reckenholz/Zurich in Switzerland) were used. The virulent strains IMBST 95041, IMBST 95031 and W 4574 are production-strains for biological control agents. Strain CH I is characterized as a less virulent strain. The strains were cultivated on Sabouraud-2%-Glucose plates at 25°C till spores could be harvested.

Characterization by microtiter plate system BIOLOG™

The microtiter plate test-system BIOLOG™ was prepared according to the manufacturer's instructions. A spore suspension in 0.2% Carageenan Type II solution with an OD_{590} of about 0.22 AU was prepared. After diluting the spore suspension tenfold for each strain SF-N and SF-P Biolog MicroPlates™ were inoculated with 100 µ l per well. The plates were incubated for 3 days at 25°C and 60% moisture. Turbidity (at 590 nm) in each well was measured by using the BiologMicroStation™ system microplate reader. For each strain the test system was replicated four times.

Results were calculated by substracting the absorbance of the reference well from each well. The reference well represents the "back-ground" absorbance from the spore suspension. Finally, the results are represented in percent-utilization by setting the second highest absorbance to 100%. Furthermore, a data base was built up by using the BiologMicroLog™ system software in order to determine the similarity of the utilization patterns between the different *B. brongniartii* strains.

Results

Carbon utilization profile

For all tested *Beauveria*-strains rather homogenous carbon utilization profiles could be determined by measuring the turbidity of each well. However, the less virulent strain CH I showed only at 7 carbon sources a remarkable different utilization pattern (Tab. 1). Strain CH I was unable to use D-ribose as carbon source while all other strains metabolized that sugar. Furthermore, the strain CH I did not grow as sufficiently as the strains IMBST 95041, IMBST 95031, IMBST 97011, IMBST 16694 and W 4574 at the carbon sources L-erythriol and α - keto glutaric acid. The sugars D-melibiose and D-melezitose and the amino-acid D-serine seem to be metabolized in a more efficient way by strain CH I than by the other *Beauveria*-strains.

Table 1: Comparison of the carbon utilization between the less virulent strain CH I (n = 4) and the virulent strains IMBST 95041, IMBST 95031, IMBST 97011, IMBST 16694 and W 4574 (n = 20, averages of virulent strains) after 3 days at 25°C

type of molecules	carbon source	carbon utilization in percent	
		less virulent strain	virulent strains
monosaccharide	D-ribose	6	59
di- and	D-melibiose	27	7
polysaccharides	D-melezitose	64	22
amino acid	D-serine	24	7
alcohol	L-erythriol	38	55
organic	α -keto glutaric acid	1	15
acids/derivatives	γ -amino butyric acid	55	30

Similarity of the carbon utilization patterns between the different* B. brongniartii *strains

Table 2 and 3 show the reidentification of *B. brongniartii* strains by comparison with a data base build up with the BiologMicroLog™ system software. Similarity is the score used in evaluating the identification. A similarity of >0.5 indicates a high degree of matches concerning the carbon utilization of a tested strain with a strain in the data base.

Except for the strain IMBST 95031 at the SF-P plates the BiologMicroLog™ system software was unable to reidentify a tested strain (Tab. 3). Similarity-values of most of the identifications were lower than 0.5. Those observations indicate that the differences in the carbon utilization patterns between the various strains are too insignificant for any clear distinction between the strains.

Table 2: Identification of *B. brongniartii* strains based on the carbon utilization of SF-N Biolog MicroPlates™ with the BiologMicroLog™ system software and a newly set up data base

strain	identification	similarity
IMBST 95031	IMBST 95041	0.360
IMBST 95031	IMBST 95041	0.269
IMBST 95041	IMBST 97011	0.214
IMBST 95041	IMBST 97011	0.225
IMBST 97011	IMBST 95041	0.470
IMBST 97011	IMBST 16994	0.731
CH I	IMBST 95041	0.442
CH I	IMBST 97011	0.451
W 4574	IMBST 95041	0.374
W 4574	IMBST 16994	0.327

Table 3: Identification of *B. brongniartii* strains based on the carbon utilization of SF-P Biolog MicroPlates™ with the BiologMicroLog™ system software and a newly set up data base

strain	identification	similarity
IMBST 95031	IMBST 95031	0.307
IMBST 95031	W 4574	0.474
IMBST 95041	IMBST 95031	0.747
IMBST 95041	W 4574	0.287
IMBST 97011	IMBST 16994	0.828
IMBST 97011	IMBST 95031	0.298
CH I	IMBST 95031	0.429
CH I	IMBST 97011	0.581
W 4574	IMBST 16994	0.685
W 4574	IMBST 16994	0.612

Discussion

Nomenclature and taxonomy of the genus *Beauveria* are still controversial. Therefore various examinations had already been made in order to characterize the spieces of this genus. Physiological studies as well as genetical (Neuveglise *et al.*, 1994) and immunological (Fargues *et al.*, 1981) techniques were used for a reliable distinction between the different *Beauveria* species. Especially the two entomopatogenic fungi *B. bassiana* and *B. brongniartii* are subjects in investigations concerning (i) strain identification (Mugnai *et al.*, 1989; Todorova *et al.*, 1998), (ii) determination of virulence (Paris & Segretain, 1975; Reineke & Zebitz, 1996) and (iii) reidentification of approved strains used as mycoinsectizides in the field (Trzebitzky & Löchelt, 1994). Summing up, it can be said that all studies were able to detect differences between the *Beauveria* species. However, because of the low numbers of tested strains it was the opinion of the authors that more research work is needed. The authors claimed that none of the observed characteristics was intense enough for a significant distinction neither between different species of *Beauveria* nor between different virulent isolates of one species.

The BIOLOG™ system proved to be a rapid, easy to handle method for the characterization of *B. brongniartii* strains. All tested *B. brongniartii* strains showed similar carbon utilization profiles. Nevertheless, the less virulent strain CH I diverged in the utilization of some carbon sources from the virulent strains. These first results indicate that the BIOLOG™ system might be a useful tool for a quick identification of virulent and less virulent isolates. However, statistical analysis of the data resulted in the fact that the differences between the various strains are insignificant for any clear strain-specific distinction. Therefore, an enlargement of the data base for better statistical verification is needed. Our results are in accordance with the working-group of Bissett (pers. communications), who supposes that the BIOLOG™ system may be useful to predict virulence and host preferences of *Beauveria* strains.

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Persistence of released *Metarhizium anisopliae* in soil and prevalence in ground and rove beetles

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Abstract: One strain of *M. anisopliae* was released as a conidial suspension in a low and a high dose ($10^9/m^2$ and $6 \times 10^{11}/m^2$) to the ground in a Lucerne field. In the control plots, the natural level of *M. anisopliae* varied between $0\text{-}3 \times 10^2$ cfu's per g of surface soil. In the plots treated with *M. anisopliae*, the number of cfu's decreased 100-1000 times during 84 weeks. Vertical distribution of cfu's was examined for all plots, however due to a low number in the control plots the cfu's were only determined down to 10 cm depth. No difference was found between the number of cfu's in the surface and the deeper layer. In the plots treated with a low dose of *M. anisopliae* the number of cfu's was 10^2 - 10^3 per g soil for both the surface layer as well for the underlying layer. In the plots with a high dose, the number of cfu's was significantly different in the surface layer from the underlying layers. The concentration was approx. 2×10^5 cfu's per g surface soil, while it was only between 10^2 -10^3 cfu's per g soil in the depth of 15-30 cm after 84 weeks.

In the treated plots 0-5.6% and 0.7-15.8% *of* respectively Carabidae and Staphylinidae were infected with *M. anisopliae*, while in the control plots 0.9-1.1% and 0-6.2% of respectively Carabidae (especially *Agonum dorsale, Carabus nemoralis* and *Amara similata)* and Staphylinidae (especially *Staphylinus* spp. and *Oxytelus* spp.) were infected. Relatively more larvae of both ground and rove beetles were infected than adults.

Key words: Carabidae, Staphylinidae, prevalence, *Metarhizium anisopliae,* persistence, vertical distribution.

Introduction

The persistence and the pathogenicity of entomopathogenic fungi in the soil ecosystem are important for the development of strategies for the biological control of soil-inhabiting insects. The entomopathogenic fungus *Metarhizium anisopliae* occurs worldwide and has been isolated from several hundred insect species, including pest insects and beneficials (Zimmermann, 1993). The occurrence of *M. anisopliae* in soils and the prevalence of the fungus in Coleoptera have been reported in studies by Steenberg *et al.,* (1995) and Vanninen (1996). It has been reported that *M. anisopliae* can persist for a long period in the surface layer of soil (Storey & Gardner, 1988; Li & Holdom, 1993). Since *M. anisopliae* is produced for microbial pest control, a knowledge of the natural occurrence of this fungus in beneficials and other non-targets is required for an evaluation of possible side effects. However, not much is known about the vertical distribution of the fungus in the soil and the dispersal of *M. anisopliae* to beneficial and other non-target insects within the soil matrix after release. The objective of this study was to elucidate dispersal of *Metarhizium anisopliae* in soil (horizontal and vertical) and among ground and rove beetles populations.

Material and methods

Persistence

In a small Lucerne field at "Snubbekorsgaard" located at the field station of The Royal Veterinary and Agricultural University 3 plots of 9 m^2 placed 7 m from each other, were treated with either 0.05% Triton X-100 or a conidial suspension of *M. anisopliae* in a low or high dose ($10^9/m^2$ or 6 x $10^{11}/m^2$). The *M. anisopliae* strain 275 (ATCC No. 90448) was produced on millet grains and conidia were harvested in 0.05% Triton X-100.

The conidial suspension was sprayed onto the ground between the rows of lucerne with a hand sprayer. Three plots in the northern end of the field were sprayed in the spring 1997 and another three plots were sprayed in the autumn. Five soil samples from each plot were taken with regular intervals from treated and untreated plots. Samples of surface soil at 0-2 cm were taken with plastic cups. Metal tubes at the lengths 7, 12, 17, 22, 27 and 32 cm were used in the same holes to take samples at respectively, 0-5, 5-10, 10-15, 15-20, 20-25 and 25-30 cm depths. Vertical distribution of cfu's were examined for all plots, however due to a low number in the control plots cfu's were only determined down to 10 cm depth. In plots treated with low and high doses cfu's were determined down to 25 and 30 cm, respectively.

One g from each soil sample was mixed with 9 ml 0.05% Triton X-100 and eventually further diluted before 0.1 ml was spread onto three plates with a selective medium (SDA with 0.5 ml l^{-1} dodine/Radspor FL (Truchem Limited, Nottingham, UK.), 50.0 ppm Chloramphenicol, 50.0 ppm Streptomycin sulphate). The plates were incubated at 23°C and cfu's (colony forming units) were counted after 5 days.

Prevalence

Seven pitfalls were inserted in the soil at the centre of each plot. Insects were collected three times a week during the period May 15 - July 9 and September 14 - November 7, 1997. In the latter period barriers were set up around the plots to prevent immigration and emigration of beetles. Insects were also been collected from 4 pitfalls in each plot (without barriers) from June 12 - July 24, 1998. From pitfalls, live beetles (mainly ground and rove beetles) were trapped and incubated individually on a moistened filter paper in 30 ml plastic cups at room temperature in the laboratory. Insects were remoistened twice a week. Dead insects incubated for minimum 10 days were observed for infection coursed by insect pathogenic fungi. Insects still alive after 3 weeks were regarded as uninfected.

Results

Persistence

In the control plots the natural level of *M. anisopliae* in the surface soil varied between 0-3 x 10^2 cfu's per g soil while in the plots treated with *M. anisopliae* the number of cfu's decreased 100-1000 times during a period of 84 weeks (Table 1). In the plots with a low dose of *M. anisopliae* cfu's decreased from 2 x 10^3 to 3 x 10^1 (in the south end) and from 6 x 10^4 to 2 x 10^2 cfu's per g soil (in the north end) (Table 1). In the plots with a high dose of *M. anisopliae* cfu's decreased from 2 x 10^6 (south end) and 6 x 10^6 (north end) to respectively 8 x 10^4 and 4 x 10^3 cfu's per g soil (Table 1). Vertical distribution of cfu's were examined for all plots, however in the control plots cfu's were not found deeper than 10 cm and there was no difference between the number of cfu's in the surface and the deeper layer. In the plots with a low dose of *M. anisopliae* cfu's had decreased from 2 x 10^3 to 2 x 10^1 per g soil in the 15-20 cm layer in the north end of the Lucerne field, while in the south end there was no difference in number of cfu's (10^2-10^3 per g soil) for both the surface layer as well for the underlying layer after 84 weeks (Data not shown).

Tabel 1. Persistence of *Metarhizium anisopliae* in the surface soil (0 - 2 cm) of a Lucerne field, cfu's per g dry soil, mean ± S.E. of 5 samples.

Time after release (weeks)	Plot without *M. anisopliae* Control	Plot with released *M. anisopliae* Low dose	Plot with released *M. anisopliae* High dose
Southern end:			
before release	$5.3 \pm 4.7 \times 10^{1}$	$1.2 \pm 0.6 \times 10^{1}$	$< 4.0 \times 10^{0}$
0*	$2.6 \pm 1.3 \times 10^{2}$	$1.7 \pm 0.6 \times 10^{3}$	$3.3 \pm 0.8 \times 10^{5}$
1	$1.2 \pm 0.7 \times 10^{1}$	$1.5 \pm 0.9 \times 10^{2}$	$1.8 \pm 0.4 \times 10^{6}$
4	$< 4.0 \times 10^{0}$	$7.0 \pm 3.6 \times 10^{2}$	$2.0 \pm 0.6 \times 10^{5}$
6	$< 4.0 \times 10^{0}$	$9.0 \pm 0.9 \times 10^{2}$	$9.4 \pm 2.5 \times 10^{5}$
13	$3.2 \pm 0.7 \times 10^{1}$	$5.2 \pm 1.4 \times 10^{2}$	$2.4 \pm 0.5 \times 10^{6}$
22	$8.0 \pm 8.0 \times 10^{0}$	$1.6 \pm 0.9 \times 10^{2}$	$6.3 \pm 1.9 \times 10^{5}$
33	$4.5 \pm 4.0 \times 10^{1}$	$9.3 \pm 5.9 \times 10^{2}$	$2.8 \pm 0.3 \times 10^{5}$
41	$8.0 \pm 8.0 \times 10^{0}$	$1.2 \pm 0.8 \times 10^{2}$	$9.1 \pm 2.2 \times 10^{4}$
67	$4.0 \pm 5.0 \times 10^{0}$	$3.0 \pm 2.2 \times 10^{1}$	$8.3 \pm 2.4 \times 10^{4}$
Northern end:			
before release	$4.3 \pm 4.3 \times 10^{0}$	$1.3 \pm 1.3 \times 10^{1}$	$1.3 \pm 0.7 \times 10^{1}$
1	$5.7 \pm 4.0 \times 10^{1}$	$2.0 \pm 0.5 \times 10^{4}$	$3.7 \pm 0.6 \times 10^{6}$
4	$4.5 \pm 3.3 \times 10^{1}$	$2.0 \pm 0.8 \times 10^{4}$	$2.8 \pm 0.7 \times 10^{6}$
6	$< 4 \times 10^{0}$	$1.5 \pm 0.6 \times 10^{4}$	$1.9 \pm 1.1 \times 10^{6}$
12	$< 4 \times 10^{0}$	$6.6 \pm 1.1 \times 10^{4}$	$5.6 \pm 2.6 \times 10^{6}$
25	$6.7 \pm 3.5 \times 10^{1}$	$2.0 \pm 0.3 \times 10^{4}$	$2.0 \pm 0.4 \times 10^{5}$
34	$< 4 \times 10^{0}$	$3.4 \pm 1.8 \times 10^{2}$	$2.6 \pm 0.7 \times 10^{5}$
44	$< 4 \times 10^{0}$	$3.3 \pm 2.5 \times 10^{3}$	$4.2 \pm 1.0 \times 10^{5}$
54	$< 4 \times 10^{0}$	$2.8 \pm 1.1 \times 10^{2}$	$4.3 \pm 1.0 \times 10^{5}$
66	$5.1 \pm 1.9 \times 10^{1}$	$5.5 \pm 1.5 \times 10^{2}$	$2.4 \pm 0.6 \times 10^{4}$
73	$3.0 \pm 2.0 \times 10^{2}$	$4.2 \pm 1.3 \times 10^{2}$	$2.8 \pm 0.5 \times 10^{5}$
84	$5.0 \pm 5.0 \times 10^{0}$	$2.2 \pm 1.1 \times 10^{2}$	$4.4 \pm 1.3 \times 10^{3}$

*after release

In the plots with a high dose released, cfu's in the north end were approx. 5×10^{3} and 4×10^{1} per g soil for respectively the 0-15 cm and 15-30 cm layers after 84 weeks. This was significant lower than the number cfu's determinated 2 weeks after treatment with the fungus. In the southern end of the field the number of cfu's decreased in the 15-25 cm layer, but was not significant different from the surface layer after 67 weeks (Data not shown).

Prevalence

In general, few insects were infected with *M. anisopliae,* and species of *Bembidion* and *Harpalus* were not infected with *M. anisopliae.* Especially species from the genera, *Amara, Agonum, Carabus, Staphylinus, Oxytelus* and *Tachyporus* were infected with *M. anisopliae.* In the northern control plot 1.1% of the Carabidae died from *M. anisopliae* infection and 0.4 and 2.6% were infected in the two treatments with respectively low and high doses (Table 2). Only 1.4% of the trapped Staphylinidae were infected with *M. anisopliae* and these were all found in the treatment with the high dose of the fungus (Table 2). In 1998 no beetles were found with *M. anisopliae* infections in the control plot and only 0.6% of Staphylinidae in the plot with low dose were infected. However in the plot with the high dose 0.7 and 1.9% of respectively Carabidae and Staphylinidae were infected (Table 2).

In the southern control plot 0.9 and 6.2% of Carabidae and Staphylinidae were infected, versus 0 and 4.0% in the low dose plot. However in the plot with a high dose 5.6 and 15.8% of respectively Carabidae and Staphylinidae were infected (Table 3). Relatively more larvae of both ground and rove beetles were infected than adults. In 1998 the prevalence was much lower and did not exceed 1.2% (Table 3). Some sampled Carabidae and Staphylinidae were naturally infected with *Beauveria bassiana* (prevalences of 0-8.5%).

Table 2. Prevalences of *Metarhizium anisopliae* in ground beetles (Carabidae) and rove beetles (Staphylinidae) from a Lucerne field (northern end)

	Control		Low dose		High dose	
	Carabidae	**Staphylinidae**	**Carabidae**	**Staphylinidae**	**Carabidae**	**Staphylinidae**
1997 summer	1.1	0	0.4	0	2.6	1.4
1998 summer	0	0	0	0.6	0.7	1.9

No. collected in 1997: Car. = 1087, Sta .= 1072. 1998; Car. = 1010; Sta. = 400

Table 3. Prevalences of *Metarhizium anisopliae* in ground beetles (Carabidae) and in rove beetles (Staphylinidae) from a Lucerne field (southern end)

	Control		Low dose		High dose	
	Carabidae	**Staphylinidae**	**Carabidae**	**Staphylinidae**	**Carabidae**	**Staphylinidae**
1997* autumn	0.9	6.2	0	4.0	5.6	15.8
1998 summer	0.3	0	0.3	0	0.3	1.2

No. collected in 1997: Car. = 486, Sta .= 343. 1998; Car. = 917; Sta. = 289.
* Barriers surrounded the plots.

Discussion

Prolonged conidial survival is an important characteristic of a successful microbial control agent and any reduction in survival reduces the effective control period. In this study 1-10% of *M. anisopliae* conidia survived the surface soil after one year. Tyni-Juslin & Vänninen (1990) reported that persistence of *M. anisopliae* and *B. bassiana* in four soil types after one year was 34% and 0.2% respectively and no effect of soil type on persistence was observed. When *M. anisopliae* was released onto the ground it was possible to detect the conidia down to 30 cm depth, however only small percentages of conidia penetrated to the lower depths. This was also observed for *B. bassiana* by Storey & Gardner (1988).
Steenberg *et al.*, (1995) found in lucerne the natural prevalence of *M. anisopliae* in over-wintering ground and rove beetles at respectively, 2.0 and 1.6%, and it was higher for *B. bassiana*. In this study the prevalence of *M. anisopliae* in the control plots (natural occurrence) and treated plots were comparable. However, when larvae were collected these were relative more susceptible to *M. anisopliae* than adults. Other factors influences the prevalence can be the climate and species of insects sampled.

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Entomophthorales as significant natural enemies of cereal aphids infesting maize in Slovakia

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Abstract: During 1997 and 1998, influence of natural enemies of cereal aphids on maize was studied in south-western Slovakia. *Metopolophium dirhodum, Rhopalosiphum padi* and *Sitobion avenae,* were found infesting maize. Aphids were present from the first days of June, and the peak of abundance was recorded during the second half of June. Entomophthorales appeared to be the major natural enemies of cereal aphids on maize during the two years of the survey. In 1997, only *Erynia neoaphidis* was found infecting aphids on maize. In 1998 this species was the main pathogen, but infections by *Conidiobolus obscurus* or *Entomophthora planchoniana* were also recorded. Compared to 1998, the more numerous rains in 1997 probably enhanced fungal propagation in aphid population (maximum infection rate of 81%). This is the first record of cereal aphid infecting Entomophthorales in Slovakia.

Key words: maize, cereal aphids, Entomophthorales

Introduction

Cereal aphids, such as *Metopolophium dirhodum* (Walker), *Sitobion avenae* (F.) and *Rhopalosiphum padi* (L.) are pests of maize in Slovakia. According to Cagá• (1991), the first aphids on the maize plants appeared in the beginning of June. After reaching the maximum population level in the end of June and in the beginning of July a rapid decrease in aphid abundance was recorded in the first ten days of July. Another outbreaks could occur in late summer and autumn.

The disappearance of cereal aphids during summer may be due to intensive pressure from predators (Visnyovsky & Rács, 1989), parasitoids (Starý, 1988; Prasli• ka & A1 Dobai, 1997), and pathogens i.e. Entomophthorales (Vickerman, Wratten, 1979). Wind (Cannon, 1986), rainfall (Lourenco & De-Oliveira, 1983), and temperatures (Honek, 1985) could also act as mortality factors.

In Slovakia, Entomophthorales attacking the aphids have been not studied yet. The aim of this study was to understand how the entomophthoralean fungi influence cereal aphid populations on maize, in relation to the climatic factors.

Material and methods

The study was carried out at the Experimental Base of the Slovak Agricultural University in Nitra (southwestern Slovakia) in the years 1997 and 1998. Aphid populations were sampled from the beginning of June (from the first appearance of aphids on maize), and samplings were conducted twice a week until the harvest. Aphids were counted *in situ* on 60 plants randomly chosen throughout the field. The number of living, fungus-killed and parasitoid-killed aphids was recorded. The fungus-killed and parasitoid-killed aphids were distinguished from each other and from living aphids by their external symptoms. Infection rate due to the

fungal infection was calculated as: Infection rate (%)= [number of fungus-killed aphids/(sum of living, parasited and infected aphids)]. A sample of the infected aphids was taken to the laboratory to determine the fungal species. Fungal species were identified according Keller (1987, 1991), and Papierok (1989). Climatic data were obtained from the Agrometeorology Station at the Slovak University of Agriculture in Nitra.

Results

Three aphid species, *Metopolophium dirhodum, Rhopalosiphum padi* and *Sitobion avenae,* were found infesting maize, the first two being the most important. Aphids were present from the first days of June, and the peak of abundance was recorded during the second half of June, in both 1997 and 1998 (Fig. 1 and Fig. 2). In 1997, 0.6 aphids per plant were recorded at the date of the first appearance of aphids (9 June) and 23% of plants were infested. Ten days later, 100% of plants were already infested. Aphid population reached the maximum on June 24 with a mean average of 91.5 aphid per plant. *M. dirhodum* and *R. padi* were the most abundant species. The population size rose at a similar rate, and 45.4 of *M. dirhodum* and 46.0 of *R. padi* were recorded on average per plant at the maximum of abundance, on 24 June. S. *avenae* reached its peak on 10 July, 3 aphids (only alates) being found on average per plant. Afterwards, aphid populations decreased rapidly. A second population peak occurred in the case of *M. dirhodum* on 25 August (with a mean average of less than 8 aphids per plant), and 40 % of plants were infested.

In 1998, 90% of plants were already infested at the date of first appearance of aphids (4 June), and 16.2 aphids per plant were recorded on average. The aphid populations reached the peak of abundance on 25 June (on average 161 aphids per plant). Four days after peak of abundance only 31 aphids per plant were recorded. *M. dirhodum* was the dominant species and its population was more numerous than in 1997, a number of 138 aphids was recorded on average per plant at the maximum of density, on 25 June. Population of *R. padi* was very low during the whole season, and on average 21 aphids per plant were found at the peak of population. *S. avenae* populations were as low as in 1997, but reached the maximum earlier (on 25 June), and on average 1,8 aphids were recorded per plant. In the summer of 1998, aphids disappeared from maize more rapidly than in 1997, and after 9 July, very few aphids were encountered. Even populations of *M. dirhodum* didn't increase at the end of August, as observed in 1997.

The first fungus-killed aphids were observed on 19 and 9 June, in 1997 and 1998 respectively. In 1997, the infection rate was 8,5% on 1 July, 24% on 3 July and reached its maximum (81%) on 10 July, i. e. two weeks after the peak of population. In 1998, the infection rate was 7% on 18 June, 22% on 25 June, and 49 % on 29 June, which was the maximum.

In 1997, only *Erynia neoaphidis* was found infecting aphids on maize. In 1998 this species was the main pathogen, but infections by *Conidiobolus obscurus* or *Entomophthora planchoniana* were also recorded. Compared to Entomophthorales, the influence of parasitoids on the decrease of aphid populations on maize was less important. The involved species belonged to the genera *Aphidius* and *Praon,* and the former genus was more frequent. The first mummies were found from the beginning of plant infestation by the aphids. The maximum number of mummies was recorded at the same time as the highest number of fungus-killed aphids, on 10 July in 1997 and 25 June in 1998. In both 1997 and 1998, the rate of parasitism never exceeded 7%. Both in 1997 and 1998, predators (i.e. Coccinellidae and Syrphidae) were observed scarcely. In conclusion, Entomophthorales appeared to be the major natural enemies of cereal aphids on maize during the two years of the survey

Fig. 2 and Fig. 4 show the climatic conditions during June 1 - September 30 at Nitra. The amount of rainfall during June 1- July 10 was 58.4 mm in 1997, and 14.1 mm in 1998. In the second half of June the amount of rainfall achieved 44.9 mm in 1997, and 3.5 mm in 1998. High average daily temperatures preceded the peak abundance of aphids in 1998, and the relative air humidity during June 20 - July 10 usually achieved 60-80% (Fig. 6) During July 5 - July 8, relative air humidity was higher than 90% in 1997 (Fig. 3).

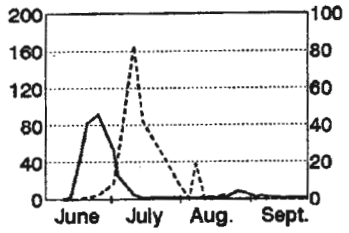

Figure 1. Number of aphids per plant (y1 axis), and infection rate (%, y2 axis) during the growing season 1997.

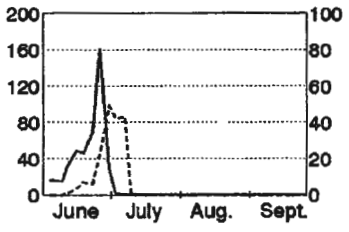

Figure 4. Number of aphids per plant (y1 axis), and infection rate (%, y2 axis) during the growing season 1998.

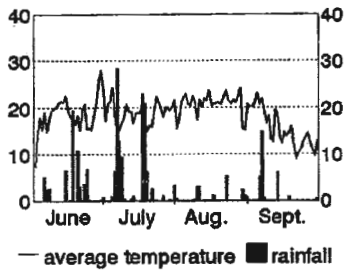

Figure 2. Average day temperature (°C, y1 axis), and rainfall (mm, y2 axis) during the growing season 1997.

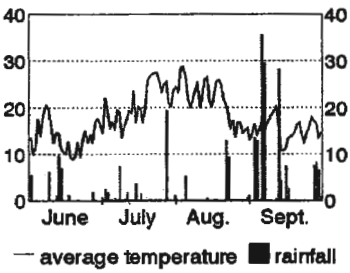

Figure 5. Average day temperature (°C, y1 axis), and rainfall during the growing season 1998.

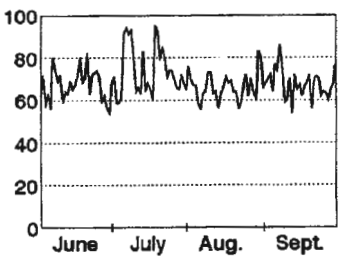

Figure 3. Relative air humidity (%) during the growing season 1997.

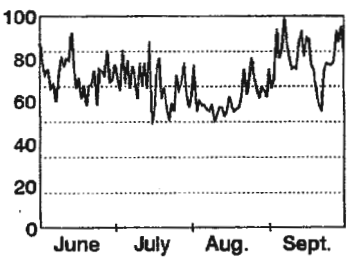

Figure 6. Relative air humidity (%) during the growing season 1998.

Discussion

Three aphid species referred to this paper are regularly found infesting maize all over the world. *M. dirhodum* and *R. padi* are two dominant species in Europe (Hand & Carrillo, 1982; Milinko *et al.*, 1983; Plewka & Pankanin-Franczyk, 1989). *R. maidis* rarely occurs in Europe. *S. avenae* is regularly present on maize plants during the growing season also in other countries, but always in very low numbers (Hand & Carrillo, 1982; Milinko *et al.*, 1983; Plewka & Pankanin-Franczyk, 1989).

As already observed in Slovakia by Cagan (1991), the first aphids appeared on maize in beginning of June and the population reached the maximum at the end of June, followed by a rapid decrease of aphid numbers. Compared to 1998, higher temperatures in 1997 positively influenced the rapid increase of the population. However the maximum number of aphids was smaller in 1997 than in 1998, whereas the infection rate by Entomophthorales reached 81% in 1997, and only 49% in 1998. Higher infection rate in 1997 was probably related to more regular and numerous rainfalls, although this situation didn't result in significant differences as regards relative humidity.

Both in 1997 and 1998 the decline of aphid population is concomitant with the rapid increase of infection by Entomophthoraler, whereas rates of parasitism and predation seemed very limited. Entomophthorales appeared indeed as the major natural enemies of cereal aphids infesting maize in Nitra area. This is the first mention of these aphid pathogens in Slovakia. Given that climatic conditions were variable from one year to another, the reason why the decline of aphid populations on maize occurred every year almost at the same time is still debatable. Such a situation resulted probably from interweaving of numerous abiotic and biotic factors.

The three entomophthoralean species found infecting cereal aphids on maize in Slovakia are known to infect cereal aphids on wheat and barley in Europe (Dean & Wilding, 1971, 1973; Dedryver, 1981; Coremans *et al.*, 1983; Papierok *et al.*, 1984, Ozino *et al.*, 1988). *Erynia neoaphidis*, which appeared like the main pathogen in our study, was showed previously to be the dominant species in more humid conditions, compared to *Entomophthora planchoniana* (Dedryver, 1981; Papierok *et al.*, 1984).

Further observations are planned in 1999 in Nitra area, with the aim to more precisely study the role of fungal enemies in population dynamics of cereal aphids infesting maize.

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Cordyceps militaris (Fr.) Link. in the outbreak of *Dendrolimus pini* L. in Lithuania

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Abstract: In average 54% (30-70%, heterogeneous data) of overwintering *Dendrolimus pini* L. larvae were found to be infected by *Cordyceps militaris* Link. in early 1996 and in the fall fruit bodies of this species were present in many places within pine moth outbreak area. In spring of 1997, in the declining outbreak of *D. pini*, this fungus was found to infect 67% of larvae. In spring of 1998, when population of *Dendrolimus pini* was low, furthermore 59% of overwintering larvae were killed with this fungus. Laboratory studies on disease transmission revealed that the contact with fungus mycelium resulted in 20%, contact with dead larvae - 10%, contact with spores - 0% infection rate of pine moth larvae.

Key words: *Cordyceps militaris*, infection, *Dendrolimus pini*, outbreak

Introduction

Meteorological conditions in Lithuania in early nineties (hot and dry summers) were favourable to the development of *Dendrolimus pini* L. Rise of this forest pest population started in 1992 and damage in about 3000 ha of forests in southern Lithuania was noticed in 1993 (Report... 1993). Pest population was increasing and expanding rapidly - in 1994 significant damage was recorded in the 10800 ha of pine forests (Report... 1994) and in 1995 outbreak area expanded to 27800 ha (Report... 1995). Fungal infection of overwintering larvae was noticed while assessing pest population early in 1996, and pilot pathogen research was assigned.

Cordyceps, with several hundred species, is the best known Ascomycete genus of insect parasites, commonly infecting larvae or pupae (Carlile & Watkinson 1994). However, it is not well studied group in insect pathology (Kaya 1998), and not so much is known about host range and impact on insect pest populations, or the transmission mechanisms.

Study therefore was designed to examine the frequency and abundance of *Cordyceps militaris* in the outbreak population of *Dendrolimus pini* and laboratory experiment was performed to check effectiveness of few possible ways of fungal infection transmission.

Material and methods

Material was collected in south Lithuania, in pine moth *(D. pini)* outbreak area. Data of 1996 was taken from pest population assessment, carried out by Forest Protection Station teams in damaged forests. 31 test plot was laid out in 1997-98 within severe pine moth outbreak area, and 8 control plots - outside damaged forests. In each plot, early in the spring, 3 to 4 samples of forest litter (2 x 0.5 m size) were taken. In total 98 samples were collected, distinguishing live and *Cordyceps militaris* infected individuals. Part of pine moth larvae, dead and mummified, have no apparent symptoms of fungal infection, but mycelium of *Cordyceps* was

always recovered from such larvae on boiled rice diet (10 g rice, 25 ml distilled water) or on live *D. pini* larvae.

Wild mycelium of *Cordyceps militaris* was successfully reared to complete fungal bodies in the laboratory on live pine moth larvae. To test the ways of infection transmission, III instar larvae of *Dendrolimus pini* were kept in Petri dishes for two days in contact with: 1) fungal mycelium, 2) spores and 3) smashed dead and dried field collected larvae, from which *Cordyceps* was recovered. After contact larvae were reared in laboratory in forest litter, imitating overwintering conditions (4°C, 95% RH).

Results and discussion

Dendrolimus pini population assessment by Forest Protection Station teams in early spring of 1996 have found 30 to 70% fungal infection in pine moth larvae. However, data was very heterogeneous and sample sizes were not indicated. In average 54% fungal infection was reported in overwintering pest population (fig. 1). In the fall scouting revealed fruit bodies of *Cordyceps militaris* L. present in many places within outbreak area.

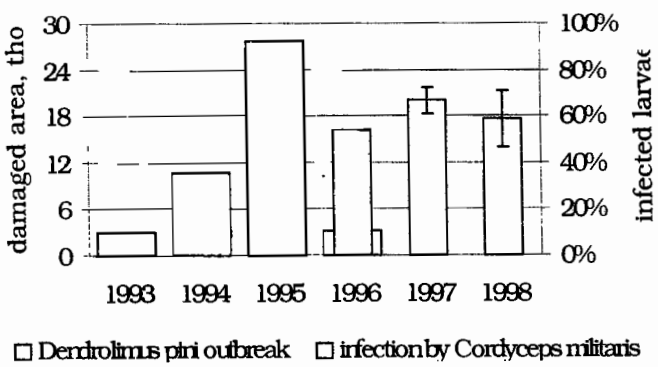

Figure *1. Cordyceps militaris* infection in overwintering larvae during *Dendrolimus pini* outbreak

66.7 ± 5.9% of overwintering *Dendrolimus pini* were found to be killed by *Cordyceps militaris* in spring 1997 (fig. 1). The outbreak was declining, pine moth occurrence (presence) in samples was 69.4 ± 4.7% (tab. 1), and mean density of successfully overwintering population in these samples was 0.3 larvae/m^2. Next year outbreak terminated, *D. pini* occurrence in samples was only 19.4 ± 4.0%, mean density - 0.4 larvae/m^2. Furthermore, 58.8 ± 11.9% of wintering larvae were infected with fungus.

There were no fungal infection found on any insect larvae or pupae in the forest litter samples outside *Dendrolimus pini* outbreak area in 1997 and 98.

Interestingly, some field collected *Cordyceps* was bearing parasite fungus *Melanospora parasitica* Tulasne; shortage of material did not allow further study.

Pilot studies on disease spread revealed that the contact with fungus mycelium resulted in 20.0 ± 6.3%, contact with dead larvae -10.0 ± 4.7%, contact with spores - 0% infection rate of overwintering larvae in laboratory conditions.

Table 1. Occurrence of *Cordyceps militaris* Link. in forest litter in the outbreak of *Dendrolimus pini* L.

Occurrence (presence)	1997	1998
Dendromimus pini	69.4 ± 4.7%	19.4 ± 4.0%
Cordyceps militaris:		
In all samples	65.3 ± 4.8%	14.3 ± 3.5%
In all samples with *D. pini*	94.1 ± 2.9%	73.7 ± 10.1%

Spread of fungal disease a year after peak damage of pine moth follow general trend of parasite and pathogen regulating role in pest outbreaks. *Dendrolimus pini is* known to be one of the hosts for *Cordyceps militaris* (Glowacka-Pilot 1974) along with other lepidopterans (Mazalaitis & Urbonas 1980); beetles (Houle *et al.*, 1987) and tipulids (Muller-Kogler 1965) are also found to be infected with this species. Fungus usually causes mortality less than 100% and our data on larvae mortality correspond to literature references; nevertheless *Cordyceps* is considered to be widely spread and important insect pest pathogen (Glowacka-Pilot 1974, Sliwa 1969).

Ways of transmission for *Cordyceps militaris* were not as widely studied as its potential in pest management. Low success of aerial way was reported (Sato *et.al.*, 1997), and this correspond to our study, were larvae could not be infected by spores.

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Temperature as a selective factor for isolation of entomopathogenic fungi from soil by means of the isnect bait method.

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Introduction

Environmental conditions, particularly temperature and humidity, are important factors in infection process and sporulation of entomopathogenic fungi.
Proposed by Zimmermann (1986) the „bait insect method" for the isolation of entomogenous fungi from soil – creates larger possibilities for investigations on species composition and frequenc of these insect pathogens in soil habitat, than the selective medium method. Using this technique more virulent isolates can be found as compared with selective medium method. When entomopathogenic fungi are isolated from soil by maens of the bait insects, temperature has an effect on the fungus and the insect as well.

The aim of this report was to established the influence of temperature on the spectrum and frequency of entomogenous fungi isolated from soil and pine litter by means of the bait insect method.

Material and methods

Samples of soil or litter were taken from arable fields, strawberry plantations and Scot pine forest litter in the vicinity of Siedlce, Poland. The soil or litter were transferred to plastic Petri dishes, each of which contained 10 or 20 larvae of *Galleria mellonella* or *Tribolium destructor*. The dishes were kept in incubators at seven different temperatures, ranging from 5°C to 35°C, with intervals of 5°C. In the second part of experiment two frameworks of temperature were used for isolation of fungi: a) 18°C and b) 28°C. At each temperature 10 Petri dishes with larvae were placed.

The first analysis of the mortality was made after 5 days, and the further ones 3 days till 24th day. Dead insects with visible mycelium were rinsed in sterile water and moved to moist chambers. Individuals showing no mycelium on the surface were superficially disinfected in 1% sodium hypochloride and after rinsing 3 times in sterile water also placed in damp chamber in order to obtain the growth of mycelium and sporulation.

Results

Four entomopathogenic fungi were isolated on larvae of *G. mellonella* from the pine litter (Fig.1). No infection was observed at 5°C and 35°C. *Beauveria bassiana* infected larvae at temperatures between 10°C and 25°C, but the highest percentage of infected larvae was observed at 25°C. *Paecilomyces farinosus* infected bait insects at the same temperatures as *B. bassiana*, but the highest mortality occurred at 20°C. *Paecilomyces fumosoroseus* seemed to be present in low abundance and was able to infect bait larvae only within the temperature range 15-25°C. *Metarhizium anisopliae* showed the greatest temperature requirements

occurring at 25°C and 30°C. Maximum mortality was reached at 30°C, at which temperature non of the other fungi caused infection.

In the case of soil from a rye field (Fig.2), where *M. anisopliae* decisively dominated, infection of *T. destructor* larvae caused by this fungus was observed at temperature between 10°C and 30°C. The highest mortality was noted at 20°C and 25°C.

In the soil from strawberry plantation where occurred two fungi: *P. fumosoroseus* and *M. anisopliae* (Fig.3) interaction between the fungal species becomes apparent. The first dominated at temperature up to 25°C, whereas over this limit *M. anisopliae* was the dominant.

In the second part of experiment, four fungal species were found in two kinds of soil (light loamy sand and alluvial silt), although their spectrum and frequency were different and depended on temperature in which larvae had a contact with soil (Fig.4 and Fig.5).
B. bassiana, M. anisopliae, M. flavoviride and *P. fumosoroseus* appeared in light loamy sand. *M. anisopliae* was the dominant species at both temperatures. At 18°C *B. bassiana, M. flavoviride* and *P. fumosoroseus* were also found. At temperature of 28°C apart from *M. anisopliae – M. flavoviride* was recorded. In alluvial silt also four fungal species appeared. *M. flavoviride* was not noted but *P. farinosus* occurred instead. All fungal species infected *G. mellonella* larvae at 18C, but *B. bassiana* was dominant. At 28C only *M. anisopliae* was observed on dead larvae.

Discussion and conclusion

Results of presented experiments as well as data from references shaw that the species *B. bassiana* and *P. farinosus* can tolerate a wide range of climatic conditions. In Canada (Widden & Parkinson 1979) and in Finland (Vanninen 1995) *B. bassiana* has been found as far north as latitude 75°. *P. farinosus* too, has a northerly distribution, being reported from Canada (Harney & Widden 1991), Finland (Vanninen 1995) and Siberia (Ogarkova & Ogarkov 1986). Fargues *et al.*, (1997) investigated the effect of temperature on growth of 65 isolates of *Beauveria bassiana* , from different geoclimatic and host origins stated, that this fungus grew at a wide temperature range from 8°C to 35°C. Optimum temperature es were generally between 25 and 28°C with several isolates exhibiting optimal growth at temperatures as low as 20°C or as high as 30°C. Also study of Roberts and Campbell (1977) and Fargues *et al.*, (1992) confirm that *B. bassiana* is mesophilic capable of growth between 6 and 35°C with optima between 20 and 30°C.

The termophily of *M. anisopliae* is indisputable as compared to *B. bassiana* and *P. farinosus*. At 10°C *M. anisopliae* takes nearly twice as long to start sporulation as *B. bassiana* (Walstad *et al.*, 1976). Miętkiewski *et al.*, (1994) showed that the optimum temperature for growth of *M. anisopliae* is 27,5°C. These data were confirmed Ferron's (1981) results who found an optimum growth at 27-28°C.

The above results showed that the temperature ranges extreme for infection discriminate development of entomopathogenic species, whereas the ranges of optimum temperatures indicate more or less thermophilic character of strains. It seems that for the Central-European climatic conditions the best way for isolation of entomopathogenic fungi from soil, is to use two ranges of temperatures 18 – 20°C and 25 – 28°C. At temperature of 18°C *B. bassiana* and *P. farinosus* were isolated more frequently while at 25 –28°C *M. anisopliae* dominated. *P. fumosoroseus* seemed to have the temperature requirements close rather to *M. anisopliae* than to *B. bassiana*.

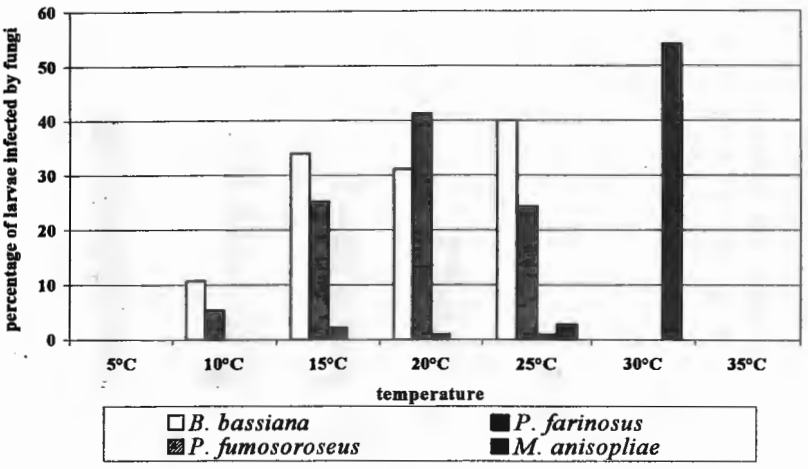

Fig. 1. Mortality (%) of *Galleria mellonella* larvae in the pine litter at different temperatures (Mię tkiewski et al. 1994)

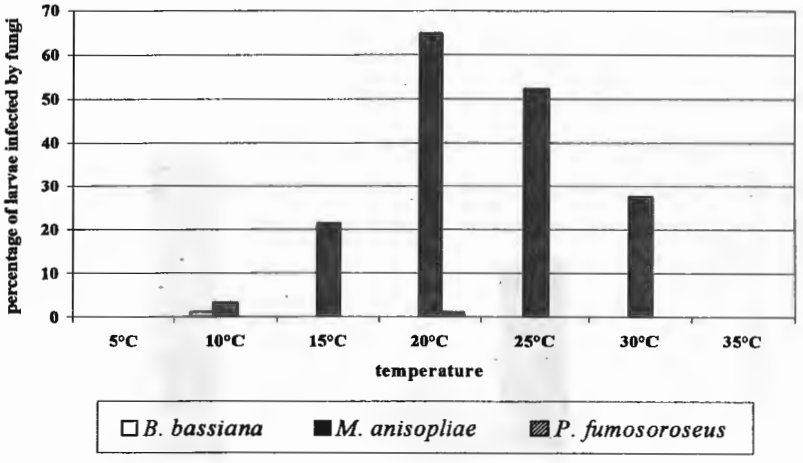

Fig. 2. Mortality (%) of *Tribolium destructor* larvae in the rye field soil at different temperatures (Mię tkiewski et al. 1992b)

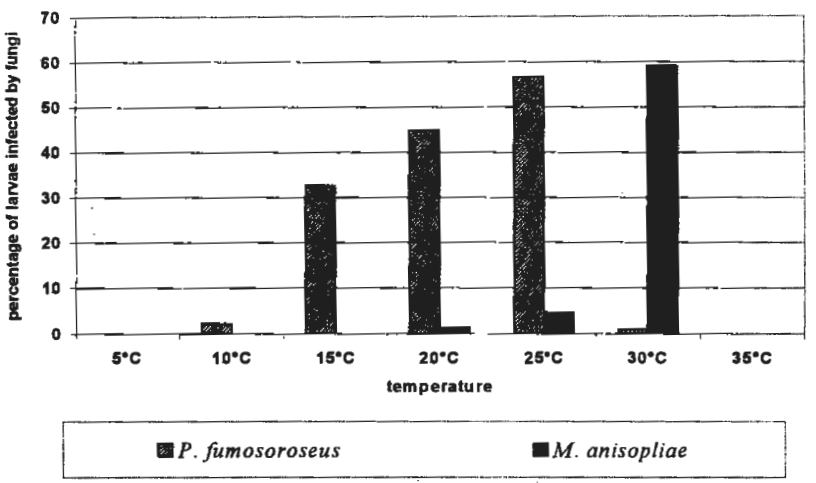

Fig. 3. Mortality (%) of *Galleria mellonella* larvae in the soil from strawberry plantation at different temperatures (Mię tkiewski., 1992a)

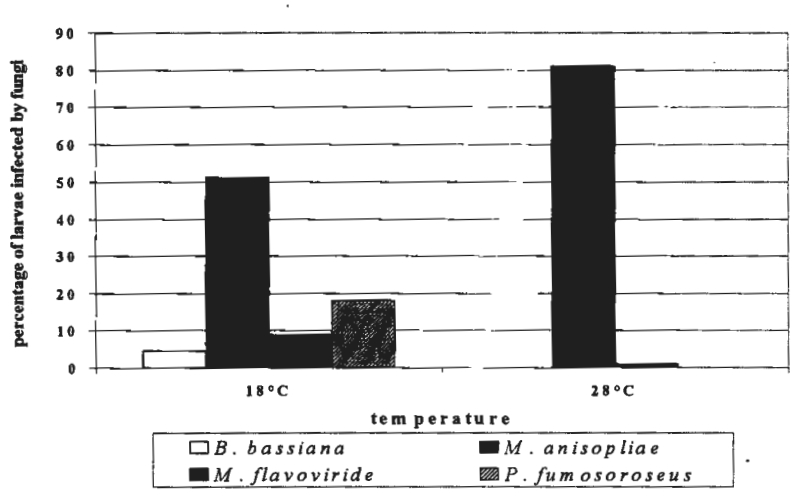

Fig. 4. Mortality (%) of *Galleria mellonella* larvae in light loamy sand at two temperatures (Tkaczuk, Mię tkiewski 1996)

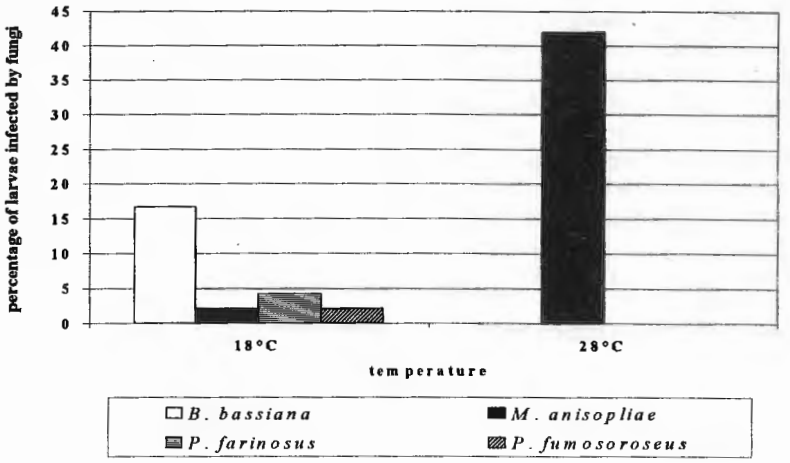

Fig. 5. Mortality (%) of *Galleria mellonella* larvae in alluvial silt at two temperatures (Tkaczuk, Mię tkiewski 1996)

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Estimation of virulence by measuring the chitinase activity in submerged culture of *Beauveria brongniartii* and *Metarhizium anisopliae*

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Abstract: Eight low, medium or high virulent *Metarhizium anisopliae* or *Beauveria brongniartii* strains were grown in submerged cultures with chitin as sole carbon source. Chitinase enzyme acitvity was measured by detecting the chitin degradation product N-acetylglucosamine (NAG) in the supernatant by HPLC-analysis. A connection between virulence and increased activity of the chitinolytic system of all tested strains could be demonstrated.

Keywords: Entomopathogenes, N-acetylglucosamine, virulence, *Beauveria brongniartii, Metarhizium anisopliae,* chitinase.

Introduction

The infection of insects by entomopathogenic fungi occurs in most cases by penetration of hyphae through the insect cuticle. The penetration process involves mechanical and enzymatic activities of the developing hypha. The relative importance of these two mechanisms is not known. However, the alterations of the culticle observed during this process have shown the important role played by enzymes such as chitinase, lipase and protease released by the germ tube during perforation of the insect cuticle (St. Leger *et al.,* 1986).

Paris & Ferron (1979) reported that lipase-negative and chitinase-negative *Beauveria brongniartii* strains were not able to infect *Melolontha melolontha.* Furthermore, preliminary studies have shown that N-acetylglucosamine (NAG) can be detected two days before larvae are killed by virulent *B. brongniartii* strains in the bioassay system (Lung & Strasser, 1999). The importance of the chitinase system focused our interest on studying the virulence of low and high virulent entomopathogenic fungi by detecting the chitin degradation product NAG in submerged culture. On the basis of these results a screening method for monitoring virulence should be established.

Material and methods

Fungal strains and maintenance

Four *Metarhizium anisopliae* strains and four *B. brongniartii* strains were isolated as follows: (i) from natural infected targets, (ii) from soil with the help of galleria bait method, or (iii) from soil by using selective medium (Tab. 1). Most of the test-strains were characterized for their virulence by standard biotests and defined as low, medium or high virulent strains. The

fungi were grown on Sabouraud-2%-Glucose agar at 25 °C until the spores were harvested with sterile 0.05% w/v Tween 80 for inoculation.

Table 1: Characterization of *Beauveria brongniartii* and *Metarhizium anisopliae* strains.

Fungi	Strain	Original host	Spore size (lengthxwidth)µm	Virulence class
Beauveria brongniartii	CH I[1]	Selective Medium	2.8 x 2.3	Low
Beauveria brongniartii	CH AN[1]	Selective Medium	3.5 x 1.7	Medium
Beauveria brongniartii	IMST 95041	*Melolontha melolontha*	3.2 x 1.8	High
Beauveria brongniartii	IMST 97011	*Melolontha melolontha*	3.1 x 1.9	High
Metarhizium anisopliae	Ma 275[2]	*Cydia pomonella*	4.9 x 2.1	High
Metarhizium anisopliae	KVL 97-1[2]	*Tenebrio molitor*	N.D.	N.D.
Metarhizium anisopliae	IMST 9601	*Phylloperta horticola*	9.4 x 3.0	High
Metarhizium anisopliae	IMST 9609	*Phylloperta horticola*	6.1 x 2.7	High

N.D. = Not detected. [1]provided by S. Keller, Switzerland. [2]provided by S. Vestergaard, Denmark.

Flask-experiments

All strains were grown in 100 ml Erlenmeyer-flasks containing 20 ml nutrient solution of 1 g l^{-1} NH_4NO_3; 1 g l^{-1} KH_2PO_4; 0.5 g l^{-1} $MgSO_4 * 7 H_2O$; 0.1 g l^{-1} NaCl; 0.13 g l^{-1} $CaCl_2 * 2 H_2O$; 0.5 ml l^{-1} trace element solution, and 2 g l^{-1} chitin (Practical grade, Sigma) as sole carbon source. With the exception of the control the flasks were inoculated with spores having a final concentration between $1.6 * 10^5$ and $5 * 10^5$ ml^{-1}. Cultures were incubated at 25°C on a gyratory shaker at 200 rpm for 8 days. The supernatant of the culture broth were measured for NAG after inoculation, 1, 2, 4, 6, and 8 days. For sampling three flasks of each strain were taken.

Analytical methods

In order to guarantee a particle free solution the supernatant was filtrated (0.2 µm) for analysis. N-acetylglucosamine was determined by HPLC with the column: AMINEX-HPX-87-H-cation exchanger with micro guard cation H^+ precolumn. The mobile phase was 4 mN H_2SO_4, the flow rate 0.5 ml/min and the temperature 30°C. The compound was detected with UV-detector at 213 nm (range -0.004; +0.036 AU).

Results

All virulent *M. anisopliae* strains showed the same or a higher amount of NAG in the supernatant than the *B. brongniartii* strains after 6 days. Most of the virulent fungal strains from both genera accumulated NAG in the supernatant with an increase of incubation time. Only the strains Ma 275 and CH AN reduced the NAG concentration after eight days. Comparing the most active M. *anisopliae* strain IMST 9601 with *B. brongniartii* IMST 95041, *M. anisopliae* accumulated 3.8 times more NAG after six days and 2.9 times more after 8 days. Regarding the NAG concentration there was a significant difference between the low or less virulent *B. brongniartii* strains CH I and CH AN and the high virulent *B. brongniartii* strain IMST 95041 ($p < 0.05$). After six days IMST 95041 showed a 4.2 times higher (0.16 mM) and after eight days a 4.5 times higher NAG concentration in the supernatant, respectively. The two *B. brongniartii* strains CH AN and IMST 97011 which are defined as moderate or high virulent strains accumulated nearly the same amount of NAG as

the low virulent CH I strain after 6 days (Fig. 1 a). After eight days incubation there was a significant difference between CH I (0.07 mM) and IMST 97011 (0.12 mM). No significant difference was detectable between the isolates CH AN and CH I.

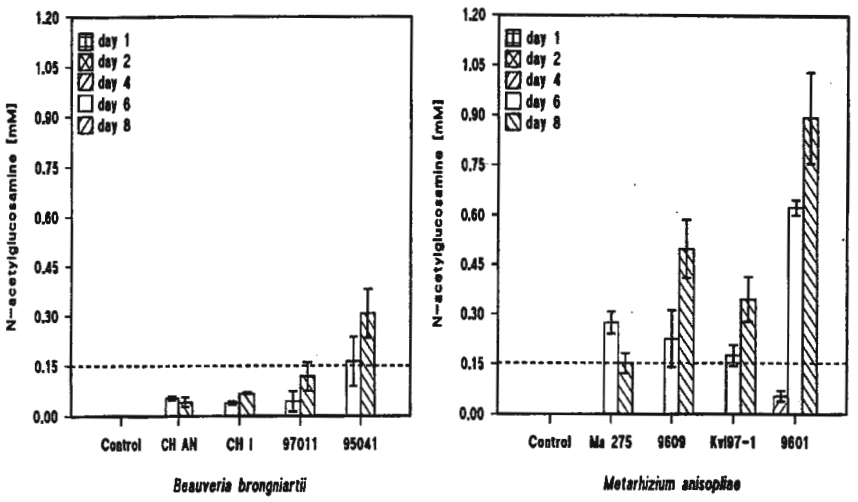

Figure 1: The detection of N-acetylglucosamine in the supernatant of (a) *Beauveria brongniartii* strains and (b) *Metarhizium anisopliae* strains.
.....:Theoretical threshold value of N-atcetylglucosamine (NAG) to identify virulent fungal strains in submerged culture (explanation see text).
Code of the strains: 97011: IMST 97011, 95041: IMST 95041 and 9601: IMST 9601.

Discussion

B. brongniartii infects specifically cockchafers *(Melolontha* spp.) but loses virulence if the fungus is cultivated on synthetic nutrient media without chitin for several generations. Futher this also occurs with *M. anisopliae,* which shows a wide host range against *Orthoptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera* and *Archnida.* This makes it necessary to prove the virulence of the strains periodically with time consuming biassays.In this work we focused on the evaluation of the exoenzyme chitinase as virulence indicator to be able to screen many *B. brongniartii* and *M. anisopliae* isolates for their virulence. Virulent entomopathogenous strains of *B. brongniartii* were used in this study to define a limit concentration of NAG in the supernatant of the submerged culture. Preliminary studies have shown that virulent *B. brongniartii* strains degraded chitin, the sole source of carbon in the culture broth, efficiently (Lung & Strasser, 1999). Both degradation products of chitin, N-acetylglucosamine (NAG) and glucosamine, could be detected in a maximum concentration of 0.42 and 0.25 mM, respectively. The authors claimed that there could be a positive correlation between an increased N-acetylglucosaminase activity and an increased mortality of the target *M. melolontha* (100% mortality after 16 days). Additionally, the faster the enzyme appeared and the higher the measured NAG concentrations were, the higher the

mortality rates was. The high virulent B. *brongniartii* strain IMST 97011 (fomer named V 11) killed 90 percent of the *M. melolontha* larvae after 10 days; NAG concentration was 0.42 mM. At the same time the moderate virulent *B. brongniartii* strain IMST 97010 (fomer named V 10) killed only 40 percent of the larvae; NAG concentration was 0.20 mM. No connection could be observed between glucosamine concentration and the virulence of the test strains.

Comparing more or less virulent M. *anisopliae* and *B. brongniartii* strains it could be demonstrated that all high virulent strains of both genera accumulated more than 0.15 MM NAG in the supernatant after six or eight days (Fig. 1a and 1b). This NAG concentration was defined as a first theoretical threshold value for estimating virulence potential of entomopathogenous fungal strains. None of the low or medium virulent strains (e.g. CH I and CH AN) were able to exceed this limit concentration.

Of course we are aware that more research is necessary to verify this argument. Essential experiments are still not finished. Currently, saprophytic imperfect fungi such as *Fusarium spp., Gliocladium roseum, Penicillium* spp., *Aspergillus* spp. and *Cladosporium cladosporoides will* be examinated for their chitinase activity in submerged culture. With the help of these tests we will get more insight how to handle the NAG concentration in submerged culture after a short incubation time. Further, strictly defined low and high virulent monospore cultures of *Beauveria* and *Metarhizium* strains will be tested under standardized submerged culture conditions. Defined fungal biomass of each strain will be incubated in a KCl-chitin solution with adding an nitrogen source. This technique makes it possible to calculate the enzyme activity of the N-acetylglucosaminidase in an more efficient way.

Acknowledgements

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Effect of magnesium ions on the pathogenicity of entomopathogenic micro-organisms applied into contaminated soil.

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Abstract: The aim of the study was to evaluate the effect of magnesium ions on the pathogenicity of entomopathogenic fungi and nematodes in soil contaminated by heavy metals. Experiment was carried out under laboratory conditions. Soil was contaminated with dissolved salts of cadmium $CdSO_4$, lead $Pb(NO_3)_2$, nickel $NiSO_4$, copper $CuSO_4 \times 7\ H_2O$ and zinc $ZnSO_4$. There were eight levels of heavy metal content in the soil. Entomopathogenic fungus *Paecilomyces fumosoroseus* and entomopathogenic nematode *Steinernema feltiae* were used in the experiment. *P. fumosoroseus* was grown on potato-dextrose-agar or on the same medium with addition of Mg ions ($MgSO_4 \times 7\ H_2O$). Ineffective juveniles of S. *feltiae* were conditioned in aqueous solution of magnesium salt ($MgSO_4 \times 7\ H_2O$). Contaminated soil was put into Petri dishes. Spores of P. *fumosoroseus* or infective juveniles of *S. feltiae* were inoculated into the soil. *Galleria mellonella* larvae were used to evaluate the pathogenicity of these micro-organisms. Soil contaminated by heavy metals significantly affected pathogenicity of *P. fumosoroseus* and S. *feltiae*. Infectivity of these micro-organisms was reduced in the soil with high level of heavy metal contamination. The magnesium ions increased the fungus and nematode infectivity against *G. mellonella* larvae in contaminated soil.

Key words: pathogenicity, entomopathogenic fungi and nematodes, magnesium ions

Introduction

Entomopathogenic fungi and nematodes are effective biological control agents against many pests. They are often applied in unfavourable condition for they survival and their efficacy against target pests. Heavy metals like cadmium, chrome or lead inhibited the growth of *P. fumosoroseus* mycelium (Jaworska *et al.*, 1997b). Entomopathogenic nematodes and fungi stimulated with manganese and magnesium ions revealed higher infectivity against tested insect *G. mellonella* L. (Jaworska *et al.*, 1997a; 1997b, 1997c).

The objective of this study was to determine the effect of magnesium ions on the pathogenicity of entomopathogenic fungi and nematodes applied into soil contaminated with heavy metals.

Material and methods

Contaminated soil

Experiment was carried out under laboratory conditions. Dusty soil, with organic matter content 16% and pH_{KCl} - 6.32, was used in the experiment. Soil was contaminated with cadmium $CdSO_4$, lead $Pb(NO_3)_2$, nickel $NiSO_4$, copper $CUSO_4 \times 7\ H_2O$ and zinc $ZnSO_4$. Dissolved salts of these metals were applied into the soil in seven different concentration. In control no heavy metal ions were added but the natural content of these metals ions was evaluated using ASA technique. There were eight levels of heavy metal content in the soil (Table 1).

Table 1. Heavy metal content in the soil

Contamination level of the soil	Heavy metal content (mg kg-1 of d.m.)				
	cadmium Cd.	lead Pb (II)	nickel Ni (II)	copper Cu (II)	zinc Zn
I (control)	1, 04	40, 86	4, 74	12,13	218, 53
II	6,17	84, 25	20, 00	33, 79	276, 57
III	11,17	114,25	35,00	53,79	326,57
IV	21,17	174, 25	65, 00	93, 79	426, 57
V	41,17	294, 25	125, 00	173, 79	626, 57
VI	81,17	534, 25	245, 00	333, 79	1026, 57
VII	161,17	1014,25	485,00	653,79	1826,57
VIII	321,17	1974,25	965,00	1293,79	3426,27

Steinernema feltiae

An English commercial strain of *Steinernema feltiae* was used in the experiment. The aqueous solutions of $MgSO_4$. 7 H_2O 160 mg/1) were prepared. Small test tubes (volume 2 cm^3) were filled with 1 cm^3 of Mg ions aqueous solution. 30 infective juveniles of *S. feltiae* were applied into the tubes. In control nematodes were kept in distilled water. Nematodes were kept in this solutions for 96 hours at 20°C. Contaminated soil was put into Petri dishes. Infective juveniles, after 96 hour contact with aqueous solution of Mg ions or distilled water, were applied into the soil at the rate of 30 per 1 Petri dish. Five *Galleria mellonella* larvae were put onto the soil. Petri dishes were stored at 25°C in the dark. The mortality of *G. mellonella* was evaluated after three days. The results were statistically studied by analysis of variance, and means were separated according to Duncan's multiple range test.

Paecilomyces fumosoroseus

Fungus *Paecilomyces fumosoroseus,* which is a Polish strain from *Leptinotarsa decemlineata.*, was tested. The fungus was kindly provided by Cecylia Bajan, Institute of Ecology, Polish Academy of Sciences, Warsaw.

The effect of Mg ions was studied using aqueous solutions of $MgSO_4$. 7 H_2O (320 mg/1) Such solutions (10 ml) were sterilised prior to their use and were applied to heat sterilised (125°C, 2 h) Petri dishes after which the glucose-potato agar standard was added (10 ml). The medium was prepared at our laboratory. Twice concentrated glucose-potato agar was prepared because then it was diluted by adding Mg ions solutions. In the control distilled water was added to obtain the same medium concentration. Than Petri dishes were inoculated with a fungus. Fungus was grown in the dark at 25°C. After 3 weeks conidia were harvested. The concentration of the conidia suspension was determined using a hemacytometer and diluted to 10^6 per ml. Soil contaminated with heavy metals ions was placed into sterile Petri dishes (diameter 10 cm). Soil was mixed with a suspensions of 1 million spores. Five *Galleria mellonella* L. larvae were put into Petri dishes. The mortality of *G. Mellonella* larvae was evaluated for six days. The experiment was replicated four times. The dishes were stored at 250C in the dark. The results were statistically studied by analysis of variance, and means were separated according to Duncan's multiple-range test.

Results and discussion

The mortality of *S. feltiae* stored in aqueous solution of Mg ions was showed in Figure 1. If the percentage of dead nematodes after 96 hours storage in Mg ions solution or distilled water is compared, it is obvious that mortality rate is higher for nematodes stored in distilled water.

The result on nematode infectivity in contaminated soil is shown in Fig. 2. Magnesium added to the solution in which the nematodes were kept increased the infectivity of *S. feltiae* against *G. mellonella* larvae. The effect of Mg ions on nematodes infectivity was also seen when the soil was contaminated with heavy metals. Infectivity of *S. feltiae* decreased with rising heavy metal content in the soil. Similarly as other entomopathogenic nematodes i.e. *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* (Jaworska *et al.*, 1996) also *Steinernema feltiae* nematodes are effectively protected from the effect of heavy metals ions when conditioned in aqueous solution of magnesium sulphate.

Differentiated heavy metal content in the soil affected the pathogenicity of *P. fumosoroseus* against *G. mellonella* larvae(Fig. 3). *P. fumosoroseus* showed higher pathogenicity in soil with low heavy metal contamination. The higher was the heavy metal content in the soil, the lower was the pathogenicity of this fungus. In the most contaminated soil the mortality of *G. mellonella* caused by *P. fumosoroseus* was low. Fungus reared on medium with Mg ions was more pathogenic than this reared on standard medium. The effect of Mg ions on *P. fumosoroseus* pathogenicity was seen in the soil with low heavy metal content. In laboratory experiment (Jaworska *et al.*, 1997c) cadmium (75 mg/1), zinc (200mg/1) and lead ions (200 mg/1), added to medium on which *P. fumosoroseus* was grown, significantly reduced the pathogenicity of this fungus. The stimulating effect of Mg ions on the entomopathogenic fungi was also observed in previous experiments (Jaworska *at al.*, 1997c; Jaworska & Ropek 1997). Statistical analysis of the influence of magnesium ions and heavy metal contamination on the infectivity of *S. feltiae* and *P. fumosoroseus* is shown in table 2. Stimulating effect of Mg ions on entomopathogenic micro-organisms infectivity is beneficial. Entomopathogenic fungi and nematodes with increased infectivity might be used on sites, where soil is polluted with heavy metals.

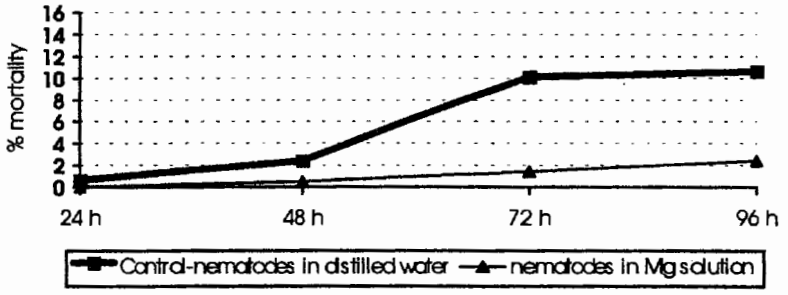

Figure 1. Mortality *of Steinernema feltiae* nematodes (%) during the 96 hour contact with magnesium ions ($MgSO_4.7\ H_2O$, 160 mg x dm^{-3})

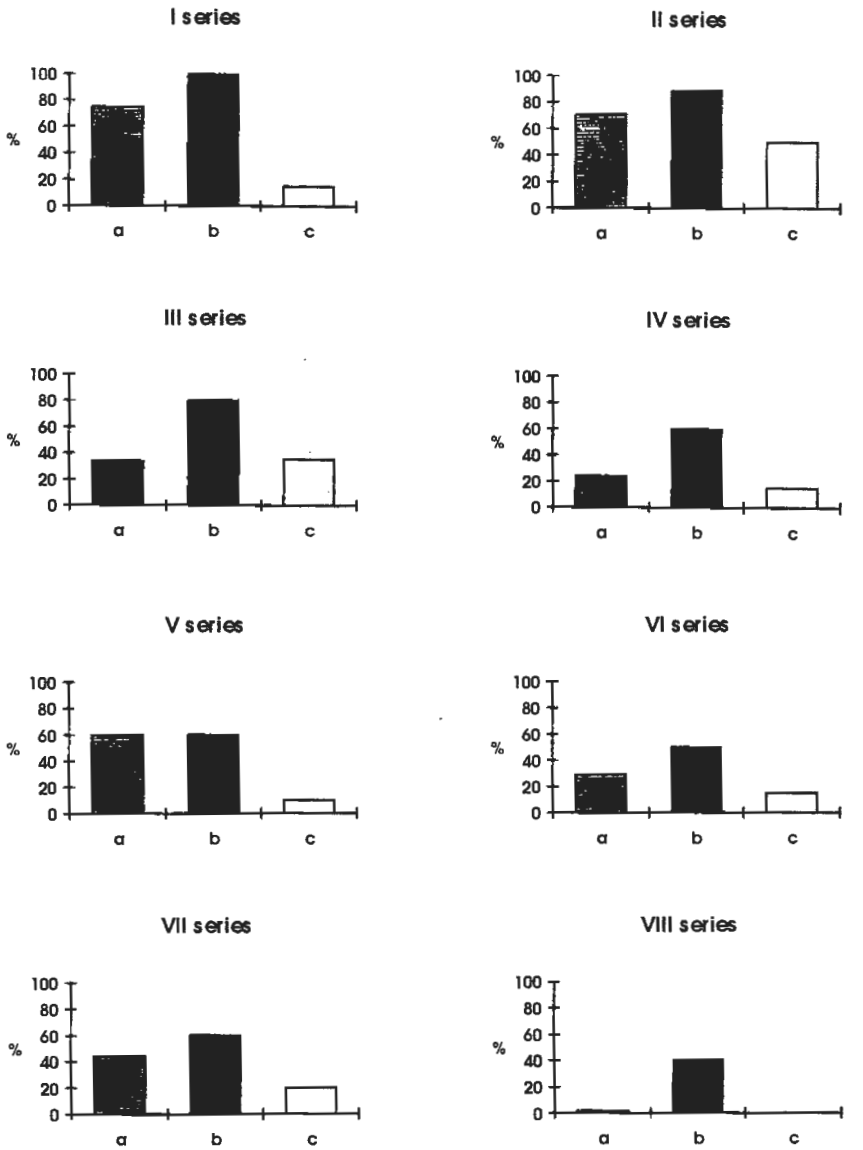

Figure 2. The influence of soil contaminated with heavy metal on pathogenicity of *Steinernema feltiae* nematodes stated by the mortality of *G. mellonella* caterpillars on the third day after aplication. a - nematodes stored in distilled water (control), b - nematodes stored in Mg ions solution, c -natural mortality of *G. mellonella* caterpillars.

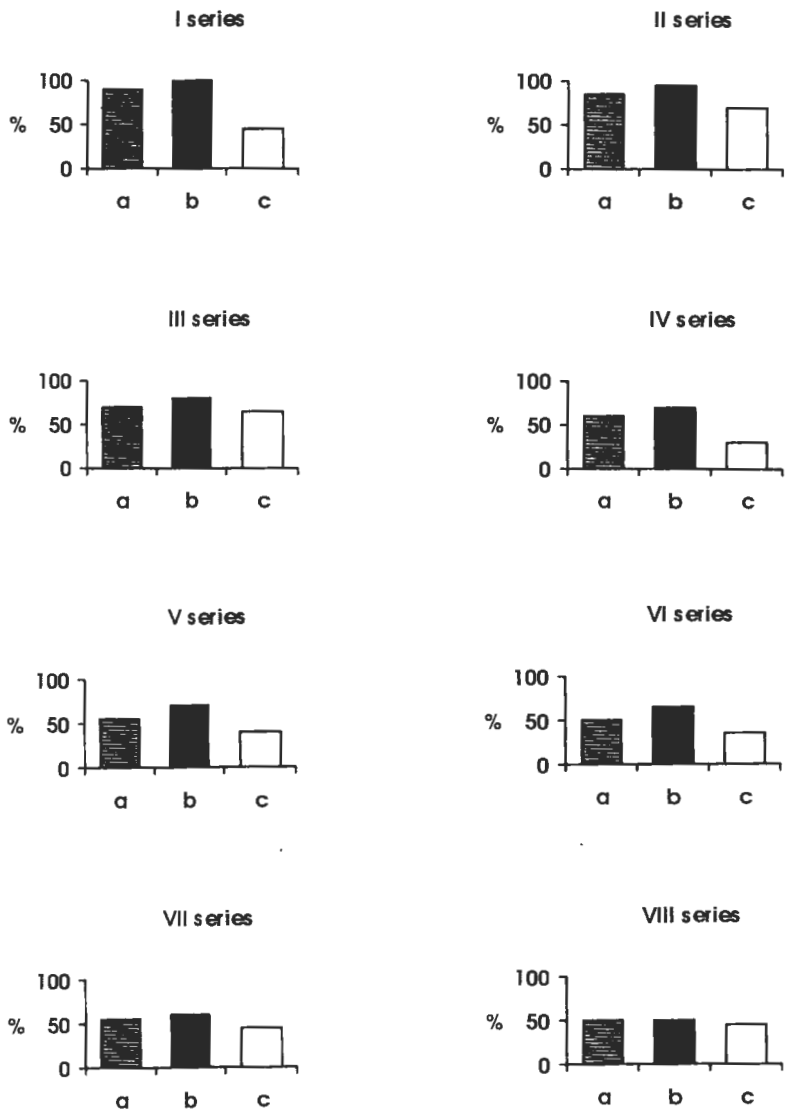

Figure 3. The influence of soil contaminated with heavy metal on pathogenicity of *Paecilomyces fumosoroseus* grown on standard medium (a) or on medium with magnesium ions (b) stated by the mortality of *G. mellonella* caterpillars (%). Control (c) - natural mortality of caterpillars.

Table 2.Statistical analysis of the influence of magnesium ions and heavy metal contamination on the infectivity of *Steinernema feltiae* and *Paecilomyces fumosoroseus*

Agents effecting micro-organisms infectivity	**Infectivity of entomopathogenic micro-organisms stated by the mortality of *G. mellonella* larvae (%)**	
	***Steinernema feltiae* (on third day)**	***P. fumosoroseus* (on sixth day)**
First agent: solution in which nematodes were kept or medium on which fungus was grown:		
• distilled water/standard medium	41.1 a*	66.2 a
• Mg ions solution/medium+Mg ions	71.4 b	77.1 b
Second agent: Heavy metal content in the soil:		
• I series	71.4 d	84.7 be
• II series	71.5 d	87.8 c
• III series	50.4 c	72.1 b
• IV series	32.1 b	52.1 a
• V series	41.6 be	55.1 a
• VI series	30.4 b	50.0 a
• VII series	40.8 be	53.3 a
• VIII series	7.9 a	48.3 a

*The Means followed by the same letters within a column are not significantly different (P.=0.05) according to Duncan multiple range test

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Accumulation of some metal ions on biomass of insect pathogenic fungus *Paecilomyces fumosoroseus*

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Abstract: The influence of 5 heavy metal ions i.e. $CdCl_2$ (50 mg x dm^{-3}), $CoCl_2$ (35 mg x dm^{-3}), $CuSO_4$ x 5 H_2O (8 mg x dm^{-3}), $Pb(NO_3)_2$ (200 mg x dm^{-3}) and $ZnCl_2$ (200 mg x dm^{-3}) on the growth of biomass entomopathogenic fungus *P. fumosoroseus* was studied. Fungus was grown on Sabauraud's medium with the addition of simple ion. It was concluded that lead, cadmium and cobalt ions are growth inhibitors of this fungus. Copper and zinc did not inhibited biomass growth. Mycelium was sampled for chemical analyses. The content of metal ions was determined by ASA technique on a Philips PU 91100X (cobalt on Iobin Yvon emission 238), following dry mineralisation of mycelium. Entomopathogenic fungus *P. fumosoroseus* has a high ability to accumulate cadmium, cobalt and copper.

Key words: heavy metal, accumulation, *P. fumosoroseus*

Introduction

Biological control methods of insect pests enables their selective elimination without polluting the environment. Active substances of insecticides are not included into a food chain, thus there are neither harmful to man and animals nor dangerous for other insects. A part of these methods is the use of insect pathogenic fungi. Muscaridine diseases caused by fungi from Fungi imperfecti class Hyphomycetes pose the main set of those diseases. Pink muscaridine disease is caused by *P. fumosoroseus.*

Insect pathogenic fungi occurred naturaly in fields and meadows. In natural environment infectivity and introduction efficacy is able to be limited by a range of soil abiotic factors. The influence of insecticides, herbicides, fungicides and mineral fertilisers present in soil has already been assessed (Bajan *et al.*, 1983).

The effect of heavy metals on insect pathogenic fungi has been little explained. The study of metal impact on *P. fumosoroseus* was conducted as a laboratory model experiment to determine effects of simple metal ions on biomass growth of fungus colony and accumulation of these heavy metals by *P. fumosoroseus* mycelium. Five metal ions were selected here such as cobalt, cadmium, copper, lead, and zinc. Concentrations of the ions were used according to the results of monitoring soil pollution near Krakow surroundings.

Material and methods

P. fumosoroseus strain from Institute of Ecology PAN (Polish Academy of Science) was used. The growth of fungi biomass took place in Erlenmeyer's flasks on liquid peptone and glucose media with addition of chosen individual metal ion. Peptone and glucose medium alone was used as control. The spawn of *P. fumosoroseus* was inoculated onto prepared sterile medium in three contemporary samplings. The fungi were incubated at the temperature of 21°C for 21

days. After that period the liquid remains together with the colony were leaked through filter paper drains (No 388, Ø 110 mm) an dried at 100°C temperature until they reached constant weight. Then the filter paper drains were weighted. The content of zinc, lead, cadmium and copper in *P. fumosoroseus* mycelium was determined after drying and burning the mycelium using the wet method. The measuring was done using ASA, Philips PU 9100X atomic measuring data station. The content of cobalt was determined using the same wet method but on Iobin Yvon emission 238 utrace apparatus. Obtained data were analysed using ANOVA procedures and Duncan's multiple test.

Results and discussion

The results of *P. fumosoroseus* growth (in grams of dry mass) are showed in Figure 1. When fungus was grown on medium alone only trace amounts heavy metals were found in the mycelium. Only the content of zinc was considerably high 5.5 mg x kg^{-1}. There was an significant influence of heavy metals ions on *P. fumosoroseus* biomass growth. Lead, cobalt and cadmium ions reduced the biomass growth. Lead ions abated the obtained biomass about 40% compared to controls on medium alone. Cobalt and cadmium ions downed obtained biomass about 20% and 10% respectively. Zinc and copper ions did not influence the biomass growth of *P. fumosoroseus*.

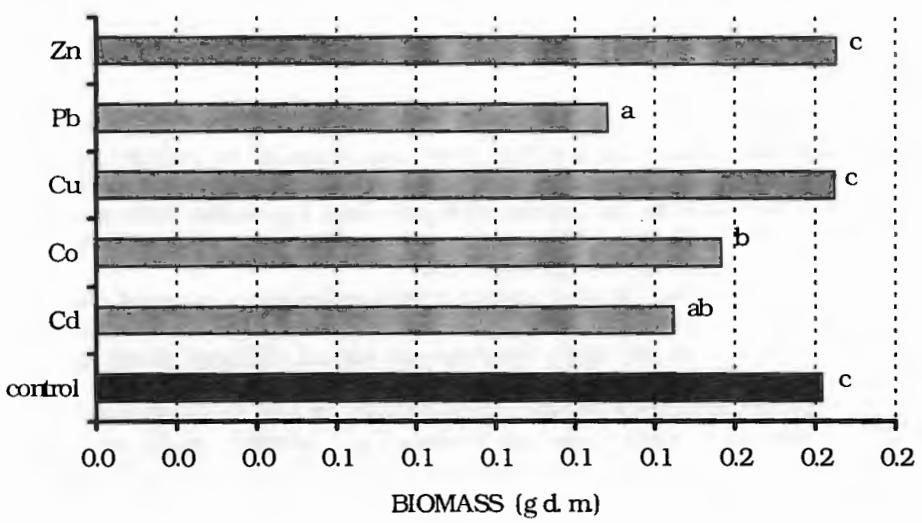

Figure 1. The influence of heavy metal ions on *P. fumosoroseus* biomass growth.

Table 1 shows the accumulation of heavy metals on biomas of insect pathogenic fungus *P. fumosoroseus*. All studied heavy metals ions were accumulated by *P. fumosoroseus*. has high ability to accumulate cadmium, cobalt and copper. The concentration of these metals in *P. fumosoroseus* mycelium was 3 to 5 times higher than in the medium. *P. fumosoroseus* did not accumulated large amounts of zinc. The concentration of zinc in mycelium was only 60 % higher than in the medium. The concentration of lead was lower in the mycelium in

comparison to the medium. *P. fumosoroseus* accumulated about 7 - 8 % of cobalt, cadmium and copper ions, which were available in the medium. The uptake of lead, which decreased the biomass growth, was considerably low. *P. fumosoroseus* accumulated only 1.6% of lead ions from the medium. Cobalt and cadmium also inhabited the biomass growth, but their uptake by *P. fumosoroseus* mycelium was much higher. It means that *P. fumosoroseus* does not accumulates heavy metals indiscriminately.

Table 1. Accumulation of heavy metals ions in *P. fumosoroseus* mycelium

	Type of medium					
	Control medium	Medium + Cd	Medium + Co	Medium + Cu	Medium +Pb	Medium +Zn
Content of heavy metals ions in the medium [m x dm^{-3}]						
Cd		50				
Co			35			
Cu				8		
Pb					200	
Zn						200
Content of heavy metals in the *P. fumosoroseus* mycelium [mg x kg^{-1}]						
Cd	0.0200	297				
Co	0.0005		114			
Cu	0.2045			28		
Pb	0.2908				169	
Zn	5.5200					325
Percentage [%] of heavy metals ions available in the medium, which were accumulated b *P. fumosoroseus*						
Cd		8.61				
Co			7.37			
Cu				7.23		
Pb					1.64	
Zn						2.97

It seems clear from the results of studies on metal ions influence on development of *P. fumosoroseus* (Jaworska *et al.*, 1996; Jaworska *et al.*, 1997; Jaworska & Gorczyca 1997), *Trichoderma viride, T. harzianum* and *T. pseudokoningii* (Jaworska & Dłużniewska, 1997), *Aspergillus flavus* (Barabasz *et al.*, 1997), and *Metharizium anisopliae* and *Beauveria bassiana* (Gorczyca 1997), that some metals appeared to be poisonous to particular fungi species. Most frequently cadmium, lead, cobalt, vanadium and chrome ions were harmful. Otherwise, Mn, Mg, Li, Cu, Al and Zn ions usually affected stimulating on variable fungi species. Some possible differences in obtained data are due to different solutions of ions in media and using various media or to specific need for suitable elements in studied species. The results of the study imply that lead, cobalt and cadmium are growth inhibitors for *P. fumosoroseus* insect pathogenic fungus and this fungus has a high ability to accumulate cadmium, cobalt and copper.

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Paecilomyces fumosoroseus: Efficacy against the cotton pests *Spodoptera littoralis* (Lepidoptera: Noctuidae), *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Aphis gossypii* (Homoptera: Aphididae) and effect of temperature on germination and mycelial growth

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Abstract: The entomopathogenic fungus *Paecilomyces fumosoroseus* (Wize) Brown & Smith is currently used mostly against whiteflies. However, the species is known also to infect other pest insects. Within a research project on biocontrol of cotton pests, we tested the effect of temperature on *P. fumosoroseus* and its efficacy against various important cotton pests. In all experiments, the isolate *P. fumosoroseus*, ARSEF 3877, was used, which was originally isolated from *Bemisia tabaci*. Bioassays were conducted against third instar larvae of Spodoptera littoralis and *Helicoverpa armigera* and against adults of *Aphis gossypii*. In first experiments at 25°C, the mortality was 76.2%, 59.6% and 95.8% in *H. armigera*, *S. littoralis* and *A. gossypii* respectively. The results showed, that *A. gossypii* is more susceptible *to P. fumosoroseus* than *H. armigera* and *S. littoralis*. In further experiments, the effect of temperature on the efficacy of the fungus against L-3 of *S. littoralis* was tested in the laboratory with 1 x 10^8 conidia/ml . The mortality and the Median Lethal Time (MLT) were studied again at 25°C, 30°C and 35°/25°C using a spore concentration of 1 x 10^8 conidia/ml. After 14 days at 25°C and 35°/25°C, the mortality was 88.5% and 84.5% with an MLT of 7.6 and 8.8 days respectively. At a constant temperature of 30°C, however, the corresponding data were 70.9% and 10.3 days. The germination of conidia was tested at 25°C, 30°C, 35°C and at an alternating temperature of 35°/25°C (8/16h). After 16h, the germination rate was 98.3%, 79.6%, 0% and 47.0% respectively. After 24 h, a nearly 100% germination was achieved at all temperatures except 35°C. The colony diameter on malt extract-peptone-agar after 14 days was 4.6 cm , 2.2 cm, 0 cm and 2.4 cm at 25°C, 30°C, 35°C and 35°/25°C.

The investigations demonstrate, that *P. fumosoroseus* is highly virulent against these three important cotton pests and that experiments on alternating day/night temperatures are essential when working with tropical pest insects.

Key words: *Paecilomyces fumosoroseus*, cotton pests, *Spodoptera littoralis*, *Helicoverpa armigera*, *Aphis gossypii*, biological control in cotton, temperature, germination, mycelial growth

Introduction

Cotton is one of the most important crops in agriculture. Therefore, all available measures are used to protect cotton plants from their main pest insects, especially the cotton leafworm *Spodoptera littoralis* (Boisd.), the budworm *Helicoverpa (Heliothis) armigera* (Hübner) and the cotton aphid *Aphis gossypii* (Glov). The development of high levels of resistance to chemical insecticides and the negative impact of pesticides on natural enemies of these pests have encouraged the development of alternative means of control within the context of

integrated pest management. In particular, the exploration for and the development of biological control methods have been intensified in the last years. Several species of entomopathogenic fungi show promise for control of these cotton pests.

Paecilomyces fumosoroseus (Wize) Brown & Smith is one of the fungal species being studied particulary for control of *B. argentifolii* (Lacey *et al.*, 1995 and Vidal *et al.*, 1997). This species was also tested against other lepidoptera like *Mamestra brassica*, *Spodoptera littoralis* (Fargues & Rodrigues, 1980) and aphids like the Russian wheat aphid, *Diuraphis anoxia* (Mesquita *et al*, 1996). It is known that the potential of fungal pathogens as biocontrol agents may be influenced by several environmental factors such as moisture, temperature, exposure to sunlight and humidity. Informations are available on the effect of temperature on germination and/or growth and entomopathogenic fungi (Müller-Kögler, 1965). In general, the temperature range for growth is between 5° and 35°C and the optimum between 20° and 30°C. Optimum temperatures for spore germination are less well documented than those for mycelial development. In this study, the pathogenic activity of one strain of *P. fumosoroseus* (ARSEF 3877) was tested against *S. littoralis* (Lep., Noctuidae), *H. armigera* (Lep., Noctuidae) and *A. gossypii* (Hom., Aphididae). In further studies, the effect of temperature on germination and mycelial growth was investigated.

Material and Methods

Fungi and insects:

The strain of *P. fumosoroseus* (ARSEF 3877) used in our investigations was originally isolated in 1992 from *Bemisia tabaci* (Gennadius) in Multan, Pakistan, and obtained by Dr. L. Lacey. Larvae of *S. littoralis* and *H. armigera* and adults of *Aphis gossypii* were obtained from AgrEvo, Germany. They were reared continuously in the laboratory at 25 ± 2°C under a photoperiod of 16:8h (L:D) on a semi-synthetic medium for the two lepidoptera and on cotton for *A. gossypii*

Bioassays

Spodoptera littoralis and *Helicoverpa armigera:*

The susceptibility of *S. littoralis* and *H. armigera* was evaluated by spraying 2 ml of 1 x 10^7 conidia/ml using a Potter tower (Burkard Ltd, England) on 6 day old third instar larvae. Three replicates of 20 larvae each were used. Control larvae were sprayed with 0,1 % Tween 80 only. After treatment and drying of the sprayed suspension for about 15 min, each larva was transferred to a separate vial with wetted filter paper and a cotton leaf disc of 2.2 cm diameter. The vials were incubated at 25 ± 1 °C, 70 ± 10% RH and 16:8h (L:D).

For *S. littoralis* the experiments were carried out at 2 constant temperatures of 25°C and 30°C and at an alternating day/night temperature of 35°/25°C, 8/16h (L:D) with 1 x 10^8 conidia/ml. Larvae were checked for mortality in 24 h intervals. Dead larvae were put in a moist chamber to check the outgrowth of the fungus.

Aphis gossypii:

P. fumosoroseus was applied using 2-ml of an aqueous suspension of conidia using a Potter tower. Five replicates of 10 adults in petri dishes (3 cm diameter) were treated with 1 concentration of 1 x 10^7 conidia/ml resulting in a dose of 3.1 x 10^3 conidia per cm^2. Control insects were treated with 2 ml of Tween 80 (0.1 %). After spraying, the petri dishes were covered with lids to maintain a saturated humidity and placed upside down in 9 cm diameter petri dishes at 25 ± 1°C and a photoperiod of 16:8 h (L:D).

After incubation for 24 h under high humidity, the lids were replaced by those with a 1 cm hole covered by a fine gauze- (0.1-mm mesh). The plates were then maintained at 25 ± 2°C and 70 ± 10% RH and a photoperiod of 16:8 h (L:D) for 7 days. The aphids were daily

monitored for mortality. Dead nymphs were placed on sterile water agar and incubated at laboratory temperature for 5-7 days to determine the outgrowth of the fungus.

Effect of temperature on germination

Three petri dishes (6 cm diameter) with malt extract-peptone agar were evenly inoculated with 0,1 ml of about 10^6 conidia/ml. After 8, 16 and 24 h at 25°, 30°, 35° and 35°/25°C (8L:16D) 3 samples of about. 1 cm^2 were taken from each plate, and the germinated and ungerminated conidia were microscopically counted. The experiment was repeated three times.

Effect of temperature on vegetative growth

Discs of 5 mm diameter from 3 day old plates of *P. fumosoroseus* were transferred to petri dishes (9 cm diameter) containing malt extract-peptone agar. The same temperatures as mentioned above were tested. The diameter of the growing colony was measured daily for 14 days. There were 4 replicates for each temperature regime. The assay was repeated three time.

Results and Discussion

The strain of *P. fumosoroseus* tested was able to infect 3rd instar larvae of *S. littoralis* and *H. armigera* as well as adults of *A. gossypii* in the laboratory. The mean mortality, the number of infected specimens and the Median Lethal Time (MLT) are listed in Table 1. Data on the bioassay at different temperatures are presented in Table 2. According to our experiments the host range of *P. fumosoroseus*, ARSEEF 3877, is fairly broad. This strain was highly virulent against *H. armigera* and *A. gossypii* and slightly virulent for *Spodoptera littoralis* 3 rd instar larvae.

In the bioassay against *S. littoralis* larvae on different temperatures, *P. fumosoroseus* caused a higher mortality at the alternating temperature of 35°/25°C than at permanently 30°C. The study on germination and growth of *P. fumosoroseus* at various temperatures indicates that this strain can tolerate high temperatures up to 35°C. Optimal germination and growth was observed by 25°C with 98.3% of germination after 16 h and 5.54 cm radial growth after 14 days. The potential of this isolate to grow and infect at temperatures of 30°C and 35°/25°C may be attributed to its geographical origin (Pakistan).

Tab. 1: Efficacy of *Paecilomyces fumosoroseus* (ARSEF 3877) against third instar larvae of *S. littoralis* and *H. armigera* and on adults of *A. gossypii*, (1 x 10^7 conidia/ml)

Insect	Mortality (% ± SE)	Mycosis (% ± SE)
S.littoralis	59.6 ± 0.9	44.0 ± 0.9
H. armigera	76.2 ± 1.9	76.6 ± 2.5
A. gossypii	95.8 ± 2.6	100

[a] Median Lethal Time (SAS, 1989)

Tab. 2: Efficacy of *Paecilomyces fumosoroseus* (ARSEF 3877) against third instar larvae of *S. littoralis* at different temperatures (1 x 10^8 conidia/ml)

Temperature	Mortality (% ± SE)[1]	MLT[2] (% ± SE)	Mycosis (% ± SE)
25°C	88.46 ± 2.5 a[3]	8.80 ± 0.2	94.76 ± 1.9 a[3]
30°C	70.86 ± 4.0 b	10.35 ± 0.2	67.31 ± 7.9 a
35°/250C	84.53 ± 2.7 a	8.85 ± 0.2	40.44 ± 10.4 b

[1] Standard error of the mean from five replications
[2]Median Lethal Time (SAS, 1989)
[3]Mean of the arcsine√ p-transformed data within a column followed by the same letter are not significant following Tukey's studentized test (P>0.05)

Tab. 3: Effect of temperature on germination and mycelial growth of *Paecilomyces fumosoroseus* (ARSEF 3877)

Temperature	Germination[2] (% ± SE)[1]	Growth[3] (cm)
25°C	98.3 ± 0.5	5.54 ± 0.1
30°C	79.6 ± 1.9	3.11 ± 0.1
35°C	0	0
35°/25°C	47.0 ± 1.7	3.28 ± 0.1

[1] Standard error of the mean from five replications
[2] Germintion after 16 h
[3]Vegetative Growth after 14 days

Conclusions

The three cotton pests Spodoptera littoralis, *Helicoverpa armigera* and *Aphis gossypii* are susceptible to the *Bemisia* derivated isolate of *P. fumosoroseus* (ARSEF 3877). These findings demonstrate that isolates from one host insect may also be highly effective against other target species. They should also be included in biocontrol programmes. The results also indicate that in our bioassays, *Aphis gossypii* adults were more susceptible than 3rd instar larvae of the two lepidoptera. Additionally, *H. armigera* was more susceptible than larvae of *S. littoralis*. This fungus is an interesting candidate for further development as a microbial control agent against these three important cotton pests. The studies on the effect of temperature on the germination, vegetative growth and the virulence against *S. littoralis* larvae demonstrated that the fungus isolate is tolerant to high temperatures and thus seems to be adapted for use in tropical and subtropical areas.

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Importance of coincidence for the efficiency of the entomopathogenic fungus *Verticillium lecanii* against *Frankliniella occidentalis*

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Introduction

The chances of success of highly virulent entomopathogenic fungi directly depend on high humidity conditions (94% RH) (Gillespie & Crawford, 1986). This is generally considered to be a sufficient coincidence for these partners.

The short period of development of Thysanoptera and its cyclic determinated change between plant and soil may lead to critical phases of incoincidence.

Material and methods

All experiments were done with the standardizised leaf or soil test respectively (Sermann, Welsch, 1997). *F. occidentalis* was obtained from a standardizised laboratory breeding. *V. lecanii* (Isolate V24) was formulated as a blastospore or conidia suspension. 5,5 ml of the spore suspension ($1{,}5 \times 10^7$ sp/ml) were applied with a fine spraying tower.

Results

1. Coincidence field
Thysanoptera are well able to overcome the occasionally incoincidence of field caused by the change of site at the different mobil stages of development of *F. occidentalis.* A similarily high mortality was obtained independently on whether spores are directly applicated or stick later on the surface of the larvae by their movement (fig.1).

2. Coincidence phases
At 25°C the mortality of the older larvae on soil was significantly smaller than at 20°C. The development phases of the thrips were shortened by 27% at a temperature of 25°C in contrast to 20°C (fig. 2) - much more than the development of the pathogen accelerated at germination (14%) or mycel development (9%) respectively. This difference was increased by using slowly germinating conidia.

3. Coincidence sensitiveness
The adults and larvae were prooved to be extraordinarily high sensitive by a quick disease development and a high mortality, whereas the pronymph- and nymph-stages showed a rather small mortality (fig. 3).

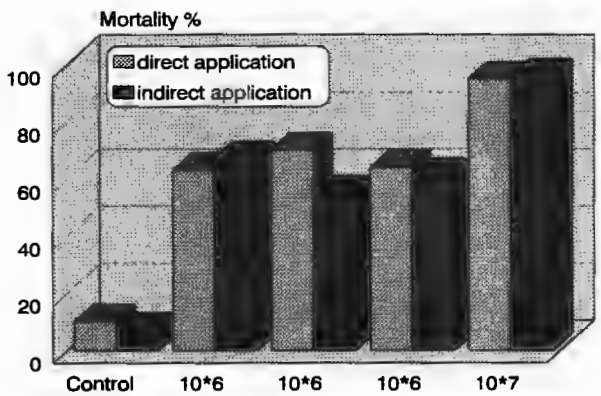

Fig.1. Mortality of *Frankliniella occidentalis* after direct or indirect application of *Verticillium lecanii* (Isolate V 24, 10^6 or 10^7 Bsp./ml) at 20°C

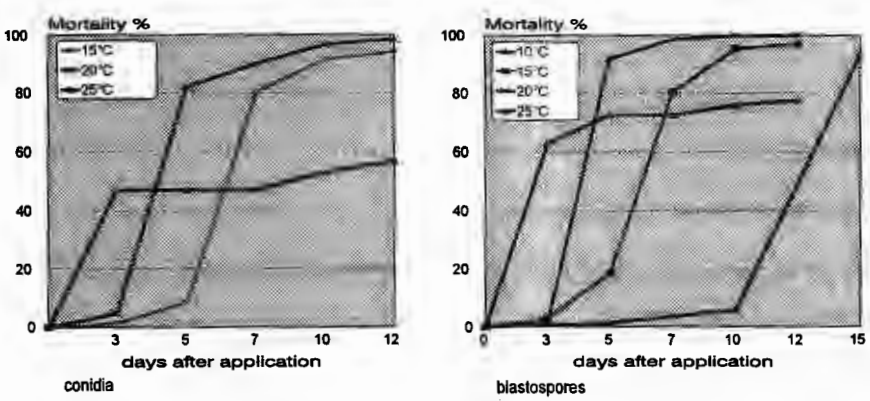

Fig.2. Mortality of *Frankliniella occidentalis* after soil application (indirect) of *Verticillium lecanii* (Isolate V 24, 10^7 Bsp./ml) at different temperature

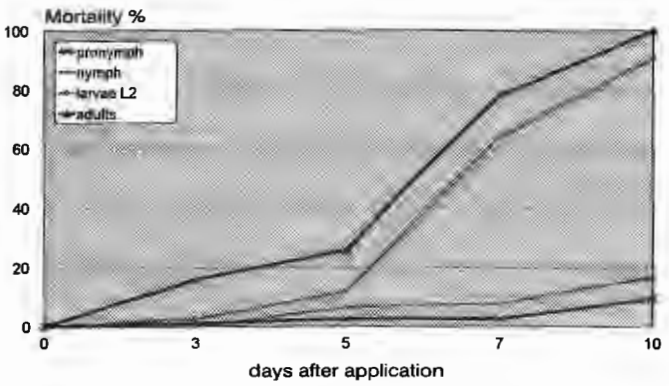

Fig. 3. Mortality of *Frankliniella occidentalis* after indirect application on leaf on larvae and adults an on soil on pronymphs and nymphs with *Verticillium lecanii* (Isolate V 24, 10^7 Bsp./ml) at 20°C

Discussion

- The partly incoincidence of host and pathogen is compensated by the high locomotion activity of Thysanoptera and has therefore no negative influence on the efficiency of the pathogen.
- Increasing temperatures cause the discrepancy of the coincidence phases and obviously lead to smaller mortality rates and may worsen the efficiency of the pathogen lastingly.
- The different sensitiveness of *F. occidentalis* at different development phases has to be evaluated as a negative aspect, but this can be compensated after the change of development phase by a later infection of the larvae or adults respectively, so that the total efficiency of *V. lecanii* is not diminished.

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Investigations on the adhesion of spores of *Verticillium lecanii* at *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae)

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Introduction

The Western Flower Thrips (WFT), *Frankliniella occidentalis*, has become a main pest in ornamental and vegetable growing. Damages caused by adult and larval stages consist mainly in mechanical destruction of flower tissues as well as in transmission of phytopathogenic viruses (Reimherr, 1989).

Due to occurring resistance against chemical compounds (Immaraju *et al.*, 1992; Broodsgard, 1994) and because of their specific life cycle which includes the cryptic nymphal stages (Mantel & Van De Vrie, 1988) conventional methods of pest control are less successful against WTF.

The entomopathogenic fungus *Verticillium lecanii* is known as a pathogen for various insects. And the use of this fungus against WTF as an alternative way has been investigated recently (Vestergaard *et al.*, 1995; Sermann *et al.*, 1998). The infectious properties of entomopathogenic fungi especially in a percentage course is correlated with the adhesion of the inoculum (Gunnarsson, 1988; Heale *et al.*, 1991). However, preconditions for a successful adhesion of spores have not been enlightened completely. The aim of the current study was to investigate the adhesive properties of both conidiae and blastospores of *V. lecanii*.

Materials and Methods

Fungal culture

The *V. lecanii* isolate V24/K2 has been separated from a mixed culture V 24 which was isolated from green aphid (*Myzus persicae*). Blastospores were raised in a culture solution consisting of 3 % malt extract and 1 % yeast extract at 25 °C under dark conditions at a shake retort. For breeding conidiae the fungus culture was inoculated on an oats-pea-flour-agar and stored at 25 °C under dark conditions for 7 to 10 days. Conidiae were washed off later on by sterile tap water and stored at - 20 °C. Thawed out at room temperature fungus suspensions were homogenised at a shaker and diluted to a density of 10^7 spores/ ml.

Insect culture

Insects were bred at bean plants (*Phaseolus vulgaris*) in a climatic chamber at 20 ±1 °C, 50 ±10 % relative humidity and 16 h light illumination.

Application of spores

Fungal spore suspensions were sprayed by assistance of a Potter tower (Burkard Manufacture Co. Ltd). The application volume was 4 ml per variant.

Observations

Observations were made by fluorescent microscopy. For this, two to three drops of fluorescent stain "Fungiqual A" (Fa. Reiner & Rembold) were added after the inoculum had been diluted. A fluorescent microscope (Leitz Metallux 3) with a system of light filters was used to observe the adhesion of spores on the objects.

Construction of the experiments

The experiments consisted of two attempts:

1. A glass surface as an inert (biologically neutral) substrate was used to exclude specific adhesion mechanisms of spores at their host's surface. In this experiment the adhesion of spores was examined in 4 variants: 0, 6, 12 and 24 h after application. For each variant the number of spores (x_1) at a certain surface (10 fields of 1 mm^2 each) was counted immediately after application (at time 0). After incubation the glass surface was washed with 50 ml water and the number of remaining spores (x_2) was established. Experiments were repeated 3 times.
2. The adhesion of both conidiae and blastospores of *V. lecanii* on the integument of alive and dead adults of *F. occidentalis* was compared. The number of stuck spores at the dorsal surface of the thorax and the caput was counted immediately after application (x_1) and 24 h later (x_2). Alive thrips were anaesthetized by cooling.

Results

Glass surface

The average number of conidiae and blastospores at the controlled surface immediately after application (x_1) varied from 51.3 to 57.3 and from 57.4 to 71.3 respectively.

Within 24 h there was a steady reduction of the adhesion of conidiae (tab. 1). After 24 h the percentage of remaining spores was 61.6 % while the reduction of spores within the first 12 h of observation was statistically not significant.

Blastospores showed an adhesion of 80.5 % after 24 h incubation. The highest loss of spores (26.6 %) was found within the first 12 h of incubation.

Tab. 1: Average difference between the number of spores at a glass surface immediately post application (x_1) and the number of adhered spores after washing at different times (x_2)

type of spores:	**conidiae**				**blastospores**			
variant (h)	0	6	12	24	0	6	12	24
number of spores at (x_1)	51.3	53.2	53.5	57.3	71.3	69.7	68.0	57.4
difference (x_1 -x_2)	9.6	13.4	14.8	22.0*	10.0	14.9*	19.0*	13.6
s	7.3	15.3	10.0	10.3	9.6	9.9	16.0	11.3
% of adhered spores	81.3	74.8	72.3	61.6	85.3	74.0	73.4	80.5

*statistically significant difference

Insects

The percentage of sticking conidiae at the integument of alive and dead thrips 24 h post application was 66.2 % and 55.7 % respectively (tab. 2). Differences in the number of

adhered spores between alive and dead thrips were statistically not significant. The adhesion of blastospores on the integument of dead thrips (68.5 %) is comparable to that of conidiae (66.2 %). Blastospores at alive insects showed the lowest percentage of adhesion (41.9 %) in comparison to all other variants.

Tab. 2: Average difference of adhered spores at the integument of dead and alive thrips at the beginning of the experiment (x_1) and 24 h post application (x_2)

parameter	**conidiae**		**blastospores**	
variant	dead	alive	dead	alive
number of spores at (x_1)	33.9	36.6	30.2	35.6
difference (x_1 -x_2)	11.5*	16.2*	9.5*	20.7*
s	6.1	7.8	5.8	8.5
% of adhered spores	66.2	55.7	68.5	41.9

*statistically significant difference

Discussion

In our own investigations the average number of blastospores and conidiae at a 1 mm^2 glass surface immediately after spraying was 66.6 and 53.8 respectively. After washing the surface 0, 6, 12 and 24 h post application a reduction of spores was observed in general. In this experiment after 24 h incubation time blastospores showed a higher adhesion than conidiae (80.5 % vs. 61.6 %). The different surface of spores of both types might be an explanation for these results.

SEM (scanning electron microscop) investigations of both types of spores showed in blastospores a clearly more smooth surface in comparison to conidiae. This surface is often covered with extra cellulare matrix (ECM). On the other hand, the ability of blastospores to germinate quickly may contribute to a better fixing. Up to date there are no literature data on this topic.

A reduction of the numbers of spores was also registered at the integument of alive and dead thrips 24 h post application. At the integument of dead insects the adhesion of both types of spores was identical. A higher adhesion of conidiae in comparison to blastspores was found at the surface of the cuticle of alive thrips. This may indicate an importance of encymatic activity of ECM (Schreiter, 1995).

The reduction of spores within the first 24 h post application is according to Sitch & Jackson (1997) an indication for a resistance of insects against the fungus. Contrary to resistant specimens, they didn't find a loss of spores at susceptible insects.
However, the own experiments on the virulence of the *V. lecanii*-isolate V24/K2 showed a thrips mortality of more than 90 % at least at day 9 post application (Wolff, 1999).

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Pathogenicity of several entomogenous fungi to some of the most serious forest insect pests in Europe

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Abstract: Different strains of the entomopathogenic fungi *Beauveria bassiana, Paecilomyces farinosus, Metarrhizium anisopliae*, and *Verticillium lecanii* were tested in laboratory with regard of their virulence against larvae of *Lymantria dispar* and *Cephaleia abietis*, and adults of *Ips typographus* and *Hylobius abietis*.

The probit analysis was used to estimate LT_{50} and to evaluate fungal virulence.
The larvae L_1 and L_{2-3} of *L. dispar* were not susceptable to the tested fungi. The only pathogenic strain 3V of *Beauveria bassiana* acted too slowly (LT_{50} - 11 and 20 days resp.).
Opposite results were recorded with *Ips typographus* - all tested strains of the fungi *B. bassiana, P. farinosus, V. lecanii*, and *M. anisopliae* acted quickly - LT_{50} from 2,6 to 5,5 days.

Only two strains of the fungi *M. anisopliae* and *B. bassiana* caused infection of *Hylobius abietis* -adults with LT_{50} 4,9 and 7,4 days resp.
Mortality of *Cephaleia abietis*-larvae due to *B. bassiana* and *P. farinosus* infection was comperatively quick -LT_{50} from 2,6 to 11,1.

The biotests indicated that hyphomycetous entomopathogens were not appropriate for control of *L. dispar*, but were promising in biocontrol of *I. typographus, C. abietis* and *H. abietis*.

Introduction

The most popular entomopathogenic hyphomycetous fungi: *Beauveria bassiana, Verticillium lecanii, Metarhizium anisopliae*, and *Paecilomyces farinosus* have broad geographical distribution and attack a wide range of hosts (Ignoffo 1967; Veen 1968; Roberts & Yendol 1971; Fargues & Remaudière 1977; Burges & Hall 1982). Numerous experiments have been made to evaluate their virulence against agricultural pests. Except of *B. bassiana* mass application in China (Feng *et al.*, 1994), little has been published about biocontrol of forest pests by fungi.

This paper reports results for laboratory test of several isolates on the forest pests *Lymantria dispar, Ips typographus, Cephaleia abietis*, and *Hylobius abietis*. There are very few records of naturally infected insects of these species (Panajotov., 1960; Majchrovicz & Yendol 1973; Neuzilova 1956; Balazy 1962; Donaubauer 1959; Kurir 1977; Urban 1967; Samsinakova & Novak 1967). Despite the rare natural spread of infections, the aim of this study was to evaluate the potential role of the fungi for biocontrol of the mentioned insects.

Materials and Methods

Fungi

The fungal strains of entomopathogenic fungi used in this study were deposited in the Institute of Entomology (ENTU), Czech Republic: isolates 43,2B, and 3V of *Beauveria bassiana*; isolates 10 and 122 of *Paecilomyces farinosus*; isolates 76 and 77 of *Verticillium lecanii*; and

Metarhizium anisopliae 1V. Conidia for the infectivity studies was prepared by growing each fungus on appropriate agar in petri dishes for 21 days at 20°C.

Insects and infectivity tests

The test with *Lymantria dispar* was carried out using larvae first and second-third instar, reared from eggs previously collected from naturally infested forests and inspected for natural pathogens. Larvae from each instar in groups of 20, in five replicates, were kept for 30 min. in culture of *Beauveria bassiana* 3V. Ten caterpillars were rinsed in sterile distilled water with 0,1% Tween 80 for counting number of spores in a Buerker 's chamber. Infected larvae were placed in glass containers and fed with fresh oak leaves replaced regularly by new ones.

The test with *Ips typographus* and hyphomycetous fungi *Verticillium lecanii* strains 76 and 77; *Paecilomyces farinosus* strains 10 and 122; *Beauveria bassiana* strains 3V and 43; and *Metarhizium anisopliae* 1V, was carried out using beetles in groups of 10 in three replicates for each fungal strain. The insects were collected in spring in spruce forests, seriously damaged by windbreak. In lab they were allowed to move free for 5 min on the surface of the fungal cultures. Ten beetles for each fungal strain were washed in distilled water with Tween for estimation of inoculum size.

Hylobius abietis adults, obtained from pine cultures were inoculated in groups of 10 allowing them

To move free for 5 min. in plates with fungal cultures of *Beauveria bassiana* strains 3V, 43, and the same reisolated from artificially infected *Ips typographus*, and *Metarhizium anisopliae* 1V.The beetles were placed in glass containers and fed with fresh pine branches, regularly replaced.

The test with *Cephaleia abietis* and fungi: *Paecilomyces farinosus* 122 and *Beauveria bassiana* 2B was aimed to estimate the role of the relative humidity (RH) on the fungal infection. The method of Ernst (1957) & Krecek (1969) with saturated salt solutions was used:

Sodium chloride (NaCl)	for 74% RH
Potassium chloride (KCl)	for 86% RH
Potassium nitrate (KNO_3)	for 94% RH
Distilled water	for 100% RH.

Diapausing larvae of *C. abietis* were collected in natural spruce stands, infected with spore powder, than placed in special glass chambers with the mentioned salt solutions at the bottom. The chambers were kept in thermostat at 25°C and darkness.

Control groups of noninfected insects were kept for comparison in all tests. Mortality was recorded

daily and mortality rates were statistically analyzed using probit analysis.

Results and Discussion

In the test with *L. dispar* the fungal strain 3V of *B.bassiana* caused prolonged mortality at doses of 3,8 x 10^7 conidia/ml. 83,6% of total mortality was recorded at the first larval instar and 76,6% - for the second-third one. The measured LT_{50} values (approximately 11 and 20 days for L_1 and L_{2-3} respectively), indicated slow rate of infection (Fig.1). Compared with the other tested forest pests, gypsy moth-larvae were less susceptible to the fungal infection. From different ways of application (spraying, dipping or dusting) only dry spores of the strain 3V of *B. bassiana* were effective. Other strains of different hyphomycetous fungi did not cause any infection (personal data). These results support the conclusion that *L. dispar* is not appropriate host for biocontrol with hyphomycetous fungi.

Majchrowicz & Yendol (1973) recorded similarly only 6 pathogenic from 21 tested to *L. dispar* fungal strains. Very promising were the results of Wasi & Hartmann (1975,1982), who reported high mortality rate in tests with larvae first and second instar in 3 to 6 day. However, it is necessary to mention that all used larvae in their experiments were preliminary sterilized. Removing the natural bacterial flora on the cuticle could facilitate the fungi for quicker penetration (Schabel, 1978) and influence the infection rate.

Effective results were obtained after application of *B. bassiana* formulations for pest control in China (Feng *et al.*, 1994), including one lymantriid species with achieved mortality of 70 to 90%.

Evidently, the variations in virulence among isolates, the method of application, and environmental conditions have been priority factors for success in biocontrol.

Mortality of the bark beetle, *Ips typographus* caused by the fungi *B. bassiana* strains 3V and 43; *P. farinosus* strains 10 and 122; *V. lecanii* strains 76 and 77; and *M. anisopliae* 1V was very high at all tested fungi (Fig. 2 Values ranged from the most pathogenic isolate *M. anisopliae* which achieved an LT_{50} of 2,6 days and total mortality of 100% in 4 days, to the least pathogenic fungus *V. lecanii* 76 and 77 which had an LT_{50} value more than 5 days and total mortality of 90%.). The estimated number of inoculum size varied from 4,6 to 6,9 x 10^5 conidia/ml.

Generally all bark beetle species are very susceptible to fungal infections (Balazy 1963; Nuorteva & Salonen 1968; Pabst & Sirokowski 1980; Doberski 1981a,b; Whitney., 1984; Prazak 1991). Hunt (1986) associated the susceptibility of coleopteror to the fungal infections with the absence of some lipids in their cuticle, which are able to prevent or decrease the rate of germinating and penetrating conidia trough the integument.

In summary, considering the mortality rates by fungi observed in laboratory, control of *Ips typographus* using hyphomycetous fungi could be promising. However, considering the isolated cases of naturally recorded mycoses, and the biological precautions, the success of field fungal implementation is under question. The most determinant factor for this is the special shelter of the bark which prevents and restricts fungal presence. Developing possibilities for transmission of spores are requisite for bark beetle-control by fungi. Transfer of the fungal spores between sexual partners, contamination of wood suitable for breeding, and contamination of hibernating places were possible and effective in experiments with transmission of *B.bassiana*-infection to *Trypodendron lineatum* (Prazak, 1991).

Further studies on possible transmission of infection might be optimistic for developing practical use of fungal pathogens against bark beetle.

Hylobius abietis adults were not significantly affected by the strains 43 of *B. bassiana* (Fig. 3), but *M. anisopliae* and *B. bassiana* 3V could be promising for control of this insect. *M. anisopliae* induced very rapid mortality to adults at applied dose of 4,9x10^6 conidia/ml - LT_{50} within 4,9 days and 100 % total mortality during 9days. Similarly virulent were the fungi *B. bassiana* and *M. anisopliae* with induced mortality of 100% in 5 and 6,8 days respectively in experiments of Walstad & Anderson (1971). The same isolates were used successfully in a field trial too.

For practical reason the control of the weevils by fungi might be difficult because of the very thick cuticle, which can restrict fungal penetration (Schabel, 1976). Other biological and ecological

perplexing factors are the short time, for which the imagos could be exposed for inoculation, as well the nonsimultaneously stage development, and the protected larval population under the bark.

The effect of various constant humidities on fungal infections was studied with *Cephaleia abietis* diapausing larvae and the fungi *P.farinosus* 122 and *B.bassiana* 2B. In the case of *B. bassiana* infection the highest mortality was detected at 100% RH, but there was not a big difference in comparison with 86%RH (LT_{50} values 7,1 and 7,4 resp.), (Fig. 4). *P. farinosus* caused more rapid infection and values of LT_{50} ranged from 1,4 at 94%RH to 2,4 at 100%RH (Fig. 5). In both fungal strains there was not a clear parallel correlation between the rising humidity (above 74%) and the rate of infection, but even at 74% RH the mortality was high.

Similar are reported results by Fargues (1982) in experiments with *B. bassiana* and potato beetle *Leptinotarsa decemlineata*. In contrary, progressive correlation between fungal infection of scolytid beetles caused by *B. bassiana*, *P.farinosus*, and *M. anisopliae* , and relative humidity was proved in reports of Doberski (1981).

In our experiment a high humidity above 90% was necessary for the fungal growth back on the cadaver of dead insects. The same result reported Ferron (1977) for *B. bassiana* infection. According Milner & Lutton (1986) 80% RH at least was necessary for *V. lecanii* growth on the insect cuticle.

In conclusion *Ips typographus*, *Cephaleia abietis*, and *Hylobius abietis* are susceptible hosts to hyphomycetous infections. Further studies and more attempts should be made for developing models of fungus-host relations, as well field implementation , regarding all biological and ecological aspects.

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Fig. 1. Infection of Lymantria dispar larvae with Beauveria bassiana

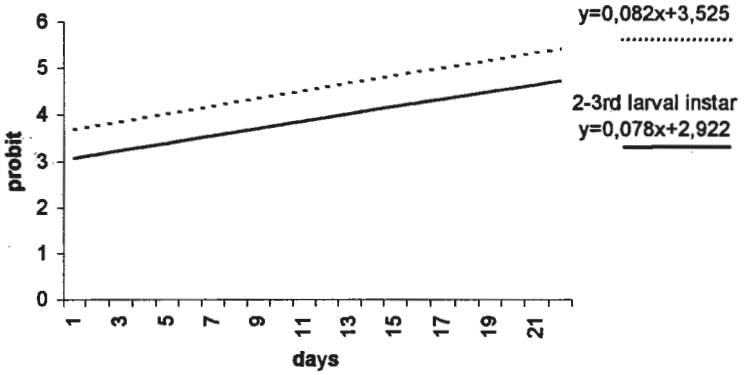


Fig. 2. Susceptibility of Ips typographus adults to infection caused by entomopathogenic fungi

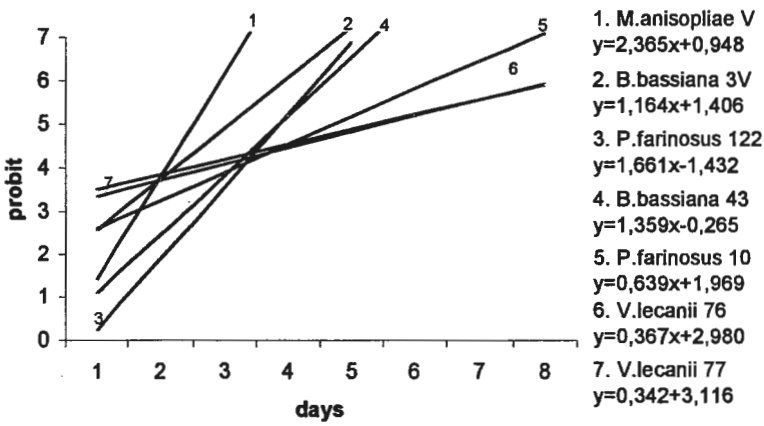


Fig. 3. Virulence of several entomopathogenic fungi on adults of Hylobius abietis

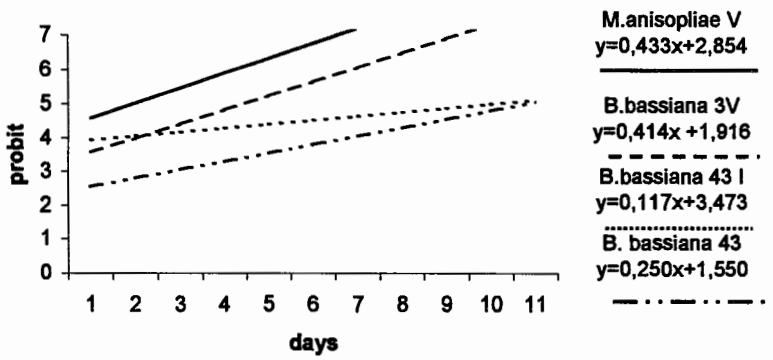


Fig. 4. Influence of RH on mycotic infection of Cephaleia abietis larvae caused by Beauveria bassiana

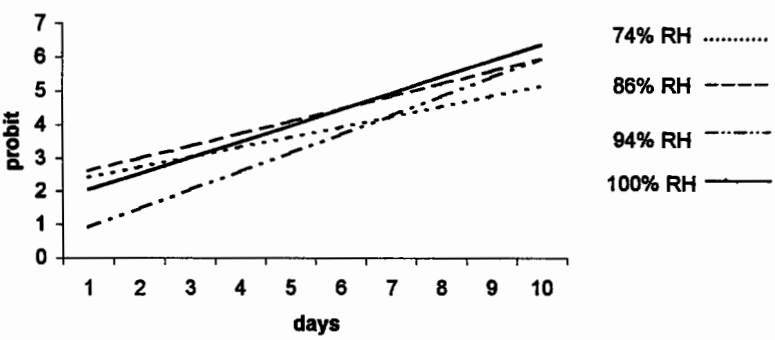
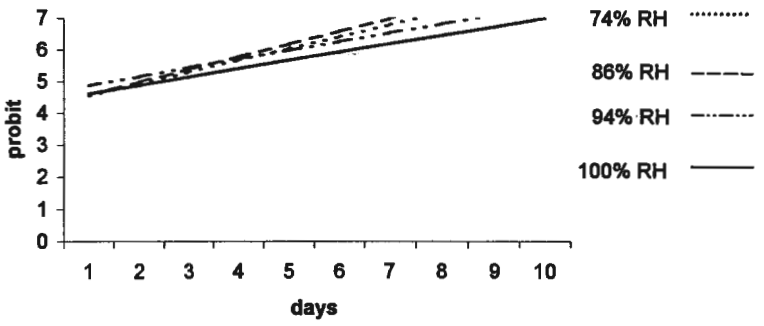


Fig. 5. Influence of RH on mycotic infection caused by Paecilomyces farinosus in Cephaleia abietis larvae



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4. Entomopathogenic viruses

Insect Pathogens and Insect Parasitic Nematodes
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Preliminary results of experiments for the use of baculoviruses in Polish forestry

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Abstract: The study was carried out in order to evaluate the insecticidal efficiency of the granulosis virus obtained from larvae of *Carpocapsa pomonella* L. against European pine shoot moth (*Rhyacionia buoliana* Schiff.). Preparation Madex 3 containing $3x10^{13}$ GIB/ml was used in laboratory and field experiments.

Under laboratory conditions, larvae of *Rh. buoliana* fed on pine shoots that were sprayed with 7 different doses of the preparation ($3 \times 10^6 - 3 \times 10^{12}$ GIB/ml). The pest mortality was evaluated 14 days after the treatment. At the same time the accumulated insect faeces were collected in order to measure feeding intensity of the observed larvae.

The results of the laboratory study demonstrated an increased mortality rate in larvae of *Rh. buoliana* that fed on pine shoots treated with Madex 3 (22.3 – 71.4%) compared to mortality in the control group (9.7%). The application of the preparation at the highest rates of the virus granules resulted in one half the feeding intensity in the observed larvae.

The results of the field trials on the application of Madex 3 ($3x10^{13}$ GIB/ml) against European pine shoot moth demonstrated reduced damage in shoots of infested pine trees.

Key words: biological control, entomopathogenic virus, *forest, Rhyacionia buoliana*

Introduction

Control of forest defoliating insects with chemical insecticides has been gradually replaced with integrated pest control methods, particularly ones associated with biological control programs that involve the use of insect parasitoids and parasites, as well as entomopathogenic microorganisms, which can suppress forest defoliator populations. Special attention has been focused on the application of insect pathogenic viruses towards the microbial control of forest insect pests.

When compared with chemical insecticides, microbial control of forest defoliators with viruses is advantageous due to its essential selectivity. The use of viruses reduces pest population numbers from economically harmful thresholds without disturbing natural balances in the forest environment. Furthermore, the use of viruses against high host densities can cause epizootics of viral disease that may spread into adjacent forest areas not yet under control. The pathogen can also surface in subsequent insect pest generations and therefore may play an important role in the natural reduction of defoliator population numbers.

Research on the role of viruses in reducing population numbers of some species of forest insect pests is currently being conducted at the Forest Research Institute in Warsaw, Poland. Observations of natural epizootics of viral diseases in populations of the most important insect pest species have been made. The impact of isolated viruses on the pests and optimum

concentrations of bio-preparations for insect control in the field have been tested under laboratory conditions. Viral introductions into pest populations have been carried out in field trials and the long term effects of the pathogens on pest numbers and population state of health have been evaluated.

The only research conducted to date in Poland on insecticidal efficiency of the granulosis virus against European pine shoot moth (*Rhyacionia buoliana* Schiff.) was carried out between 1996 and 1998 by the Forest Research Institute. Larvae of *Rh. buoliana* feed within buds of Scots pine (*Pinus sylvestris* L.) resulting in deformations of both apical and lateral shoots followed by abnormalities in tree development.

Materials and Methods

The insecticidal efficiency of the granulosis virus obtained from larvae of the codling moth (*Carpocapsa pomonella* L.) against *Rh. buoliana* was evaluated under laboratory conditions. The virus preparation Madex 3 (Andermatt Biocontrol, Switzerland) at the rate of 3×10^{13} GIB/ml was used in the experiments. The survey was carried out in April, during the period when larvae of *Rh. buoliana* generally leave pine buds after overwintering and move towards new pine buds to start their spring feeding. Pine shoots (10-15 cm long) infested with *Rh. buoliana* were collected in 7-9 year-old Scots pine plantation in the Jablonna Forest District near Warsaw in the central part of Poland.

Individual pine shoots were placed in separate glass containers and foil collars (approximately 5 cm diameter) were placed underneath buds to accumulate faeces produced by feeding larvae. The pine shoots were sprayed with 7 different doses of Madex 3 at rates from 3×10^{6} to 3×10^{12} GIB/ml using a laboratory sprayer. Each rate was applied to 45 (3 by 15) infested pine shoots and approximately 10 ml of the suspension per treatment was used. Forty-five larvae that infested non-treated shoots were considered as the control group. Mortality evaluation and faeces collection (from foil collars and infested buds) were carried out 14 days after treatment. Observations on the presence of virus granules in dead larvae were conducted using a light microscope (phase contrast, 1000x magnification).

Field experiments were conducted in a Scots pine plantation, established on a burned over area in 1992 (Forest District Jablonna), where 70% of trees were infested by the European pine shoot moth. Madex 3 at the dose of 0.1 l/ha with adjuvant NuFilm 17 (10 ml/10 l of applied liquid) was used during the experiments.

The first experiment was carried out in April, after white web sites, which sheltered spring-feeding larvae, were observed in pine shoots. The second experiment was carried out in July, following the first observations of male *Rh. buoliana* capture in pheromone traps and after finding newly laid eggs on pine shoots.

Apical and lateral shoots in the two upper whorls of 30 pine trees were sprayed using a laboratory sprayer. Thirty non-sprayed pine trees were used as the control group. The efficiency of the April treatment was evaluated in the following June and the efficiency of the July treatment was evaluated in September of the same year. The number of damaged pine shoots (apical and lateral in the two upper whorls) in both treated and non-treated groups of *Rh. buoliana* infested trees was determined.

Results

Analysis of numbers of *Rh. buoliana* larvae in pine buds treated with the virus demonstrated that the highest mortality (71.4%) was obtained in the group that fed on shoots sprayed at the highest rate of the preparation (3×10^{12} GIB/ml). In the groups that fed on shoots sprayed at rates of $3 \times 10^{9} - 3 \times 10^{11}$ GIB/ml the mortality reached 53.7 – 63.8%. Fewer dead insects were found in the buds of shoots treated at the rates of 3×10^{6} and 3×10^{8} GIB/ml. The mortality in these groups amounted 22.3-46.3%. The lowest mortality rate (9.7%) was observed in the control group of larvae that fed on non-sprayed shoots (Tab. 1).

Table 1. Mortality of *Rhyacionia buoliana* larvae after 14 days of feeding on pine shoots treated with Madex 3

Rate of Preparation (GIB/ml)	Mortality (%)
3×10^{6}	22.3
3×10^{7}	34.5
3×10^{8}	46.3
3×10^{9}	53.7
3×10^{10}	60.2
3×10^{11}	63.8
3×10^{12}	71.4
Control	9.7

Microscopic observations indicated numerous virus granules in dead larvae that had fed on the treated pine shoots, but no such granules were observed in dead larvae that had fed on non-sprayed shoots. It appeared that parasitoids (*Ichneumonidae*) killed 4 larvae in control group, but the cause of death of others in this group has not been determined.

The intensity of *Rh. buoliana* feeding is shown in Table 2 which indicates the total mean weight of faeces produced per larva during 14 days of feeding on virus-treated and non-treated pine shoots.

Table 2. Total mean weight of faeces of *Rhyacionia buoliana* larvae produced during 14 days of feeding on pine shoots treated with Madex 3

Rate of Preparation (GIB/ml)	Mean Weight of Faeces (mg)
3×10^6	57,23
3×10^7	54,17
3×10^8	51,26
3×10^9	42,27
3×10^{10}	28,06
3×10^{11}	25,48
3×10^{12}	22.98
control	61.52

The lowest total mean faeces weight (22.98 – 28.06 mg/larva) was produced by larvae that fed on shoots treated at highest rates of preparation (3×10^{10} – 3×10^{12} GIB/ml). Larvae that fed on shoots sprayed at lower rates (3×10^6 to 3×10^9 GIB/ml) produced on average approximately twice as many faeces (42.27 - 57.23 mg/larva) when compared with the groups treated at higher rates. Larvae that fed on non-treated shoots produced the highest mean total amount of faeces (61.52 mg/larva) in comparison with all treated groups (Tab. 2).

The results of field trials demonstrated that the application of the preparation Madex 3 reduced the number of damaged by *Rh. buoliana* pine shoots in infested trees (Tab. 3).

Table 3. The effects *Rhyacionia buoliana* control under field conditions

Sample Period	Treatment	Damaged Shoots (%)	
		Apical	Lateral
April	Madex 3	46	34
	Control	80	71
July	Madex 3	6	4
	Control	48	31

The evaluation of April treatment effects demonstrated that in sprayed trees 46% of apical shoots and 34% of lateral shoots were affected by European pine moth, while in non-treated trees damage of that kind was observed in 80% and 71% of apical and lateral shoots, respectively. The effects of July treatment indicated that the frequency of injured by *Rh. buoliana* apical and lateral shoots in the group of virus treated trees was 6% and 4%, respectively. More damage was found in the shoots of non-treated trees where the pest infested 48% of apical shoots and 31% of lateral shoots.

Discussion

The area of pine plantations and young forest stands in Poland affected by *Rh. buoliana* has increased to 30 thousand hectares (Kolk A. et al. 1998). Research on the use of parasitoids and parasites in reducing pest populations has been conducted in many European countries. However, according to results reported to date on pathology of European pine shoot moth, entomopathogenic microorganisms affecting this species have not been fully identified. In the seventies, Huber (1978) demonstrated the ability of the granulosis virus isolated from the codling moth to affect larvae of *Rh. buoliana*. The natural granulosis in this species was reported from Canada only in 1983 when it was recognised in 26 of 85 surveyed *Rh. buoliana* larvae (Burke, J. & Percy, J. 1983).

The present research was conducted in order to evaluate the effectiveness of the *C. pomonella* virus formulated as Madex 3 in reducing numbers of *Rh. buoliana*. Virus preparations that consist of *C. pomonella* virus have been produced in numerous countries and applied to control the codling moth and other tortricid pests in orchards (Falcon L. A. & Huber J., 1991). Such preparations have not yet been used to control insect pests in Polish forests. The experiments reported on here are the most up to date to examine possibility of using Madex 3 in the biological control of *Rh. buoliana*.

The results of the laboratory experiments demonstrated increased mortality rates in the European pine shoot moth that fed on pine shoots treated with the bio-preparation (22.3 – 71.4%) when compared with the mortality observed in the control group (9.7%). The obtained results confirm the previous observations of Huber (1978) who showed that the CpGV virus applied at the rate of approximately 10^4 GIB/ml caused 50% mortality in European pine shoot moth population. The application of Madex 3 at the highest rates of the virus granules resulted in one half the feeding intensity in the observed larvae.

The results of the field trials demonstrated the effectiveness of the preparation Madex 3 in the control of *Rh. buoliana* resulting in constituent pine shoot protection in treated trees. Sprayings applied in July, when *Rh. buoliana* larvae generally start hatching, appeared to be more efficient than the treatment applied in April. These results suggest controlling *Rh. buoliana* larvae in the summer, when the duration of pest exposure to control agents is longer than in the spring.

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Persistence and accumulation of *Malacosoma neustria* nuclear polyhedrosis virus in the ecosystem after virus application

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Abstract: *Malacosoma neustria* nuclear polyhedrosis virus (Mn NPV) is a potential agent for the control of the European tent caterpillar *Malacosoma neustria* (Lasiocampidae). Latvian isolates of Mn NPV were used as a source of virus insecticide. Environmentally safe matrix materials were used as additives. Tested additives had good stickerability to the plants, wettability of dispersible dry formulations, stability against solar radiation. An optimized method of DNA-DNA hybridization was used for determination and evaluation of the amount of virus polyhedrae on plant surface. The results of specific DNA-DNA hybridization and bioassay demonstrate that tested virus insecticide formulations secure the persistence of virus 3 weeks after spraying.

Key words: *Malacosoma neustria,* nuclear polyhedrosis virus, persistence, biological control

Introduction

Baculoviruses represent a natural component of the ecosystem and have great potential in integrated pest control. Baculoviruses including nuclear polyhedrosis viruses (NPVs) are considered to be safe bioinsecticides and have been formulated and applied as biological insecticides against pest populations (Huber, 1986, Entwistle, 1998). The nuclear polyhedrosis viruses have different capabilities of surviving in abiotic environment. NPVs described are inactivated by various environmental factors such as sunlight, high summer temperature, humidity and rainfall. The most important virus-inactivating factor in abiotic environment is sunlight, especially UV which may reduce the viability of the virus to one or a few days (Ignoffo *et all.,* 1977, Jaques, 1977). At plant level, the impact of solar UV is influenced by crop architecture and position of virus on the crop. Richards & Payne (1982) show that populations of granulovirus capsules remaine constant in cabbage field over 7 days. During the past two decades several natural and organic chemicals have been evaluated as sunlight protectants (Jaques, 1977, Young & Yearian 1974, Shapiro, 1989). Rainfall is also an important factor to disperse viruses on trees (Cunningham, Entwistle, 1981). The inactivation rate of viruses may be slowed down using various additives- stickers and UV protectants to the virus preparations.

A virus insecticide was developed and applied as a control agent against *Malacosoma neustria* populations in various regions of Latvia. During the recent years different virusinsecticide formulations have been developed and tested. We have estimated the persistence and accumulation of NPV in biocenosis and in the *M. neustria* population after application of elaborated virus formulations.

The aim of our studies was to estimate persistence of Mn NPV after treating apple-trees with virus insecticide formulations developed in Laboratory of Experimental Entomology of Institute of Biology, University of Latvia. We compared the persistence of polyhedrae and Mn NPV activity in different formulations after exposing them in the environment.

Materials and methods

Viruses and additives

Mn NPV isolated in Latvia (Jankevica, Cudare, Ose, 1998) was used as the basis of the virus insecticide. Six additives used in the formulations were tested: polyglucine, molasses of peat (experimental product, Institute of Wood Chemistry, Latvia), lysine KKL (Factory for producing lysine, Latvia), a by-product of citric acid production (Institute of Microbiology, Latvia), Belkosine M (Russia) and bentonite (Sigma). In previous experiments, most of the tested additives (conc 0.5%) gave good wettability of dispersible dry formulations as well as stability against solar radiation, promoted adhesion to the plants, and retained virus activity in the environment for 22 days (Jankevica, Zarins, 1997).

Virus application and collecting leaf probes

Apple-trees were sprayed with virus preparations ($2x10^7$ polyhedra/ml, 50 litres/ha) using air-blast sprayer, the concentration of tested additives was 2%. The virus was exposed on foliage. During the experiment, the average daily temperature was 12.5 - 18.0°C. Table 1 shows some meteorological characteristics. Two hours, 7, 14, 21, 28 and 80 days after virus application and exposure in the environment leaves were randomly collected for bioassays and 200 discs (10 mm diameter) were cut for DNA-DNA hybridization.

Table 1. Meteorological conditions during experiment

Amount of precipitations per week, mm				Σ precipitation during 80 days, mm	Σ hours of sun shining during 80 days, h
Weeks					
		III	IV		
36.3	7.2	3.8	58.3	278	540.9

Bioassay

Persistence of virus viability after exposing them in the environment was tested using a bioassay. Third instar larvae of *M. neustria* reared on artificial diets were used for the bioassay. Experiments were repeated 5 times (20 larvae in each replica). Virus-sprayed leaves (100 cm^2) were homogenized and added to diet (50 ml). Leaves sprayed with water were added to diet and used as a control. Virus activity was expressed as the percentage of corrected mortality caused by the virus.

DNA-DNA hybridization

We used a modified method of DNA-DNA spot-hybridization for the detection of Mn NPV and the evaluation of the amount of polyhedrae on leaf surfaces after exposure in the environment. We used ^{32}P-labelled DNA probe capable to detect Mn NPV recommended by Sharipo (Sharipo, 1991). 40 leaf discs were washed and analyzed for each sample with DNA-DNA hybridization followed the protocol (Sharipo, 1991). The experiments were repeated 5 times.

Results and discussion

Efficiency of virus preparations containing different additives was 84 to 96% before, and 60 to 80% and 15 to 51% after exposing the leaves in environment for 14 and 28 days, respectively. The calculated amount of polyhedrae that sticks to the treated plant leaves was 5000-7000 polyhedrae/cm. The loss of polyhedrae during the first week determined by the

specific DNA-DNA hybridization on apple leaves varied between 20 to 65% in variants with additives; in the control (virus in water suspension without additives) the loss was 81%. The loss of polyhedrae in the control depends on the amount of precipitation per week. We determined that in the control 99,8% of polyhedrae were lost during 28 days. The use of the additives: lysine KKL, polyglucine, by-product of citric acid production and molasses of peat increased the persistence of the polyhedra on leaves 7, 6, 4, and 3 times, respectively. In general, percentage of larval mortality of the larvae fed on the leaves exposed 14 and 21 days was 2.5 to 3.5 times higher to that in the control (Fig. 1).

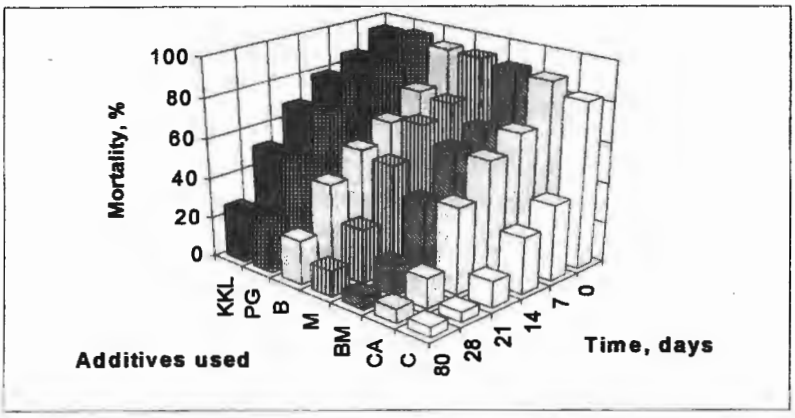

Figure 1. Efficiency of virus preparations depending on additives used and time exposure in environment. PG- polyglucine, M- molasses of peat, KKL- lysine KKL, CA- by-product of citric acid production, BM - Belkosine M and B- bentonite, C- control- virus water suspension

Application of additives exert the influence of environmental factors. This is similar to the results obtained in laboratory experiments with simulated rain (Jankevica *et all.*,1998 b), were tested additives exerted the influence of rainfall. After 28 days exposure the amount of polyhedra more than 240 polyhedrae on 1 cm² leaf surface exceeds determined LD_{50} - 55 polyhedra/larvae for 2nd instar *M. neustria* larvae (Jankevica *et all.*, 1998 a) and is enough to infect insects in the population. Actively feeding 3rd and 4th instar larvae got a sufficient dose of viruses (LD_{50} of 985 polyhedrae, Jankevica *et all,*1998 a), when eating up 5-10 cm² of the leaves, treated and exposed for 28 days. Our results correspond to the conclusions of Cunningham & Entwistle (Cunningham, Entwistle,1981) that various additives - stickers and LTV protectants reduce the negative impact of rainfall and UV on virus activity.

Results presented show that additives used secure Mn NPV activity in viral preparations used to control *Malacosoma neustria.*

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5. Entomopathogenic bacteria

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Susceptibility of newly emerged adults of *Leptinotarsa decemlineata* (Say) to *Bacillus thuringiensis* ssp. *tenebrionis*

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Abstract: *Bacillus thuringiensis* ssp. *tenebrionis* (BTT), a well known microbial insecticide, which is used as a biological control agent against young larvae of Colorado potato beetle (CPB), was tested against freshly emerged (< 24 h) CPB imagines. Our results showed, that BTT caused a high mortality to the young CPBs. This is an interesting effect, however the practical importance in agriculture is only small.

Key words: *Leptinotarsa decemlineata, Bacillus thuringiensis* ssp. *tenebrionis,* young imagines, feeding activity, mortality, egg production

Introduction

The coleopteran active strain of the *Bacillus thuringiensis* was discovered in 1982 at the Institut for Biological Control in Darmstadt: *Bacillus thuringiensis ssp. tenebrionis* (in short: BTT) [Krieg *et al.,* 1984].

Now the commercial products of BTT are used for biological control against the Colorado potato beetle (in short: CPB) in Europe and North America.

It is known, that the microbial insecticide is efficient against the younger instars (L_1 and L_2) [Krieg *et al.,* 1984, Zehnder & Gelernter, 1989, Keller & Langenbruch, 1993]. The older larvae (L_3 and L_4) are less susceptible. If old beetles receive BTT treated leaves, only feeding inhibition and stop of oviposition can be observed. This effect is reversible, if the CPBs are supplied with untreated potato leaves again. The BTT cause no mortality to the beetles.[Krieg *et al.,* 1984]

Maini *et al.,* (1994) reported about susceptibility of CPB adults to BTT. They observed high mortality by the imagines, if they fed BTT sprayed potato leaves. It is important to know that the observed beetles were freshly emerged (<24 h).

In experiments with young beetles (<24 h) Langenbruch (1996) had the same results as Maim et al. (1994). Because of a very low amount of consumed leaves, it was not clear whether mortality was due to BTT, or due to starvation.

This paper is about the newest investigations.

Material and methods

In August 1997 potted plants of *Solanum tuberosum* 'Aula' were sprayed with 1 % Novodor® (Abbott), a commercial preparation of *Bacillus thuringiensis ssp. tenebrionis* (active ingredient 2 % BTT). The control potato plants were treated only with distilled water. After drying the leaves were put into insect cages (35 x 40 x 50 cm). Ten CPBs (<24 h) were set in every cage, selected randomly. The CPB strain tested was collected from potato fields near Darmstadt and than reared for several generations on potatoes in a greenhouse of the institute.

The following treatments were carried out in three replications:

"Control": the beetles fed on water treated plants for the whole duration of the experiment (12 days)

"Hunger control": after a preperiod of 3 days starvation the imagines were supplied with water treated plants for the rest of the experiment (12 days)

"3 days BTT": the adults received BTT sprayed potato plants for the first 3 days, then water treated plants till the end of the observation (9 days)

" 12 days BTT": the beetles received BTT treated plants for 12 days

The following table shows the conditions during the investigation.

Table 1. The conditions in the greenhouse during the experiment

Temperature (°C)		Relative humidity (%)		Photoperiod (h)	
Day	Night	Day	Night	Day	Night
25 ± 4	19 ± 4	50 ± 15	68 ± 22	16	8

Results and discussion

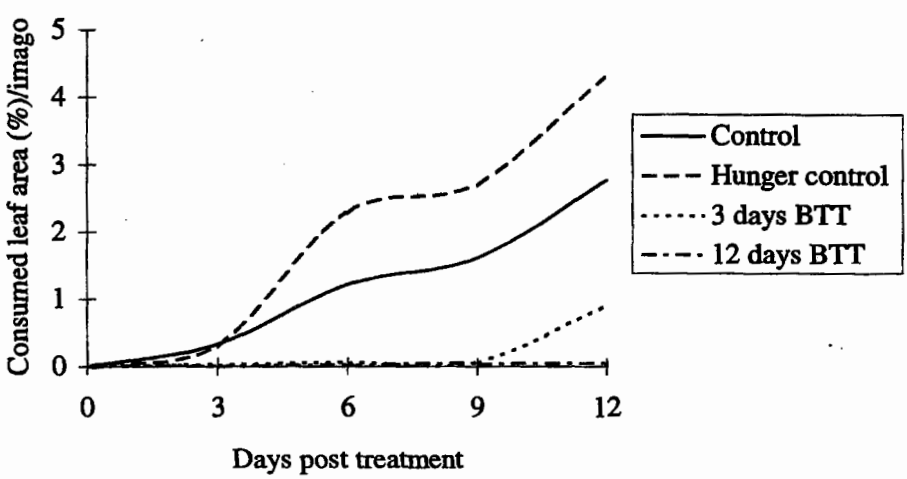

Fig. 1 . The cumulative feeding activity* / imago (%)
* in % of total leaf area

As fig. 1 shows, the feeding activity by "Control" and "Hunger control" was undisturbed. The 3 days preperiod of starvation by "Hunger control" had no negative effect. The feeding activity within the "3 days BTT" treatment was depressed compared with the "Control". Only after 9 days the surviving beetles started to consume the leaves again (even if they received

untreated leaves between the 3. and 12. day). The imagines of the "12 days BTT" treatment fed all the time only a minimum amount of the leaves.

For the statistical analysis we used the MiniStat statistical programm (Vargha, 1999). We used the Tukey-Kramer pairwise comparison of means. After 12 days there was a 95 % significant difference between "Control" and "3 days BTT", and between "Control" and "12 days BTT". The significant difference between "Hunger control" and "12 days BTT" was 99 % (k = 4, df = 8).

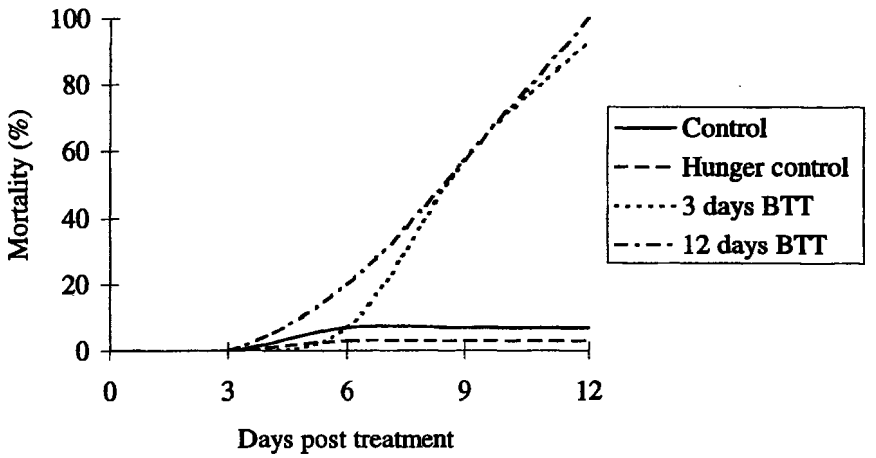

Fig. 2. Trend of cumulative mortality (%)

Fig. 2 shows the trends of cumulative mortality. At the 12. day the mortality by both BTT treaments were over 90 %. The question is: Is the mortality due to BTT toxin, or due to an antifeedant effect of BTT?

By the Tukey-Kramer pairwise comparison of means (k = 4, df = 8) there is a 99 % significant difference between the following treatments: "Control" and "3 days BTT", "Control and "12 days BTT", "Hunger control" and "3 days BTT", "Hunger control" and "12 days BTT". There is no mortality in the treatment "Hunger control", so the significant difference at the end of the experiment between the treatments "Hunger control" and "3 days BTT" shows, that the mortality in the latter treatment is due to BTT. The adults of the "3 days BTT" treatment have not only be deterred from feeding but must have fed some parts of BTT treated leaves in the first 3 days, otherwise there would not have been any significant difference in the mortality between "Hunger control" and "3 days BTT" treatments.

The egg production/cage was also observed (see fig. 3). Because of the mortality we calculated a corrected egg production/cage (from 10 living CPBs).

As fig. 3 shows the egg production in "Control" and "Hunger control" started after 6 days. Because of the 100 % mortality by the "12 days BTT" treatment, there was no egg production. By the "3 days BTT" treatment the living imagines (7%) started with a small egg production after 9 days.

The statistical analysis of the data showed, that the egg production between "Hunger control" and "3 days BTT" was significantly different (95%). The difference between the "Hunger control" and "12 days BTT" treatments was significant, too (99 %).

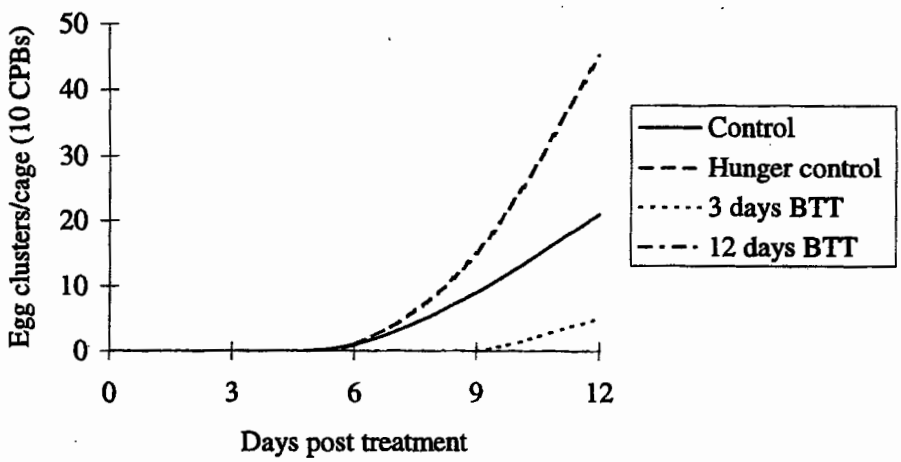

Fig. 3. The corrrected egg production / cage*
* produced egg clusters / cage (corrected to 10 beetles)

Conclusions

• The mortality found was caused by the consumed BTT treated leaves (and not by starvation!), so freshly emerged adults (< 24 h) of CPB are susceptible to BTT
• The results show an interesting effect, however the practical importance in agriculture is only small, because only young beetles are susceptible and they do not emerge all together

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Biobit XL (*Bacillus thuringiensis* Berliner subsp. *kurstaki*) in controlling ECB (*Ostrinia nubilalis* Hübner) on corn in East Croatia

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Abstract: Corn is grown in Croatia on 400 000 ha, and average yield is 4,5 t/ha. The majority of cornfields are settled in eastern part of Croatia. European corn borer (*Ostrinia nubilalis* Hübner) is constantly present pest at our cornfield. Intensity of attack of ECB in last 3 years was 42,75% in average. Here is important to stress that 40% of corn production is in monoculture. This study showed differences in attack intensity of ECB between untreated plots and plots treated with Biobit XL (3 l/ha) - the preparation on the base of *Bacillus thuringiensis* Berliner subsp. *kurstaki*. Controlling was done in 1995 year on silage corn (hybrids Marista and Stira) and 1997 on grain corn (hybrid Decalb).
On silage corn, attack intensity on untreated plots was 100%, while on treated plots it was 59%. Hybrid Decalb, sown in monoculture, had 83% attack intensity in untreated plots and 35% on treated plots. On silage corn, 1,61 tunnels and 1,79 larvae per plant was determined on untreated plots and 0,64 tunnels and 0,01 larvae per plant on treated plots. On grain corn 2,01 tunnels and 0,4 larvae per plant was determined on untreated plots and 0,54 tunnels and 0,01 larvae on treated plots. Average length of damage on both treatments was 3,5 cm per plant on untreated plots and 1,02 cm on treated plots.
Grain yield on Decalb hybrid was 32% higher on treated than on untreated plots.
Controlling of ECB with Biobit XL showed good results, better yield, lower number of tunnels and larvae, lower length of damage, and it could be applied in integrated pest management.

Key words: corn, biological control, *Bacillus thuringiensis* Berliner

Introduction

Corn is the main cereal in Croatia which grows on 400 000 ha, mostly in eastern parts of the country. The main pest on corn is European Corn Borer (*Ostrinia nubilalis* Hübner) which is constantly present at our cornfield. Average intensity of attack for the period 1971 - 1998 was 37,68%; the lowest attack intensity was recorded in the year 1979 (2,32%) and the highest was in the 1987 (98,44%), (Ivezić & Raspudić, 1997). Intensity of attack of ECB in last 3 years was 42,75% in average (Ivezić *et al.*, 1998). Here is important to stress that 40% of corn production is in monoculture. Controlling of ECB are not implemented in Croatia regulary. In the 1988 Ivezić at al. (1988) were recorded reduction in attack intensity by using preparation on the base of *Bacillus thuringiensis*.
The aim of this study was to determine differences between untreated and treated plots with biological preparation Biobit XL on the base of *Bacillus thuringiensis* Berliner subsp. kurstaki, by using it on silage and grain corn.

Material and methods

Efficacy of Biobit XL on the base of *Bacillus thuringisensis* subsp. kurstaki was recorded in 1995 on two hybrids of silage corn – Marista and Stira. Those hybrids were sown after harvesting of wheat and barley. The trials were settled on IPK Osijek, locality Velika Branjevina.

The same trials were done in 1997 year, on IPK Osijek, locality Bare Čepin, but this time with grain corn, Hybrid Decalb. This hybrid was sown in April. Both trials were done in five replicates.

Preparation Biobit XL was used for controlling ECB, in the dose of 3 l per ha, and application was done using hand spray K-15.

Application of the preparation was done in the beginning of August 1995 in the silage corn, and hybrid Decalb was treated with the preparation on 28th of July 1997. This time of application was determined with occurrence of first to third stages of larvae and with occurrence of small holes at the top leaves of the corn plant.

Dissection of corn was done before harvest, 25th of September 1995, and 23rd of September 1997. 20 plants in five replicates were examined randomly. Attack intensity, number and position of tunnels and larvae length of damage was determined. Yield was determined in t/ha, with 14% of moisture. The results were evaluated by using ANOVA and Lsd test. Coefficient of efficacy of preparation Biobit XL was presented.

Results and discussion

Intensity of attack of ECB was lower in treated (both silage and grain) corn in compare to untreated plots. Percentage of attack intensity is shown in fig. 1.

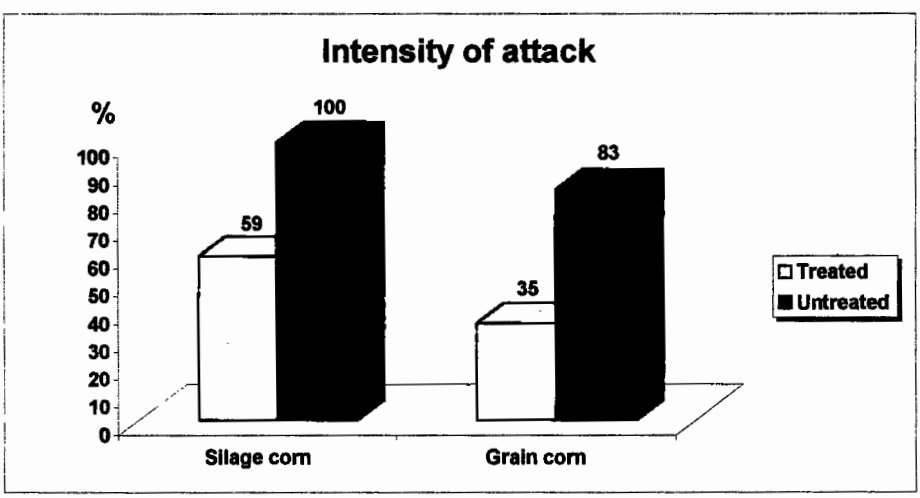

Figure 1. Intensity of attack of ECB.

Similar results were obtained by Ivezić *et al*, (1998). They had trials with three different time of application of the same preparation Biobit XL, and they found out lower intensity of attack in all three treatments in compare to untreated plots.
Biobit XL decreased total number of tunnels and larvae per corn plant in treated plots, which is visible in fig. 2.

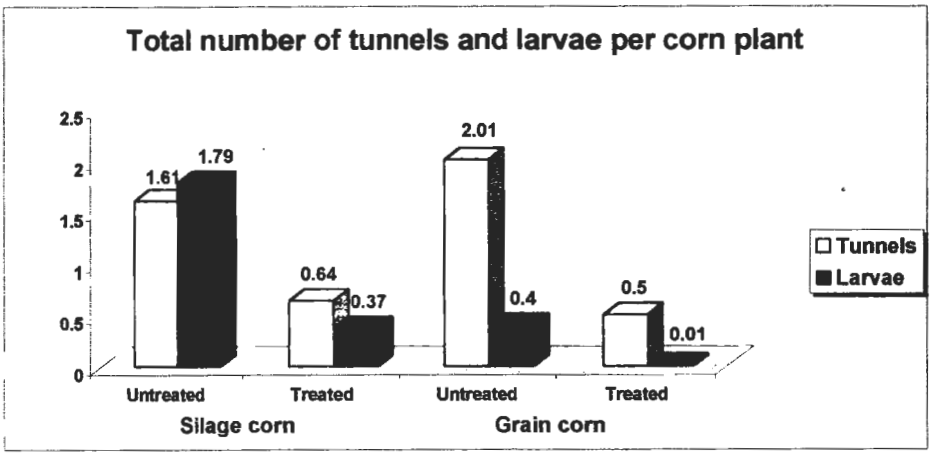

Figure 2. Total number of tunnels and larvae per corn plant.

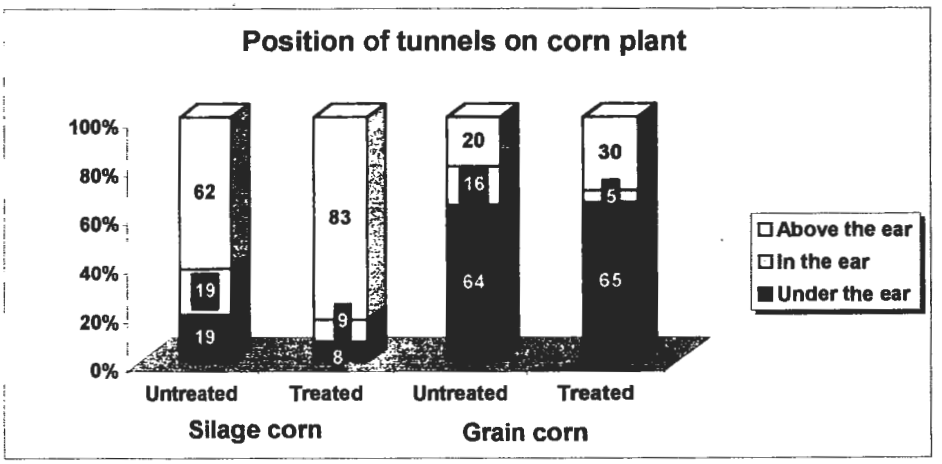

Figure 3. Position of tunnels per corn plant.

It was detected that majority of tunnels on plant of silage corn was above the ear, in opposite to grain corn plant where tunnels appeared mostly under the ear (fig.3). Position of the larvae is presented in fig. 4.

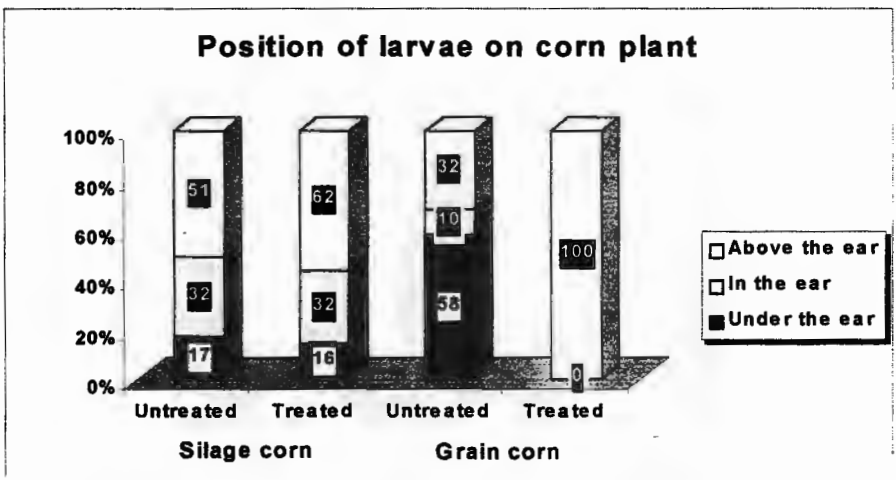

Figure 4. Position of larvae on corn plant

Length of damage also decreased in treated corn (fig. 5).

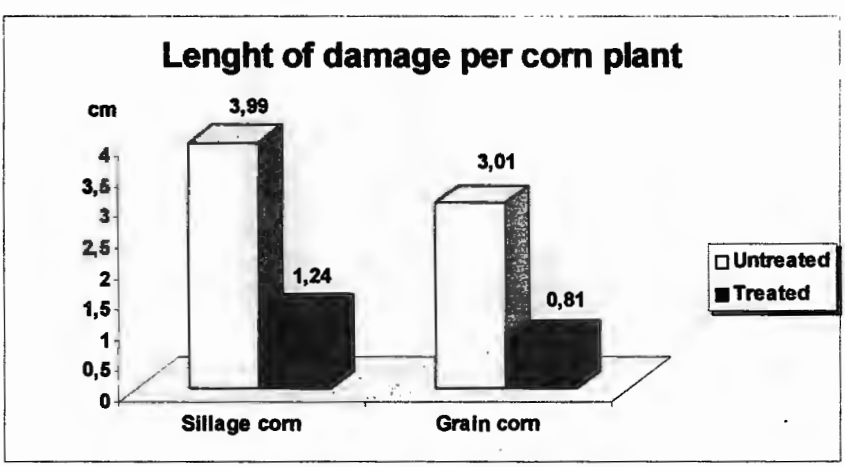

Figure 5. Length of damage per corn plant

ANOVA and Lsd test showed very high significant differences in attack intensity between untreated and treated corn (table 1).

Table 1. Intensity of attack and corn yield.

Treatment	Intensity of attack ECB (%)	Yield (t/ha)
Untreated	83	9,34
Treated	35**	13,68

** Very high significance (0,01)
* High significance (0,05)

Lsd 0,05 = 14,67 Lsd 0,05 = 3,49
Lsd 0,01 = 20,58 Lsd 0,01 = 4,89

Preparation Biobit XL on the base of *Bacillus thuringiensis* subsp. kurstaki had in 1995, on the silage corn, 41% coefficient of efficacy. In 1997 on grain corn coefficient of efficacy was 58%. All this parameters shows that biological preparation Biobit XL is appropriate for controlling of ECB, with very high efficacy and ecologically acceptable.

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Expression of *cryIVA* and *cryIVD* genes of *Bacillus thuringiensis* in baculovirus expression system

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Abstract: Baculoviruses cause disease in larval insects and generally have a narrow host specificity. These properties make baculoviruses potentially significant biological agents for the control of the insect pests. To increase their usefulness as effective biological insecticides, it is desirable to alter these viruses genetically to be more virulent. During the infection, production of proteins that interfere specifically with insect metabolism or metamorphosis, such as hormones, enzymes and toxins might enhance the pathogenicity of these viruses.

Genes (5.5 kb) encoding the *CryIVA* and *CryIVD* proteins of *Bacillus thuringiensis* subsp. *israelensis* were inserted into the baculovirus *Autographa californica* nuclear polihedrosis virus so that gene expression was under the control of the strong, very late polyhedrin gene promoter. Selection and purification of novel recombinant baculovirus (*Ac*NPV/*Ac*RN2) were performed by plaque assay. Expression of these proteins (134 and 72 kDa) in *Spodoptera frugiperda* cells was determined by 8% SDS-PAGE. The engineered recombinant baculovirus provides a convenient source of *CryIVA* and *CryIVD* insecticidal crystal proteins for various studies.

Key words: *cryIVA*, *cryIVD*, gene expression, baculovirus

Introduction

There is a number of viruses which can be used for insect control. Among these, baculoviruses are specific for only arthropods. Since they are natural subjects, they do not effect ecological equilibrium dramatically (Demirbağ *et al.*, 1997). However, these viruses have a relatively slow speed of action, because baculovirus-infected larvae continue feeding for long periods of time, inflicting nearly as much damage as uninfected insects (Hunter *et al.*, 1984). Through genetic engineering it may be possible to improve the insecticidal properties of these viruses. Production of insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis* might improve the speed of action and virulence of baculoviruses.

Insecticidal crystal proteins (ICPs) of B. *thuringiensis* are among the best studied biological insecticides (Höfte & Whiteley, 1989). These proteins, synthesized as large crystals during sporulation of the bacterium, are toxic to larvae from Lepidoptera, Diptera and Coleoptera and, therefore potentially interesting as pest control agents. When ingested by larvae, these crystals solubilize in the midgut and release proteins (protoxins) ranging from 70 to 130 kDa (Lilley *et al.*, 1980; Nagamatsu *et al.*; 1984). These mature toxins bind receptors present on epithelial cells of the midgut of susceptible insects (Hofman *et al.*, 1988) and probably force pores in the membrane of these cells (Knowles & Ellar, 1987; Slatin *et al.*, 1990), leading to cell lysis and finally to death of the larvae.

In order to investigate whether the *CryIVA* and *CryIVD* ICPs are potentially useful to improve the pathogenicity of baculoviruses and to provide a convenient source of *CryIVA* and

CryIVD ICPs for various studies, a recombinant baculovirus expression system was employed.

Material and methods

Insect cells and viruses

The *Spodoptera frugiperda* (*Sf*-9) cell-line was used, and maintained at 28°C in Grace's insect medium (Invitrogen) supplemented with 10% fetal bovine serum. Linearized Bac-N-Blue DNA (*Bsu*36 I digested DNA of *Autographa californica* nuclear polyhedrosus virus, Invitrogen) was used to generate the recombinant baculovirus in transfection. *Autographa californica* nuclear polyhedrosus virus (*Ac*NPV, Invitrogen) was used as wild type virus.

Plasmid vectors and bacterial strains

cryIVA and *cryIVD* genes were derived from pHE4-AD (a gift of Dr. Arieh Zaritsky, Department of Life Sciences, Ben-Gurion University of the Negey, Beer-Sheva 84105, Israel) which contains the full-length of these genes as a *Nco* I - *Pst* I fragment (5568 bp). pAMP 18 was used as an intermediate plasmid for transfering the *cryIVA* and *cryIVD* genes into the baculovirus transfer vector. pBlueBac4.5 (Invitrogen) was also used as basic transfer vector for the construction of recombinant baculovirus. *Escherichia coli* JM109 strain was used to transform plasmid vectors.

Construction of the baculovirus transfer vector pAcRN2

Plasmid DNA's were isolated from JM109 as described by Ausubel *et al.*, (1990). Plasmid pHE4-AD was digested with *Nco* I and *Pst* I, and the fragment containing the coding sequence of the ICPs (5.5 kb) was purified from a 0.8% agarose gel using Geneclean II Kit (Bio 101). To obtain a suitable restriction site in front of *Nco* I site, this fagment was subcloned into *Nco* I - *Pst* I restricted pAMP 18. The resultant clone, nicknamed pRN2, contains *Bgl* II, *Sma* I, *Kpn* I, *Sac* I and *EcoR* I sites in front of *Nco* I site. New plasmid DNA was recovered from transformed *E. coli* JM109 cells. The insert was further excited from pRN2 by *Sac* I - *Pst* I, and 5.5 kb fragment was cloned into *Sac* I - *Pst* I restricted pBlueBac4.5. This resulted in recombinant baculovirus transfer vector, p*Ac*RN2.

Infection and transfection of cells

Wild-type *Ac*NPV DNA (Bac-N-Blue) and constructed baculovirus transfer vector DNA (p*Ac*RN2) were cotransfected into *Sf*-9 cells using Lipofection (Invitrogen) in serum free medium, essentially as described by OReilly *et al.*, (1992). Cotransfection of these DNA's into *Sf*-9 cells resulted in allelic replacement of the wild-type polyhedrin gene of *Ac*NPV with the *cryIVA* and *cryIVD* genes under the control of the abundantly expressed polyhedrin promoter. Recombinant viruses (*Ac*NPV/RN2) were selected by plaque assay as blue plaques after staining with X-gal. Plaque purification was carried out four times to ensure virus homogeneity. A stock of recombinant virus was developed and viral titer (pfu/ml) was calculated by plaque assay.

Expression of recombinant protein

To monitor the synthesis of *CryIVA* and *CryIVD* in *Ac*NPV/RN2-infected cells, *S. frugiperda* cells in a 6-well plate (1 x 10^6 cells/well, Corning) were infected with wild-type and recombinant virus (10 pfu/cell). Uninfected (as control) and wild- type infected cells were harvested at 48 h.p.i, however, recombinant virus-infected cells were harvested at 24, 48 and 72 h.p.i., and lysed by detergent lysis method (Invitrogen).

Proteins extracted as described by OReilly *et al.*, (1992) and electrophoresed on 8% SDS-PAGE at 30 mA according to Laemmli (1970) with a 20 µl of sample volume, and stained with silver nitrate as described by Sambrok *et al.*, (1989).

Results

Construction of recombinant baculovirus, AcNPV/RN2

In this study, ICP genes (*cryIVA* and *cryIVD*) of *B. thuringiensis* subsp. *israelensis* were expressed in *Spodoptera frugiperda* cells using a baculovirus, *Autographa californica* nuclear polyhedrosis virus.

The ICP genes (5.5 kb) in pHE4-AD were first transferred into the intermediate plasmid, pAMP 18, and then the resulting clone, intermediate transfer vector, was named as pRN2 (8.2 kb) (Figure 1). In this way we added suitable restriction sites in front of *Nco* I site of the ICP genes for transferring the fragment into the baculovirus transfer vector.

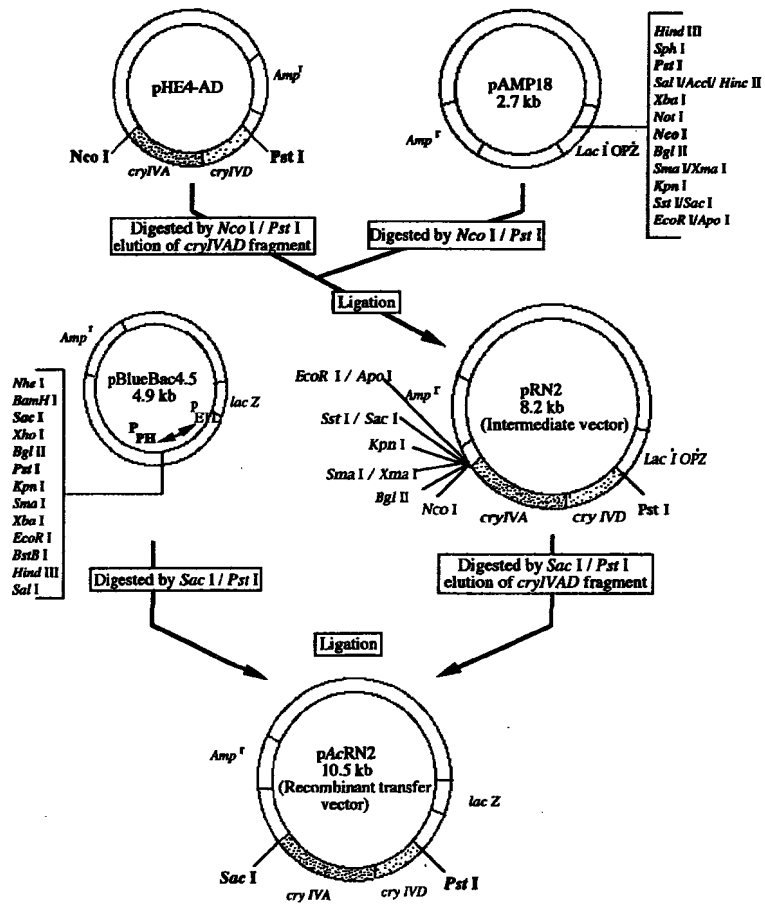

Figure 1: Construction of recombinant baculovirus transfer vector, p*Ac*RN2. First the ICP gene sequences in the pHE4-AD were transferred into the pAMP 18. Then the *Sac* I-*Pst* I fragment of ICP gene sequences in the pRN2 were transferred into the baculovirus transfer vector, pBlueBac4.5, in front of polyhedrin promoter. Finally, recombinant transfer vector p*Ac*RN2 was formed

The *Sac* I - *Pst* I fragment of ICP genes in the pRN2 were transferred into the baculovirus transfer vector, pBlueBac4.5, in front of polyhedrin promoter and the resulting transfer vector was named as p*Ac*RN2 (Figure 1). The ICP gene sequences in the clones were confirmed by agarose gel electrophoresis. Figure 2 indicates that p*Ac*RN2 contained the ICP gene sequences. The transfer vector pBlueBac4.5, contains, in addition to a unique cloning site downstream the polyhedrine promoter, a gene cassette containing *lac Z*, which is expressed in infected insect cells and drives the expression of the marker gene.

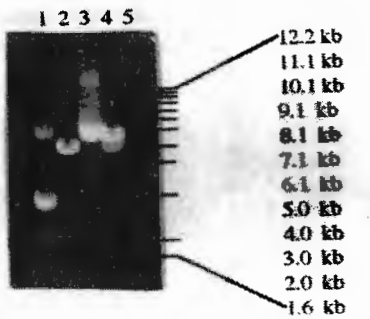

Figure 2: Detection of ICP genes insertion in the p*Ac*RN2. pBlueBac4.5 and p*Ac*RN2 were digested with *Sac* I-*Pst* I enzymes and electroporesed in 1% agarose jel. Lanes 1, pBlueBac4.5 DNA; 2, pBlueBac4.5 DNA digested with *Sac* I-*Pst* I; 3, p*Ac*RN2 DNA; 4, p*Ac*RN2 DNA digested with *Sac* I-*Pst* I; 5, Marker DNA

Since the baculovirus genome is very large (about 130 kb), recombinant viruses containing foreign genes can only be obtained by recombination in host cells cotransfected with the wild-type viral DNA and a recombinant transfer plasmid vector containing the foreign gene. So the p*Ac*RN2 transfer vector DNA containing the ICP genes and linearized DNA of wild type *Ac*NPV, Bac-N-Blue DNA, were cotransfected into *S. frugiperda* cells by the protocol outlined in Invitrogen catalog (Figure 3). After cotransfection, recombination events that transfer the ICP genes from the transfer vector to the polyhedrin gene site of the *Ac*NPV genome DNA were accomplished. The p*Ac*RN2 DNA and *Ac*NPV DNA were succesfully cotransfected into *S. frugiperda* cells and multiplied. The recombinant virus was selected and characterized by plaque assay in the presence of X-gal. The blue-colored plaques without the polyhedral inclusion bodies were isolated as recombinant viruses, and named *Ac*NPV/RN2.

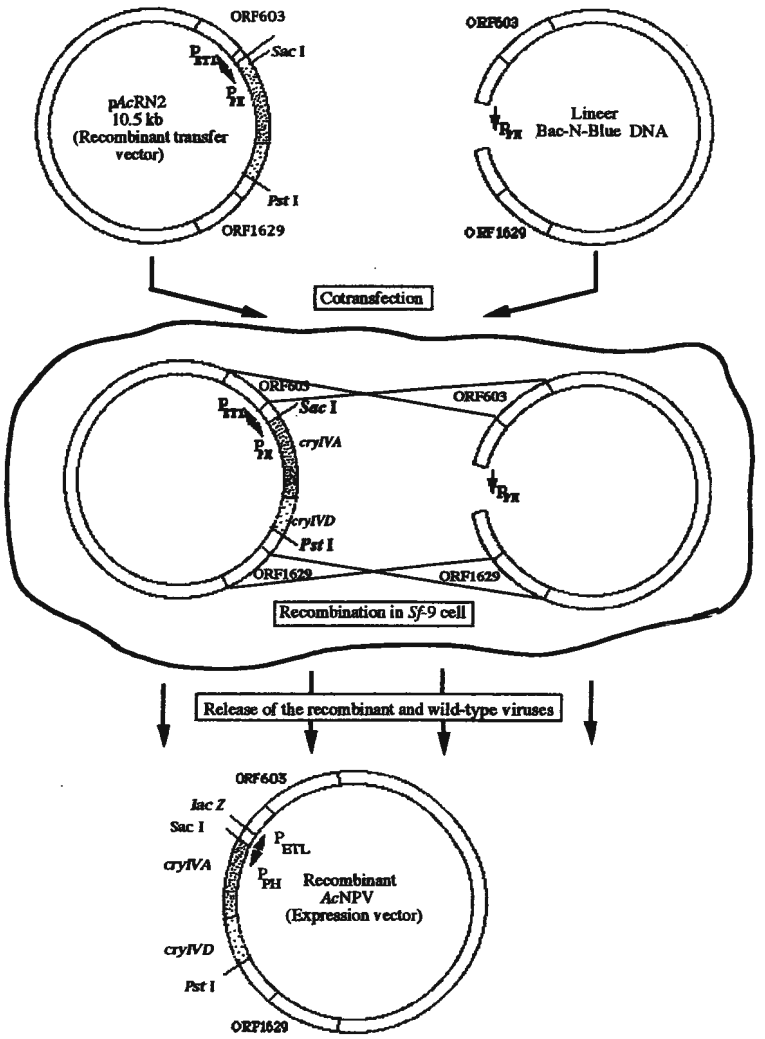

Figure 3: Construction scheme of recombinant virus *Ac*NPV/RN2 by cotransfection with *Ac*NPV DNA and p*Ac*RN2 plasmid

Expression of ICPs in* S. frugiperda *cells

S. frugiperda cells, infected with wild type *Ac*NPV, were harvested at 48 h p.i. and cells infected with recombinant virus (*Ac*NPV/RN2) were harvested at 24, 48 and 72 h p.i., and analysed by 8% SDS-polyacrycamide gel electrophoresis (SDS-PAGE). The cells, infected with *Ac*NPV/RN2 polyhedrin (28 kDa) was absent, instead, proteins of approximately 72 and 134 kDa was observed (Figure 4).

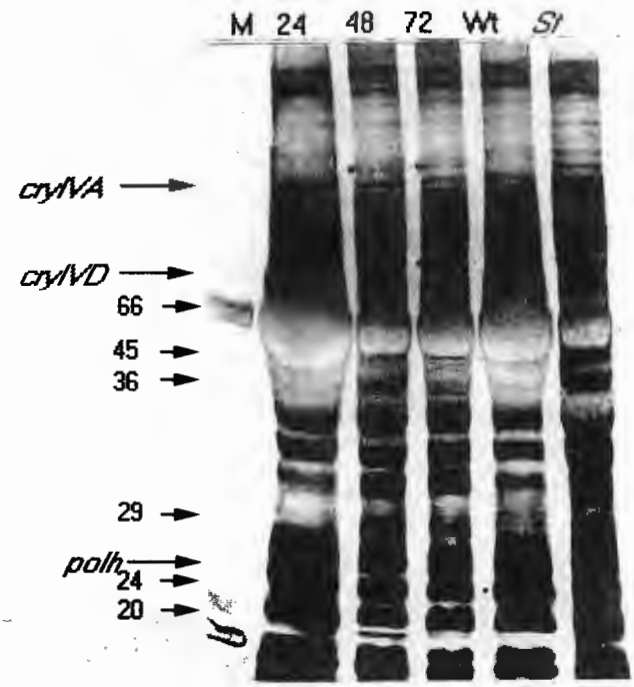

Figure 4: SDS-PAGE analysis for detection of ICPs produced by *S. frugiperda* cells infected with *Ac*NPV/RN2. *Sf* uninfected; Wt: infected with wild type *Ac*NPV; 24, 48 and 72: infected with recombinant virus, *Ac*NPV/RNl (numbers indicated the time of harvest post infection); M: molecular weight markers.

Discussion

Baculoviruses are insect pathogens with a relatively slow speed of action and this limits their use as control agents of pests. Baculovirus-infected larvae continue feeding for long periods of time, inflicting nearly as much damage as uninfected insects (Hunter *et al.*, 1984). One strategy, therefore, has been to develop viruses which immobilize and kill their insect hosts more quickly. Many reports have shown success in improving the insecticidal effectiveness, of insecticidal proteins, neurotoxins or juvenile hormone inhibitors (Carbonell *et al.*, 1988; Heinz *et al.*, 1995).

The hyperactivity of the baculovirus polyhedrin promoter has been widely exploited to express foreign genes (Luckow & Summers, 1988; Luckow, 1991). The *polh* gene is dispensable for virus replication and the expression of a foreign gene is based on the allelic replacement of the *polh* gene (O'Reilly *et al.*, 1992; King & Possee, 1992)

In this study we performed the expression of the ICP genes of *Bacillus thuringiensis* subsp. *israelensis* recombined in a baculovirus, p*Ac*RN2. The genes *cryIVA* and *cryIVD*, encoding 134- and 72 k-Da proteins, respectively, were cloned together into the baculovirus transfer vector, pBlueBac4.5, resulting in pRN2. The Virus DNA, Bac-N-Blue, was cotransfected and recombined with the ICP gene of pRN2 in the *S. frugiperda* cells. Recombination occured between the homologous sequences. The recombinant virus was

selected and characterized by plaque assay containing X-gal. Blue color and no polyhedra formation plaques were selected as recombinant viruses. One best selected recombinant virus was named p*Ac*RN2. These observations indicated that the pRN2 subclone and the Bac-N-Blue DNAs were successfully recombined within the *S. frugiperda* cells and multiplied. A similar construction was reported by Merryweather *et al.*, (1990) wherein they cloned *B. thuringiensis* subsp. *kurstaki* HD-73 endotoxin (ICP) gene in *Ac*NPV.

The ICPs in insect cell produced by *Ac*NPV/RN2 showed 134 and 72 kDa on 8% SDS polyacrylamide gels by silver nitrate stain (Figure 4). The titer of the *Ac*NPV/RN2 in infected cells was $1.4x10^8$ pfu/ml at 7 days postinfection.

The expression and recombination of ICP genes in baculovirus expression system described here may inform the basis for continued isolate, characterize and test for biologic activity of this protein.

Acknowledgements

This research was supported by a grant from Karadeniz Technical University and TUBITAK (Turkish research council) research budget. We gratefully acknowledge Dr. Zaritsky for his great contribution with pHE4-AD including *cryIVA* and *cryIVD* genes.

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New mosquitocidal *Bacillus thuringiensis* strains and non larvicidal *Bacillus thuringiensis* var. *israelensis* strains

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Abstract: *Bacillus thuringiensis* (*B.t.*) strains were tested for toxicity towards *Aedes aegypti, Anopheles stephensi* and *Culex pipiens* larvae. Two strains HBt18 and DBt230, not flagellated and serologically untypable respectively, were found to be larvicidal, both present toxins related to already known toxins. Besides, two strains LBt97 and LBt155 serotyped as *israelensis* were found not toxic towards mosquito larvae. The latter strains had a different crystal structure and protein content than that of mosquitocidal *B.t.i.* strains

Key words: *Bacillus thuringiensis*, mosquitocidal activity, protein content, immunological relationships, non-toxic *B.t.i.* strains.

Introduction

Since the discovery of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) (Goldberg and Margalit, 1977) and *B. sphaericus* (Kellen *et al.*, 1964), much effort has been devoted to the search of strains presenting different toxins than that of *B.t.i.* ans *B. sphaericus* or displaying higher toxicity towards mosquito larvae. This has been reinforced since intensive use of *B. sphaericus* for mosquito control led, in certain circumstances, to larval resistance. One way to delay or even prevent this is to vary the use of control methods or bacterial toxins used

According to Ragni *et al.* (1996), a number of strains belonging to *B.t.* var. *morrisoni*, *B.t.* var. *thompsoni*, *B.t.* var. *malaysiensis*, *B.t.* var. *canadensis* and auto-agglutinating strains were found as toxic as *B.t.i.*, but all with toxins were all similar to *B.t.i.* ones. Only a few mosquitocidal strains with different toxins than *B.t.i.* are known. *B.t.* var. *medellin* and *B.t.* var. *jegathesan* are nearly as toxic (0-10 less toxic) as *B.t.i.* while *B.t.* var. *darmstadiensis* is 10-100 times less toxic than *B.t.i.* depending on the mosquito species tested.

A search for new mosquitocidal strains was conducted by testing *B.t.* strains from the collection at The Royal Veterinary and Agricultural University, Denmark. Bioassays on *Aedes aegypti, Anopheles stephensi* and *Culex pipiens*, protein profile and immunological relationships of these strains with known toxins were performed at the Pasteur Institute, France.

Materials and methods

All bacterial strains were from The Royal Veterinary and Agricultural University, Denmark, except 16a which was stored at the Pasteur Institute, France. The origins and serotypes of the five strains are listed in table 1. Strain 16a (*B.t.i.*) was used as reference. All strains were grown in UG medium (1% glucose) and underwent shaking until cell lysis. The final whole cultures (FWC) were kept at 4°C until use.

Table 1. *Bacillus thuringiensis* strains, serotype and source.

Strain	Serovar	Serotype	Source
16a	*israelensis*	H 14	French Guyana
HBt18	NF(a)	-	Grass foliage, Holland
DBt230	UTY(b)	-	Cauliflower, Denmark
LBt97	*israelensis*	H 14	Pine needles, Lithuania
LBt155	*israelensis*	H 14	*Limantria dispar* (alive), Lithuania

(a): NF: Not flagellated. (b): UTY: Untypable by reference H antisera of available 67 serotypes.

Crystal morphology of HBt18 and LBt155 was assessed by transmission electron microscopy as described by Damgaard *et al.* (1996). Protein content was determined by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% acrylamide gels) using concentrated FWC, or purified crystals and a LMW electrophoresis calibration kit (Pharmacia). Proteins were stained by Coomassie-blue. Proteins were transferred onto Hybond-C nitrocellulose membrane and detected immunologically with the Amersham ECL (enhanced luminescence) Western blotting kit. Rabbit antibodies directed against *B.t.i.*, *B.t.* var. *jegathesan*, and *B.t.* var. *medellin* total purified crystals were used for detection. Bioassays were carried out on three different mosquito species: *Ae. aegypti* (strain Bora-Bora), *An. stephensi* (strain ST15), and *C. pipiens pipiens* (strain Montpellier) as described by Ragni *et al.* (1996). Preliminary results allowed giving lethal dosages (LD) LD50 and LD90 from one set of bioassays.

Results

Clear irregular shaped crystals could be seen in all the *B.t.* FWCs. As revealed by electron-microscopy, HBt18 strain produced big crystals with several parts differing in shape and density (Figure 1.2). LBt155 strain produce a small angular crystal with a homogeneous density (Figure 1.1).

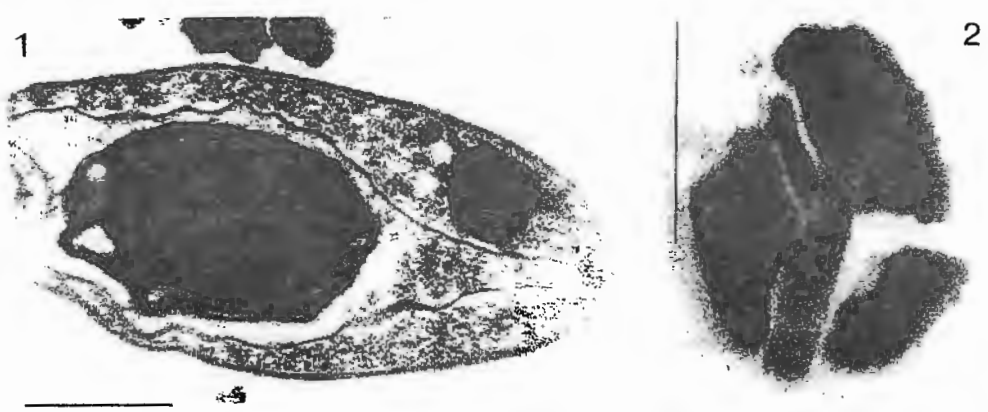

Figure 1. Electron microscopy of *Bacillus thuringiensis* isolates.
Bar represents 500nm. **(1)** LBt155, whole cell **(2)** HBt18, crystal.

Larvae of *Ae. aegypti*, *An. stephensi* or *C. pipiens* were subjected to 1x10-2 x FWC dilutions of *B.t.i.* LBt97 and LBt155 strains: no mortality was recorded even though these strains belonged to the serovar *israelensis* and that they clearly produced parasporal crystals. Moreover, 1 µg/ml of *B.t.i.*

LBt97 purified crystals (concentration 1000 times higher than the LD50 of a *B.t.i.* toxic strain) were non toxic to *Ae. aegypti* larvae. DBt230 strain was tested against *Ae. aegypti* with a LC50 estimated around 5x10-4 FWC dilution. Larvicidal activity of HBt18 strain was similar to that of *B.t.i.* 16a strain (Table 2).

Table 2. Comparison of the larvicidal activity of HBt18 and 16a FWC on three mosquito species.

	Aedes aegypti		*Anopheles stephensi*		*Culex pipiens*	
Strain	LD50(a)	LD90	LD50	LD90	LD50	LD90
16a	2.60x10-6 (2.29-2.94)	4.90x10-6 (4.13-5.82)	4.60x10-5 (4.02-5.25)	9.07x10-5 (7.27-11.32)	3.69x10-6 (3.26-4.18)	6.93x10-6 (5.79-8.30)
HBt18	4.20x10-6 (3.71-4.76)	8.76x10-6 (7.30-10.51)	2.10x10-5 (1.84-2.39)	3.59x10-5 (3.04-4.25)	1.14x10-5 (0.99-1.33)	2.33x10-5 (1.82-2.99)

(a): Lethal dosages (LD) are expressed in dilutions of final whole culture. 95% confidence limits are shown in brackets.

The protein content was analysed on SDS-PAGE (Figure 2). Proteins of purified crystals from strain 16a (Figure 2A) were those expected from *B.t.i.* Polypeptides of FWC from LBt97 and LBt155 *B.t.i.* strains were clearly dissimilar and different from that of *B.t.i.* (Figure 2B). Purified crystals of LBt97 strain (Figure 2A) displayed polypeptides of 68 and 56 kDa. HBt18 strain produced at least 6 polypeptides ranging from 90 to 26 kDa with two major bands at 75 and 55 kDa (Figure 2C). Strain DBt230 (data not shown) showed a different profile (110, 64 and 42 kDa) than *B.t.i.*.

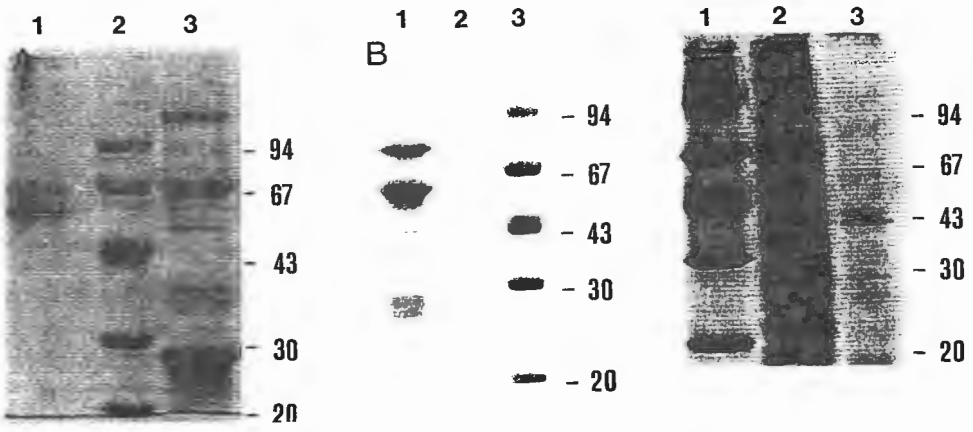

Figure 2. Protein analysis in final whole cultures (FWC) and purified crystals from *Bacillus thuringiensis* strains. **(A)** lane 1, 11 µg purified crystals of LBt97 strain; lane 2, LMW Pharmacia kit; lane 3, 20 µg purified crystals 16a strain. **(B)** lane 1, 10xHBt18 strain; lane 2, blank; lane 3, kit.**(C)** lane 1, kit; lane 2,10xFWC LBt97 strain; lane 3, 10xFWC LBt155 strain.

Immunological relationships of the protein components of crystals or FWCs of *B.t.* strains were studied (except for LBt155 strain) using *B.t.i.*, *B.t.* var. *jegathesan*, and *B.t.* var. *medellin* total crystal antibodies. Preliminary results showed that HBt18 strain cross reacted clearly with *B.t.* var. *medellin* and *B.t.* var. *jegathesan* proteins of 70-90 kDa and faintly with *B.t.i.* at 28, 67 and over 100 kDa. DBt230 reacted with all three antibodies at 70 kDa and bands at over100 kDa. Crystals of LBt97 cross reacted with *B.t.i.* and *B.t.* var. *jegathesan* at *ca.* 70 kDa and over100 kDa. All these results have to be confirmed.

Discussion

From a large *B. thuringiensis* screening this study had focussed on 4 *B.t.* strains: HBt18, DBt230, LBt97 and LBt155.

The HBt18 non flagellated strain produced multiple proteins which correlated with the picture obtained by electron microscopy, showing that its crystals were composed of several components of various shape and density. HBt18 strain showed high mosquitocidal activity, similar to that of *B.t.i.* 16a strain, while the untypable DBt230 strain showed only moderate activity. They both reacted clearly with known antibodies directed against mosquitocidal toxins. Consequently further studies have to be performed with these 2 strains.

LBt97 and LBt155 *B.t.i.* strains were isolated in Lithuania from pine needles and *Lymantria dispar*, respectively. Both have different protein profiles, and also different from what is normally found in *B.t.i.* strains. They both produced crystals smaller than usually seen, with a homogeneous electron density. Although they were serotyped as *israelensis* none of them showed any mosquitocidal activity, even when purified crystals were tested. This lack of toxicity of crystalliferous *B.t.i.* strains has never been reported. Immunological studies with LBt97 strain should be performed with antibodies raised against all individual mosquitocidal toxins. Indeed, the Cry11A protein from *B.t.i.* is one of the proteins responsible for the major mosquitocidal effect (Chilcott and Ellar, 1988), and it would be worthwhile to see whether LBt97 would cross react preferentially with this protein. A molecular comparison of LBt97 and a standard *B.t.i.* strain would allow a better understanding of their differences.

These results support the fact that there is a great diversity of mosquitocidal strains within *B.t.* species, and that finding new toxic strains is still up-to-date. The results also confirm that serological classification shows its failure in detecting toxicity even within serotype H14, and that reliable information should also included toxicity or toxin genes.

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Natural occurrence of *Bacillus thuringiensis* in Lithuanian forest ecosystems

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Abstract: The natural occurrence *of Bacillus thuringiensis* in Lithuanian forest ecosystems was investigated in foliage samples from birch, spruce and pine, in lepidopteran larvae of *Lymantria dispar* and *Dendrolimus pini*, and in litter samples from birch and pine stands. *B. thuringiensis* was isolated from all sampled material. The average frequency of *B. thuringiensis* in the different materials varied between 0.71 and 0.99 of the total *B. cereus/B. thuringiensis* populations with the lowest frequency found on pine needles and the highest on birch foliage. More than 55% *of* the tested *B. thuringiensis* strains from each *of* the samples, foliage, litter, and *D. pini* larvae were active against *Pieris brassicae* larvae, but surprisingly less than 10% of the *B. thuringiensis* strains from *L. dispar* larvae were active against *P. brassicae*. Serotyping of selected *B. thuringiensis* strains showed that the majority (about one third) belonged to serovar *kurstaki* and another third were non-motile. Other serotypes were present in low numbers. More than 90% of the *kurstaki* strains had lepidopteran activity, but unexpectedly only one out *of* four *aizawai* strains were Lepidoptera active. The studies document the presence of a large unexplored resource of *B. thuringiensis* in the Lithuanian forest environment.

Key words: *Bacillus thuringiensis*, natural occurrence, forest ecosystems, litter, lepidopteran larvae, foliage

Introduction

Approximately one third of the total area in Lithuania is forest. Conifers account for 62% of the forest area while the broad-leaved, birch and aspen account for 33%. Important forest defoliators are the lepidopterans *Dendrolimus pini, Lymantria monacha* and *Lymantria dispar*. The insecticidal bacterium, *Bacillus thuringiensis*, is presently the most used microorganism for insect pest control. It is common in different soils throughout the world (Martin & Travers, 1989) and have been isolated from different insects (Chilcott & Wigley, 1993), and more recently from the phylloplane of different deciduous and conifer trees as well as other plants (Smith & Couche, 1991; Ohba, 1996; Damgaard *et al.*, 1997; Damgaard *et al.*, 1998). In order to get more information on the natural occurring *B. thuringiensis* population in different Lithuanian forest ecosystems we isolated *B. thuringiensis* from foliage, lepidopteran larvae living of the foliage, and litter from the birch/L. *dispar* system and the conifer (pine and spruce)/*D. pini* system. A number of selected strains were characterised by qualitative toxicity tests against a susceptible lepidopteran species (*Pieris brassicae)* and by serotyping.

Material and methods

Sampling

All sampling was performed in forest plots that were never previously sprayed with *B. thuringiensis*. Samples of birch twigs and live *L. dispar* larvae were taken in the beginning of July 1997 together with litter from beneath the birch trees in a birch stand in Siluté (West Lithuania). Live and fungus killed (*Cordyceps* spp.) larvae of *D. pini* and litter were sampled from a pine stand in the Druskininkai area (South Lithuania) in April, and during summer 1997 pine and spruce needles were sampled twice from other stands in the same area.

***Isolation of* Bacillus thuringiensis**

From each examined foliage and litter sample, a 3 g subsample was blended in 10 ml PBS for 3 x 30 sec in a Stomacher 80 Lab-blender at maximum speed. Lepidopteran larvae were homogenised singly in a glass tissue grinder with varying amounts of PBS, depending on the size of the larva. Leaf and litter extracts and insect homogenates were centrifuged (20 min, 4300 x g) and the resulting pellets resuspended in 0.5-3.0 ml of PBS and heat-treated for 30 min at 65°C (Smith & Couche, 1991). Tenfold serial dilutions of the suspensions were plated on T3 sporulation agar (Travers *et al.*, 1987) and incubated at 30°C for 24 hours. Colonies with a diameter of more than 2 mm and having a rugose, "ice-crystal" appearance and were selected and subcultured on T3 until sporulation. Strains were examined under a phase-contrast microscope and colonies containing crystal inclusions were assigned to *B. thuringiensis* and the others classified as *Bacillus cereus*. A number of strains was randomly selected for further characterisation by serotyping and qualitative toxicity tests.

H Serotyping

The currently recognised antisera were kindly supplied by Dr. M. M. Lecadet, Institut Pasteur, Paris, France, and the serotyping was performed as described by de Barjac (1981).

Qualitative toxicity tests of the strains

The *B. thuringiensis* strains were tested for *per os* insecticidal activity against larvae of *Pieris brassicae* (Lepidoptera), a species that is susceptible to most of the Lepidoptera-active cry-genes (van Frankenhuyzen & Nystrom, 1999). Bacteria were grown in 25 ml of T3-sporulation medium for 5 days at 30°C on a rotary shaker and sporulation examined by phase-contrast microscopy before use. Larvae (30 neonates per strain) were fed an artificial agar-based diet (Damgaard *et al.*, 1996). The bacterial broth was incorporated into molten diet at a rate of 30 ml per g diet. The larvae were incubated at 20°C and the mortality measured after 5 days. Strains were considered toxic if they caused more than 80% corrected mortality.

Results and discussion

B. thuringiensis was isolated from all the sample types at average frequencies between 0.71 (pine needles) and 0.99 (birch foliage) out of the total *B. cereus/B. thuringiensis* populations. These are very high frequencies compared to the frequencies found in other studies (Chilcott & Wigley, 1993; Damgaard *et al.*, 1997).

Table 1. Average densities of *Bacillus thuringiensis* in different foliage and litter samples

Sample	No. of samples	Mean spores/g	SE
Birch foliage	8	3.5×10^{1}	$\pm 9.5 \times 10^{0}$
Litter (Birch)	4	1.8×10^{4}	$\pm 9.3 \times 10^{3}$
Spruce needles	7	5.4×10^{1}	$\pm 2.6 \times 10^{1}$
Pine needles	11	3.0×10^{0}	$\pm 8.7 \times 10^{-1}$
Litter (pine)	4	2.3×10^{2}	$\pm 1.2 \times 10^{2}$

The average density of *B. thuringiensis* measured as spores per g material was estimated for the foliage and the litter samples and is shown in Table 1. The lowest average density was found in pine needles with 3.0 spores/g while the highest average density (1.8×10^{4} spores/g) was found in litter from beneath birch trees, a difference of 10,000. In general, the lowest numbers were found on the foliage samples. The low density of spores found in the pine litter compared with the birch litter samples was most likely due to the difference in sampling time, since the pine litter was sampled in April where it was still snow and frost.

A total of 184 strains were selected for toxicity test against *P. brassicae* larvae. At least 55% of the strains from the foliage and litter samples as well as *D. pini* were active against *P. brassicae*. Surprisingly, less than 10% of the strains from *L. dispar* had *P. brassicae* activity. Thus the hypothesis that the specific *B. thuringiensis* population is correlated to the dominating insect species present and/or feeding in the environment (Kaelin *et al.*, 1994; Damgaard *et al.*, 1997; Damgaard *et al.*, 1998) could not be confirmed for the strains isolated from *L. dispar*.

Table 2. Serotype, origin and *Pieris brassicae* activity of selected *Bacillus thuringiensis* strains.

Serotype	No. of strains	Strains isolated from	*Pieris* activity*	
			+ (no.)	- (no.)
aizawai	4	birch foliage, litter (pine)	1	3
israelensis	2	*L. dispar*, pine needles	0	2
kenyae	1	litter (birch)	0	1
kurstaki	26	all sample types	24	2
sumiyoshiensis	5	birch foliage, *D. pini*	5	0
thuringiensis	1	*L. dispar*	1	0
tolworthi	1	*D. pini*	0	1
non-motile	20	all sample types except pine needles	8	12
untypeable	7	birch foliage, *L. dispar, D. pini*	2	5
Total	67		41	26

*-: not active against *P. brassicae* larvae. +: more than 80% corrected mortality after 5 days

As seen in Table 2 approximately one third of the serotyped strains belonged to the serotype *kurstaki* and almost another third were non-motile (lacking the flagella needed for serotyping). Other serotypes were present as well but were less abundant. Seven strains were untypeable by the currently recognised antisera and could potential represent new serotypes. The *kurstaki* strains had almost exclusively lepidopteran activity, a phenomenon also seen in

other investigations (Ohba, 1996; Damgaard *et al.*, 1997). Only one of the four *aizawai* strains had lepidopteran activity, which was more unexpected since this serotype is often, like *kurstaki*, correlated with lepidopteran activity (Obha & Aratake, 1994). Thus the Lithuanian forest environment contain a large and diverse *B. thuringiensis* population which is still to be explored.

Acknowledgements

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6. Protozoa

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Evaluation of the potential of a microsporidian isolate for the biological control of *Lymantria dispar* L.

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Abstract: A German microsporidian isolate was fed to laboratory reared gypsy moth (*Lymantria dispar* L.) larvae. Larvae were either kept individually in diet cups or in small populations with 10 larvae per cup to investigate the influence of the infection on bionomical parameters.

The larval development was significantly retarded after infection with microsporidia. Infected males reached higher pupal mass compared to healthy males, whereas the pupal mass of infected females was significantly lower than the control in small populations. No significant differences were observed between individually reared healthy and infected female pupae. The overall frass dry mass of infected and healthy larvae did not differ significantly. Larval and pupal mortality varied considerably.

The results will be discussed in respect of the potential of this microsporidian isolate for a regulation of gypsy moth population density.

Key words: *Lymantria dispar* L., Microsporidia, Biological Control, Nosema

Introduction

In European forests, Gypsy moth (*Lymantria dispar* L.) is an economically and ecologically important defoliator of deciduous trees with periodically occurring outbreaks (Schwerdtfeger 1981). Interactions between insects and their pathogens affect the periodic insect population cycles (Anderson & May 1980). They can result in a low population density beneath the damage treshold (Hochberg 1989). As obligate parasitic protozoa, microsporidia were shown to have a significant impact on the natural regulation of insect populations (Franz & Huger 1971, Lipa & Madziara-Borudiewicz 1976, Wilson 1981, McManus *et al.*, 1989). Microsporidia were a main mortality factor in Russian Gypsy moth populations and caused a retarded larval development as well as a reduced fecundity (Zelinskaya 1980). The pathology of microsporidia in *Lymantria dispar* was described by David & Pilarska (1988) and Pilarska & Vavra (1991).

The objective of this project was to assess the potential of microsporidia in the regulation of the population density of Gypsy moth. Therefore the sublethal effects of the German microsporidian isolate on individual Gypsy moth larvae and a small laboratory population were analysed.

Material and Methods

Insect rearing

The German microsporidian isolate was originally isolated from a German strain of Gypsy moth in 1993 (Linde & Rappl 1994), fresh spores were produced in 1998 and stored in liquid nitrogen. Gypsy moth (*Lymantria dispar*) egg masses were obtained from USDA-APHIS (New Jersey Standard Strain). Larvae were reared in the laboratory on artificial diet (Odell

and Rollinson 1966) at 18-24°C, 50 % RH. Third instar larvae were individually infected by feeding 1 µl spore suspension on a diet cube.

Larvae infected with 10^3 spores/µl were kept individually in diet cups, whereas the dosage was $2x10^3$ or $2x10^4$ spores per larva in small populations with 10 larvae per cup. Larval mass, developmental stage and health status were recorded every second day. Once a week, frass was removed from small populations and dried at 80°C. After pupation resp. adult hatch, the infection levels were examined.

Data analysis

For the purpose of data analysis, means of daily larval mass, daily dry weight of feces, and length of development were compared using t-test. Data of female pupal mass were analysed with one-way analysis of variance with means compared using Student/Newman/Keuls test.

Results and Discussion

Effects on the host insect

The infection of Gypsy moth larvae with a German microsporidian isolate affected the length of larval development, larval and pupal mass, dry mass of frass, mortality, and egg production of the host insects. Compared to healthy larvae, the infection with microsporidia resulted in a significantly prolonged larval and pupal stage in individual cups as well as in small populations (Fig. 1). Corresponding to other authors, our results confirm the retarded larval development of infected compared to healthy larvae for the German microsporidian isolate (Linde *et al.*, 1996, Mitchell & Cali 1994, Jeffords *et al.*, unpubl. data, and Onstad *et al.*, unpubl. data).

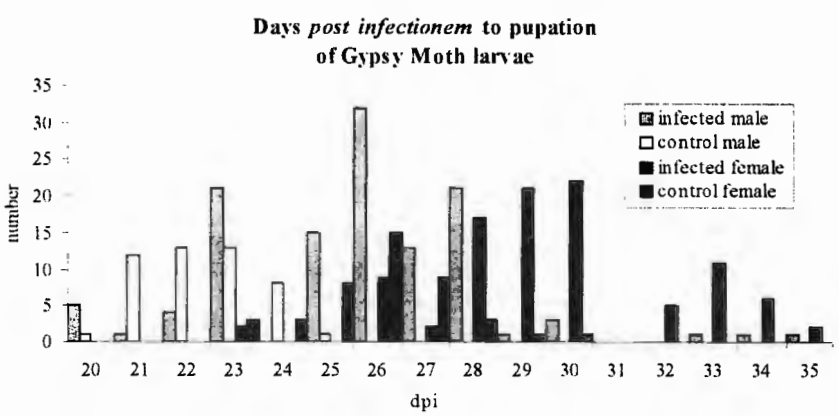

Fig. 1: Days post infectionem to pupation of Gypsy moth larvae infected with a German microsporidian isolate

Until pupation, healthy larvae had a significantly higher mass than infected larvae, but only male hosts reached a significantly higher pupal mass than the control (Fig. 2). However, healthy and infected females from individual cups did not differ in pupal mass, whereas in small populations the pupal mass of infected females was significantly lower than the controls (Fig. 3). Larvae, kept individually and infected with 10^3 spores/µl reached a significantly higher pupal mass than larvae from small populations, infected with $2x10^4$ spores/µl. Control

larvae in small populations produced a significantly higher frass dry mass per day and larva than infected larvae. After day 18-20 p.i. the relation was inverse. Finally, the overall dry mass of feces was similar: 56 mg produced by infected and 53-64 mg by healthy larvae.

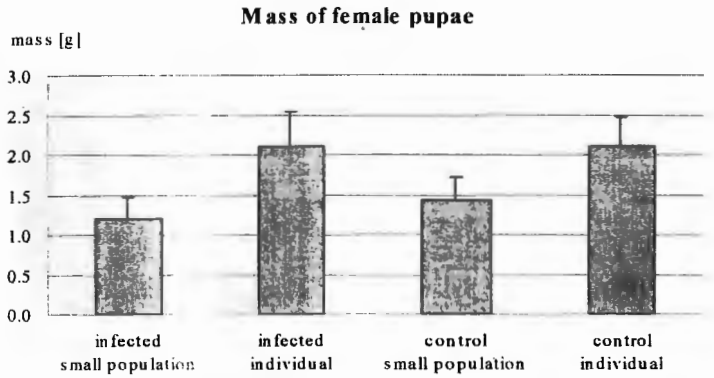

Fig. 2: Mass of female Gypsy moth pupae infected with a German microsporidian isolate (mean ± standard deviation)

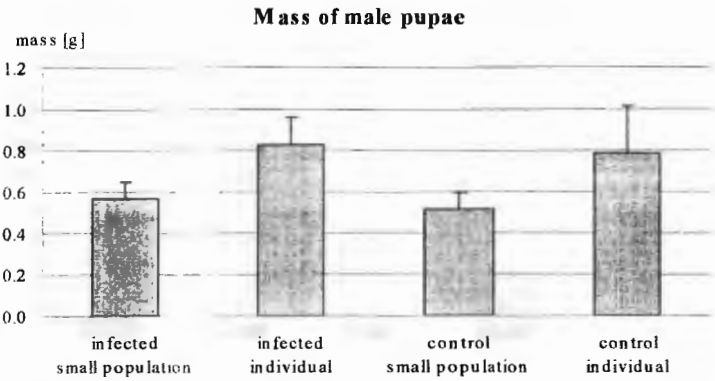

Fig. 3: Mass of male Gypsy moth pupae infected with a German microsporidian isolate (mean ± standard deviation)

The stage-specific mortality of the infected host insects varied considerably. Among individually reared larvae infected with 10^3 spores/µl, mortality was 5 %; whereas 12 % died as prepupae and 36 % as pupae. In contrast, 94 % of the hosts infected with the tenfold dose died during the larval stage, 4 % in the pupal stage (Fig. 4). In small populations, infected with $2x10^4$ spores/µl, larval mortality ranged from 0 to 10 % and pupal mortality from 23 to 83 %, apparently not depending on the spore dose (Fig. 5). Until day 15 *post infectionem* 0 to

2 % of the infected larvae had died. Linde *et al.*, (1996) recorded 8 % mortality among individually kept larvae, each infected with $5x10^5$ spores of the same isolate.

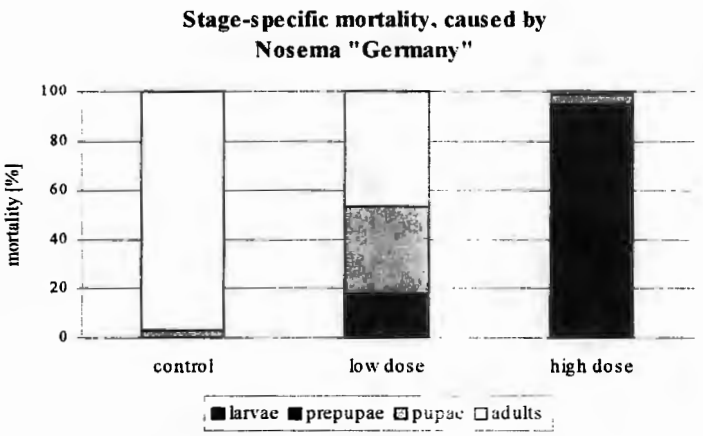

Fig. 4: Mortality of individually reared Gypsy moths infected with a German microsporidian isolate

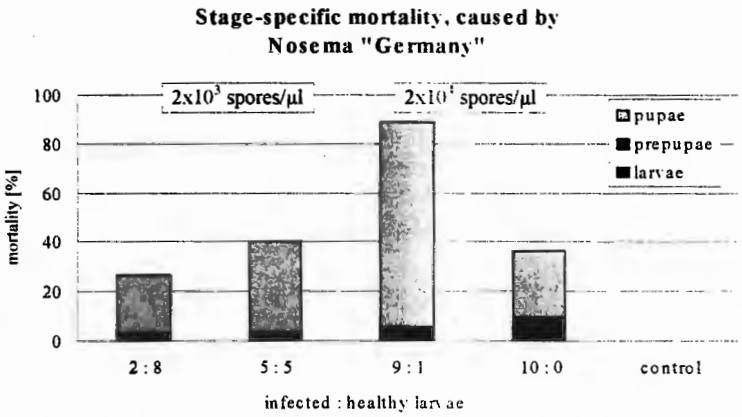

Fig. 5: Mortality of Gypsy moths from small populations infected with a German microsporidian isolate

Conclusions

Our results demonstrate that an infection with the German microsporidian isolate significantly affects the development of Gypsy moth. Increased mortality following infection can cause a longer lasting reduction of the population density. To ensure a long-term control of Gypsy moth populations, an effective horizontal and vertical transmission of the pathogen is required. Thus, data of horizontal transmission are needed, and investigations about the persistence of spores during the latency period are necessary.

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Preliminary results on the occurrence of microsporidia of gypsy moth (*Lymantria dispar* L.) from different forest habitats of Poland

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Abstract: In Poland oak forests cover only 6.2 % of the total forested area and gypsy moth does not belong to dangerous forest pests. Between 1960 and 1998 around 4 300 ha were sprayed to control gypsy moth. Outbreak centres of gypsy moth are usually localised in the south-west and north-east parts of the state. In 1997 an isolate of *Nosema* sp. was found in gypsy moth larvae from Biebrza Marshes (Biebrza National Park) in the north-east of Poland. Microsporidia infected 91 % of larvae. Spores fulfilled salivary glands of the heavily infected larvae. In 1998 gypsy moth larvae and pupae were collected from 1 location in the southern, from 1 location in the north-east and from 1 location in eastern Poland. Dissections of the larvae were done and the percent of infected specimens collected from different locations was determined. The infectivity of the Biebrza isolate was checked. The microsporidia isolate did not kill the gypsy moth larvae but prolonged the larval development.

Key words: Microsporidia of gypsy moth, prevalence in gypsy moth populations, infectivity of microsporidia

Introduction

At present the gypsy moth (*Lymantria dispar* L.) is not a dangerous pest in Polish forests. In 1960-98 it appeared in an area of 34, 000 ha and around 4, 300 ha was controlled during that time (Burzynski *et al.*, 1960-98). The main reason for the situation has been, that deciduous stands cover only 22.7 % and oak stands or mixed stands with oaks in species composition cover only 6.2 % of the total forested area in Poland. However, in order to increase the percentage of deciduous species in the total forested area, the restitution of stands is done in Poland and it could be a reason for any change in the status of the gypsy moth in the future.

The purpose of the studies is the estimation of the healthiness of gypsy moth larvae from a few populations in Poland with a special attention given to the estimation of the microsporidia prevalence in different populations and their possible importance as a natural enemy of the gypsy moth.

Material and Methods

Larvae collection

Forth, fifth and sixth instar gypsy moth larvae were collected. In 1997 larvae were collected from one location: the Biebrza National Park in the north-east of Poland. In 1998 larvae were collected from three locations: the Biebrza NP, the Biała Podlaska Forest Inspectorate in eastern Poland and the Rudziniec FI in southern Poland. Larvae collected in 1997 and larvae collected in 1998 from the Biała Podlaska FI and the Rudziniec FI were picked up from their host plants once - between June 10th and July 10th. Larvae collected in 1998 in the Biebrza NP were picked up from burlap banded host trees three times a week between June 10th and July

10th. After collection the most of the gypsy moth larvae were kept frozen in -18°C until dissection.

Table 1. Description of gypsy moth locations

Location/Year		Host plant	No. of collected larvae	Population density
Biebrza NP	1997	alder and birch	101	high (in the small area where larvae were collected)[1]
	1998	alder, birch, oak and hornbeam	23	low (73 egg masses/ha)[2]
Rudziniec FI, 1998		oak, birch, larch and pine	128	high (up to 100 4th - 6th instar larvae/ one 6 year old tree), (spraying operation after larvae collection)[3]
Biała Podlaska FI, 1998		willow	67	low (67 larvae were all the larvae collected in the area 100 m^2)

[1]- Bystrowski C., 1997, personal communication;
[2]- Sukovata L., 1998, personal communication;
[3]- Dudik W., 1998, Spraying Operation Report

Sectioning & microscopical analyses

Sectioning and microscopical (contrast phase microscopy) analyses of collected gypsy moth larvae were done. The presence of parasitoids larvae (*Tachinidae* and *Ichneumonidae*) inside gypsy moth larvae and *Parasetigena silvestris* eggs on the gypsy moth larval integument as well as the presence of nematodes in the midgut lumen were noted. Some of the gypsy moth larval tissues were inspected for the presence of microsporidia. The salivary gland, fat body and midgut of every larva were inspected, as well as gonads, Malphigian tubules and muscles of some larvae. The presence of virus polyhedrons and fungus structures in the inspected tissues was also noted. Giemsa staining method with Weiser modification, (Weiser, 1977) was used to find the amount of nuclei in spores.

Infectivity of the Biebrza isolate

The impact of the microsporidia isolate found in the Biebrza NP on NJSS third instar gypsy moth larvae was estimated. Spore suspensions in a few concentrations were prepared. Suspensions (0,1 ml) were smeared evenly on the surface of a gypsy moth artificial diet (ODell *et al.*, 1985; Odell *et al.*, 1997) in Petri dishes. Larvae (5 larvae per one Petri dish in 10-12 repetitions per one concentration) were put on the infected diet. Larvae after 24 hours of a starvation were used in the experiment. After 72 hours feeding on an infected diet, larvae had been transferred to Petri dishes with a fresh diet. Later during the experiment the diet was changed every 4-5 days. The mortality of larvae and the rate of larval development were checked twice a week until pupation.

Results and discussion

Prevalence of microsporidia and other natural enemies

Microsporidia were present in every sampled gypsy moth population. The highest prevalence of microsporidia among gypsy moth larvae was observed in the Biebrza NP population. In

1997, 91 % of larvae was infected and 55 % was heavily infected. In 1998, 22 % of larvae was infected and 10 % was heavily infected. In 16 % of individuals from Rudziniec FI and in 3 % of individuals from Biała Podlaska FI, few spores of microsporidia were found and only one larva from Biała Podlaska FI was heavily infected with microsporidia.

Table 2. Pathogens and parasitoids of gypsy moth larvae collected from 3 locations in Poland

Location	Year	n[1]	Prevalence of natural enemies of the gypsy moth [%]					
			m[2]	v[3]	p[4]	f[5]	n[6]	hl[7]
Biebrza NP.	1997	101	91 (55)*	40	43	2	0	1
	1998	23	22 (10)*	4	17	17	0	40
Rudziniec FI	1998	67	16 (0)*	25	55	4	0	38
Biała Podlaska FI	1998	128	3 (1)*	29	60	2	17	21

n[1]- No. of larvae, m[2]- microsporidia, v[3]- viruses, p[4]- parasitoids, f[5]- fungi, n[6]- nematodes, hl[7]- healthy larvae; *- first position: the percent of infected larvae; second position: the percent of heavily infected larvae (in brackets)

Spores of microsporidia found in gypsy moth larvae from Biebrza NP in 1997 and in 1998 were *Nosema* type (two nuclei). The most attacked tissue was salivary gland and fat body. The salivary gland of the heavily infected larvae was fulfilled with spores. Fat body was usually less infected tissue than salivary gland. Spores were found also in midgut, muscles and gonads.

In 1976-78 spores of *Nosema lymantriae* Weiser were found in gypsy moth dead larvae (Glowacka, 1982) collected in the Biebrza NP territory. The most infected tissue was salivary gland, fulfilled with spores. It suggests, that the microsporidium can be a permanent component of the Biebrza Marshes ecosystem.

Table 3. Single and group attacking strategy of natural enemies on the gypsy moth larvae

Location	Year	n[1]	Frequency of natural enemies attacking the gypsy moth larvae separately and in groups [%]									
			m[2]	v[3]	p[4]	f[5]	n[6]	mp[7]	mv[8]	pv[9]	mpv[10]	hl[11]
Biebrza	1997	101	32	1	5	2	0	21	24	0	14	1
	1998	23	22	4	17	17	0	0	0	0	0	40
Rudziniec	1998	67	3	0	21	4	0	9	0	21	4	38
Biała Podlaska	1998	128	2	10	30	2	16	0	1	18	0	21

n[1] - No. of larvae, m[2] - microsporidia, v[3] - viruses, p[4] - parasitoids, f[5] - fungi, n[6] - nematodes, mp[7] - microsporidia and parasitoids, mv[8] - microsporidia and viruses, pv[9] - parasitoids and viruses, mpv[10] - microsporidia, parasitoids and viruses, hl[11] - healthy larvae

A strong effect of parasitoids on gypsy moth larvae from Rudziniec FI was observed: parasitoids larvae were present in 55 and 60 % of gypsy moth larvae from Rudziniec and Biała Podlaska FI respectively. The majority of parasitoids found in larvae from all locations

belonged to *Tachinidae* family. Nematodes and fungi were practically of no importance as natural enemies of the gypsy moth larvae from sampled locations. The population density of different kinds of gypsy moth natural enemies varied in three locations.

Natural enemies attacked gypsy moth larvae separately or in groups (Table 3). The impact of a group of three kinds of natural enemies (microsporidia, viruses and parasitoids simultaneously) on gypsy moth larvae was observed in two of the four cases, where the population density of gypsy moth was high. A similar phenomenon was observed in one of two factors group: microsporidia and parasitoids. When the population density of the gypsy moth was low, insects were affected with natural enemies, which attacked larvae rather separately than in groups. The attacking strategy of natural enemies seems to be dependent on the population density of gypsy moth.

Infectivity of the Biebrza isolate

The microsporidia isolate from the Biebrza NP did not kill the gypsy moth larvae even when it was used in the highest concentration (Table 4).

Table 4. Results of infection of third instar NJSS gypsy moth larvae with microsporidia spore suspensions from Biebrza NP after 18 days

Concentration [spores/ cm^2]	No. of larvae	Mortality [%]	Frequency of different stages [%]		
			Larvae	Prepupae	Pupae
0	50	0	24	17	59
2.4×10^{-1}	60	0	23	20	57
2.4×10^{0}	60	2	31	22	45
2.4×10^{1}	60	8	37	13	42
2.4×10^{2}	60	0	45	7	48
2.4×10^{3}	51	4	58	15	23
2.4×10^{4}	50	0	72	26	2
2.4×10^{5}	51	2	88	4	6

After 18 days, 24 % of larvae among the control individuals and 88 % of larvae among individuals treated with spores at the highest concentration was found. Similarly, 59 % of pupae among the control individuals and 6 % of pupae among individuals treated with spores at the highest concentration was noted. The new isolate prolonged the larval development.

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Study of gypsy moth microsporidia transmission by *Glyptapanteles liparidis* and *Cotesia melanoscela* (Hym.: Braconidae)

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Abstract: The study is bringing a preliminary results about ability of parasitoids to transport microsporidia infection in to host (larvae) body via ovipositor. The gypsy moth *Lymantria dispar* (L.) larvae were exposed to *Glyptapanteles liparidis* (Bouché). The ovipositor of *Glyptapanteles* females was contaminated by water suspension of microsporidia *Vairimorpha lymantriae* (Wieser) in two different doses 2.5 x 10^6 and 2.5 x 10^3 . Two exposure periods (2 h and 24 h) were used for contact between host larvae and parasitoid females. One of the larvae set was exposed manually. *V. lymantriae* infection was not occurred in the larvae body as a result of transmission. The parasitism level of gypsy moth larvae exposed by parasitoids with contaminated and uncontaminated (control) ovipositor was not different significantly. The total larval mortality showed same figure. Also the influence of different time or procedure of exposing did not show significant differences into results.

Gypsy moth *L. dispar* larvae were exposed to *Cotesia melanoscela* (Ratzeburg) females contaminated by *V. lymantriae. Two* methods of contamination (ovipositor and exposure to infected hosts) were tested. The exposure periods was 24 h. *V. lymantriae* infection was occurred int 5-20% of gypsy moth larvae, which did not cause the mortality of them. The method of contamination by infected larvae exposure showed the highest influence on the occurrence of *V. lymantriae* in the gypsy moth larvae. The significant differences was not occurred in parasite emergence (and total larval mortality) from contaminated or uncontaminated parasitoids sets.

Key words: gypsy moth, microsporidia, braconids, transmission

Introduction

Gypsy moth (*Lymantria dispar,* L.) *is* one of the most injurious factors of the oak forests in Slovakia. The larvae of the pest prefer *Quercus cerris* as a host-plant. Few thousands of hectares of forests is heavily defoliated by gypsy moth in south and south-west part of Slovakia every 6-9 years. The population dynamics of the pest is regulated by pathogens, parasitoids and predators. Entomopathogenic micro-organisms and undetermined causes are the most significant factors that influence pest's abundance (from 50 to 70% of mortality). Highest mortality is caused by NPV (Zubrik, Novotny 1995). Microsporidia, specifically the genera *Nosema* and *Vairimorpha* are quite common in natural population of the pest.

The microbial insecticides, mostly on the base of *Bacilus thuringiensis* and viruses are used in biological control of the pest in Slovakia. After the areal application, an effective transmitting the pathogens from a local point is desirable. A great advantage of the pathogens is that they can affect large areas within a short time after an application. Parasites and predators could play an important role in this problem. They have often been cited as a important vectors of insect pathogens (Raimo *et al.,* 1997, Bell et. *al.,* 1974).

The objective of this study has shown, that a parasitoids of the gypsy moth have the ability to transmit microsporidia and can successfully infect gypsy moth larvae.

Material and methods

Two parastitoids of gypsy moth were used in this study. It was *Glyptapanteles liparidis* (Ratzeburg) and *Cotesia melanoscela* (Ratzeburg).

Cotesia melanoscela is an oligophagous larval parasite, which produce 2 generations per year (Crossman 1922). We used *C. melanoscela* adults, which were reared from the field-collected (in spring 1998) cocoons of the second generation.

Glyptapanteles liparidis is an gregarious, oligophagous larval parasite of gypsy moth. In Slovakia, It can have 2 generation per year. This species over-winters in *Dendrolimus pini* or in some other over wintering hosts (Schedl 1936). We obtained colonies of *G. liparidis* from the laboratory of University of Vienna.

Colonies of both species were maintained in the laboratory at 14L:l0D and at 15°C /10°C and supplied with a honey as a food source (Schopf 1991).

Gypsy moth larvae were reared from field -collected eggs (from Rumania) and were reared under long day condition (16L:8D) at 20°C in Petri dishes on an artificial diet (modified tobacco horn worm diet) (Bell *et al.*, 1991).

The microsporidia *Vairimorpha lymantria* originated from Slovakia was used as a pathogen. The microsporidia inoculum was prepared from Slovakia originated spore material via host larvae in laboratory of Illinois Natural History Survey. For our laboratory experiments with parasitoids was prepared the water suspension of spores.

There was used one method of contaminating females by tests with *G. liparidis.* Ovipositor of the parasite was manually contaminated with the *Vairimorpha lymantria* suspension (water suspension) of a known concentration (two different doses was used). Host larvae (in second stage) were exposed to parasitoids for 2 and 24 hours and one group of the larvae was exposed to infected parasitoid wasp manually. The control group consisted of larvae that were not exposed to parasitoids.

Two methods of contaminating females by tests with *C. melanoscela* was used in our experiments. First one: manually contaminating the ovipositor of the parasite with microsporidia suspension. Second one: exposing the parasitoids females to infected larvae. Host larvae (one set of larvae in premoult in to 2 stage and one set in second stage) were exposed to parastitoids 24 hours. 4 repetition were made.

After exposing to the parasitoids host's larvae were reared 31 days or until emergence of parasitoids or until death. Larvae were examined daily and reason of the mortality were determined.

Manual contamination of ovipositor

After female parasite were cold-anaesthetised (by -7°C for 30 second), their ovipositor were brushed with a cotton swab dipped in a microsporidia suspension with doses 2.5 x 10^3 spores per ml and 2.5 x 10^6 spores per ml by *G. liparidis* experiments and 2.5 x 10^6 spores per ml by *C. melansoscela* experiments. Four contaminated females and four males were placed in a 2 1 plastic container together with 20 larvae of *Lymantria dispar.* After being exposed to these parasitoids, larvae were removed from the container and were placed in 30 ml plastic cups (one larvae per cup) and reared on artificial diet.

Contamination by exposure to infected hosts

50 specimens of the second stage gypsy moth larvae were placed on contaminated food (contaminated with microsporidia water suspension 2.5 x 10^6 spores per ml) and reared on it 4 days. After them they were removed on uncontaminated diet and reared 2 days. Six days after the larvae had been infected 10 contaminated larvae were placed in 2 1 plastic box together with four males and females of *C. melanoscela.* Exposure time was 24 hours. Subsequently contaminated parasitic wasp were removed from a box and placed in other 21 box with 25

larvae in premoult into the 2nd instar (or 25 larvae in second instar respectively). Exposure time was again 24 hours. After exposure to the contaminated wasp, larvae were removed and placed in 30 ml plastic cups (one larva per cup) and were reared on artificial diet.

Results and discussion

C. melanoscela and *G. liparidis* have been chosen for this experiment, because they are very important natural enemies of gypsy moth not only in Slovakia but also in all European regions (Coulson 1981, Coulson *et al.*, 1986). They have a very big reproductive capacity, they are relatively easy reared in the laboratory and they have a similar mechanism of oviposition.

This study has been our first step in more detailed research of the possibility to transmit the pathogens by parasitoids.

Glipapanteles liparidis

V. lymantria infection was not occurred in the larvae body as a result of transmission, but another entomopathogens were observed in death larvae bodies as a NPV, septicaemia bacteria and entomopathogenic fungus. Only NPV (in 6%) caused mortality of larvae primarily. All other microbial were present in larvae body not as a primary pathogens. The reason of multiplication of micro-organisms as a bacteria or fungus was optimum condition in death larvae body. Except of influence of entomopathogenic mikroorganismus the larvae mortality includes also mortality by stress, caused after the repeated ovipositor of females the parasitoids (table 1).

Table 1: Gypsy moth larval mortality in percent (by *Glyptapanteles liparidis).*

	Control *	**larvae exposed to contaminated females of *G. liparidis***						**larvae exposed to uncontaminated females of *G. liparidis***		
		$2{,}5 \times 10^3$			$2{,}5 \times 10^6$					
Expose time		2 h.	24 h.	man.	2 h.	24 h.	man.	2 h.	24 h.	man.
Parasite emer.	-	5	30	30	30	55	45	20	25	60
mort. b path.	0	0	0	30	5	40	20	30	20	15
total mortality	0	5	5	60	35	95	65	50	45	75
total larvae in test	20	20	20	20	20	20	20	20	20	20
		a	b	c	a	b	c	a	b	c
Control* = larvae not exposed to *G. liparidis* a,b,c = not sign. 5% level										

The mortality by in larvae exposed to females of parasitic wasp for 2, 24 hours or manually was not significant different, but it increase from 2 hour exposed larvae to manually exposed larvae (table 1). The similar methods of contamination has been used in the study of Raimo *et al.*, in 1977 with the similar results.

The parasitism level of gypsy moth larvae exposed to parasitoids with contaminated and uncontaminated ovipositor was not different significantly. The same results was determined by Raimo et al. in 1977. Total 33.3 % of larvae were parasitized and we obtained 572 cocoons of *G. liparidis* (it approximately 9.5 pieces of cocoons per one parasitized larvae). Parasitism

at the rate of 48% could by found in gypsy moth population at a low density site in central Europe (Schopg, Hoch 1997).

The total mortality reached 51.0%. 50% of mortality was occurred 30 days after start of the experiment (fig. 1).

The 2nd stage larvae of the host were exposed to females of *G. liparidis*. From total amount of 60 parasitised larvae, from 49 were hatched parasitoids in late 4th instar (table 3). More parasitoids emerged generally from 4th instar hosts that from other instars also in experiments of Schopf & Hoch (1997). They mentioned , that the 5th and 6th instar as a final instar was also not rare in their experiments. If the hosts larvae are parasitized during premoult into the 2nd instar, parasitoid larvae emerged at the end of the host's 4th instar (Schopf, Steinberger, 1996).

Table 2.: Parasite emergence, larval mortality (in percent for all tests with *C. melanoscela)* caused by pathogens or undetermined reasons and infected larvae by microsporidia.

Replicate	**Control Larvae not exposed to *C. melanoscela***			**Larvae exposed to uncontaminated females of *C. melanoscela***			**Larvae exposed to *C. melanoscela* with contaminated ovipositor**			**Larvae exposed to *C. melanoscela* exposed to infected host**		
	mort.	paras.	micr.	mort.	paras.	micr.	mort.	paras.	micr.	mort.	paras.	micr.
1	0.0	-	0.0	8.0	72.0	0.0	16.0	80.0	8.0	8.0	88.0	8.0
2	0.0	-	0.0	12.0	68.0	4.0	4.0	92.0	8.0	28.0	60.0	8.0
3	4.0	-	0.0	12.0	68.0	8.0	32.0	52.0	8.0	40.0	52.0	24.0
4	12.0	-	0.0	8.0	80.0	4.0	16.0	80.0	8.0	36.0	36.0	20.0
				a	b	c	a	b	c	a	b	c
a,b,c = not sign 0.05%,												

Cotesia melanoscela

V. lymatriae infection was occurred from 4-24% of gypsy moth larvae, but the infection was not so strong to be able to be a reason of the mortality. In the experiments of Raimo *et al.*, (1977) *C. melanoscela* was capable of vectoring lethal doses of NPV to gypsy moth larvae.

The visibly difference in larval mortality caused by pathogens and undetermined reasons between larvae exposed to uncontaminated and contaminated parasitoids was occurred. Highest mortality was occurred by group of larvae exposed to *C. melanoscela* exposed to infected host (table 2). Total mortality of the pest reached 87.3%. The 50% mortality was occurred 16 days after the start of the experiment. *C. melanoscela* declared its very high potential of the ovipositing a quite large number of larvae during a short time. Mortality by *C. melanoscela* varied from 15-90!% in experiments of Raimo *et al.*, (1977) (fig 1).

The significant differences was not occurred in parasite emergence from contaminated and uncontaminated parasitoids sets.

Larvae in premoult in to the second instar and the 2nd stage larvae of the host were exposed to females of *G. liparidis*. Total parasitization was higher in the first group - 76.6%, in second group there was parasitized 61%. In first group: from totally number of 115 parasitized larvae from 104 were hatched parasitoids in 3rd instar. In second group: from totally number of 92 parasitized larvae from 88 were hatched parasitoids in 3rd instar. Generally the *C. melanoscela* by the same condition parasitized twice as much larvae as *G. liparidis* and the development of the parasitic wasp was much shorter than by *G. liparidis*. *C.*

melanoscela adults hatched from the 3 [th] instar of gypsy moth mostly - *G. liparidis* prefer 4[th] instar (table 3).

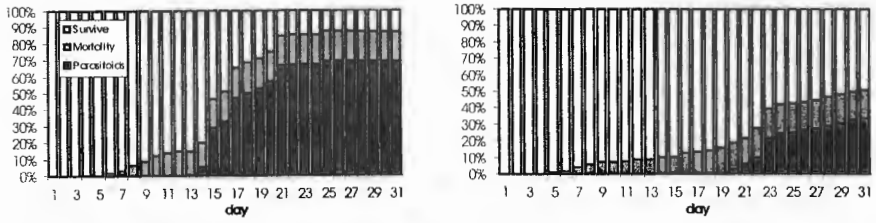

Figure 1. Development of mortality in all test with *C. melanoscela* (left) and *G. liparidis* (right).

Table 3. Duration of the development of *C. melanoscela* and *G. liparidis* in host larvae until parasitoids were emerged from gypsy moth larvae.

Species of parasitoid	nl	instar of ovipositing	n2	Final instar			
				2[nd] instar	3[rd] instar	4[th] instar	5[th] instar
C. melanoscela	115	premoult into the 2 instar	11	15.0 ± 0.0			
			104		18.3 ± 2.6		
C. melanoscela	92	L2	88		15.8 ± 1.8		
			4			25.8 ± 1.3	
G. liparidis	60	L2	8		20.5 ± 0.7		
			49			24.1 ± 2.5	
			3				30.3 ± 0.9

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Morphometrical comparison of various microsporidian isolates from the Gypsy Moth, *Lymantria dispar* L.

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Abstract: Several isolates of microsporidia (Protozoa) were collected from Gypsy moth populations throughout Europe in the last decade. For a complete description of these isolates and to facilitate the quick identification in the light microscope, spore measurements are necessary. The aim of this study was to provide morphometrical data for 11 microsporidian isolates belonging to the genera *Nosema* and *Endoreticulatus*. The isolates originate from Gypsy Moth populations in Germany, Portugal, Poland, Austria, Slovak Republic, Czech Republic, Hungary, and Bulgaria. All isolates were fed to a laboratory rearing of Gypsy Moth larvae. Fresh spores, isolated from larvae, were measured using the LEICA image analysis system Quantimet 500. Data were processed in Excel and analysed using different statistical programmes. Significant differences were found among several of the *Nosema*-isolates, partially corresponding to the results of the ultrastructural findings. The practical value for the differentiation of the isolates is discussed.

Key words: gypsy moth, microsporidia, morphometrical characteristics

Introduction

In an international, cooperative approach to find a pathogen for the biological control of Gypsy Moth (*Lymantria dispar* L.), several isolates of microsporidia (Protozoa) were collected throughout Europe in the last decade. In addition to research focussing on the effects of the microsporidian infection on the insect and experiments on host specificity, an evaluation of the taxonomic position of the isolates is necessary.

The identification of the isolates, using light- and electron microscopy of the ultrastructure and morphology of developmental stages, and studies on the host specificity are under investigation by different working groups in Europe and the USA (Linde & Rappl, 1994, Solter *et al.*, 1997, Maddox *et al.*, 1999). At least three different genera (*Nosema, Vairimorpha,* and *Endoreticulatus*) have been found. For a complete description of the isolates and to facilitate the quick identification in the light microscope, spore measurements are necessary. The aim of this study was to provide morphometrical data for the comparison of 11 microsporidian isolates, belonging to the genera *Nosema* and *Endoreticulatus*. The isolates originate from Gypsy Moth populations in Germany, Portugal, Poland, Austria, Slovak Republic, Hungary, and Bulgaria.

Material and Methods

All isolates were fed to a laboratory rearing of Gypsy Moth in Eberswalde to obtain fresh spores. Gypsy Moth larvae were fed artificial diet and reared under identical conditions. Spores were recovered from infected target tissues by homogenization and washed in distilled

water. A drop of spore suspension was placed on an agar cushion on a microscopic slide (Vavra & Maddox, 1976). No less than 150 spores were measured using the LEICA image analysis system Quantimet 500 and a magnification of 1,600. Data were processed in Microsoft Excel 97 and analyzed using Statistica v. 5. Cluster analysis was used to produce dendrograms of *Nosema* and *Endoreticulatus* isolates based on four morphometrical parameters - length, width, length/width ratio, and volume of spores.

Results and discussion

Nosema-*isolates*

Significant differences were found among the group of the *Nosema*-isolates including one isolate from Poland (Biebrza Marshes), one from Germany (Northern Bavaria), one from Hungary (Turisvandi), two from Bulgaria (Veslec and Levishte), one from Austria (Ebergassen), one from Portugal (Lisbon) and one from Slovakia (Businee). There were no significant correlations between their morphometrical characteristics (table 1).

Table 1. Metrical characteristics of the *Nosema* isolates

	POL.	GER.	HUNG.	VESL.(BG)	LEV.(BG)	EBERG.(AT)	PORT.	BUS.(SK)
Length	5.06	5.16	4.95	4.84	4.98	4.61	4.65	4.89
Width	2.00	2.33	2.11	2.18	2.21	2.27	2.20	2.19
L/W	2.55	2.24	2.37	2.24	2.28	2.04	2.13	2.25
Volume	10.70	14.84	11.68	12.07	12.87	12.62	11.88	12.43

Cluster analysis (fig. 1) showed that the *Nosema* isolate from Germany is very different from the other isolates. This corresponds to the results obtained on its development, ultrastructure of developmental stages, and tissue specificity. The other *Nosema* – isolates represent a monolyte group in which the *Nosema* from Poland showed less similarity.

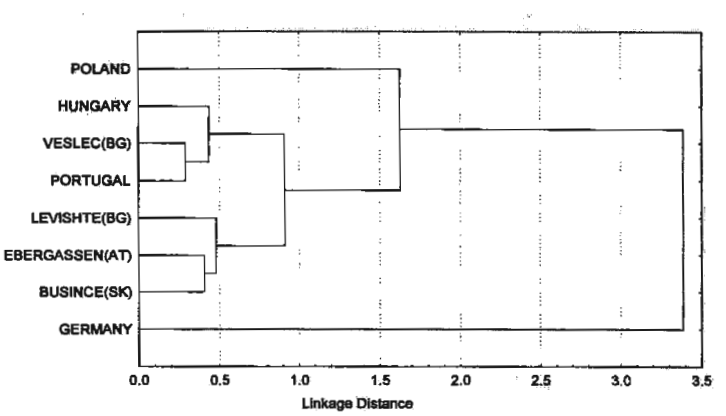

Fig. 1. Tree diagram for eight *Nosema* isolates based on their metrical indexes (length, width, length/width, volume)

Morphometrical analysis of the *Nosema* isolate from Pavlovce (Slovakia) confirmed the presence of a big and a small type of spores as seen in the light microscope. Cluster analysis clearly distinguished two groups of spores (table 2 and fig. 2).

Table 2. Descriptive statistics for clusters analysis of *Nosema* from Pavlovce

Cluster 1(contains 74 cases)				**Cluster 2(contains 76 cases)**			
	Mean	S.D.	Variance		Mean	S.D.	Variance
Length	5.19	0.55	0.30	L	4.88	0.49	0.24
Width	2.34	0.16	0.03	W	2.00	0.15	0.02
L/W	2.23	0.34	0.12	L/W	2.47	0.37	0.14
Volume	14.85	1.92	3.70	V	10.15	1.30	1.68

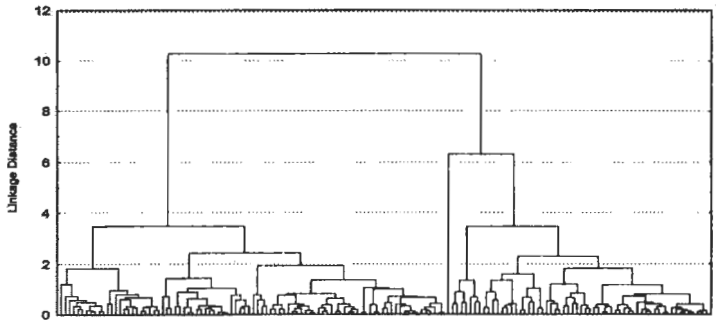

Fig. 2. Tree diagram for *Nosema* from Pavlovce based on its metrical indexes (length, width, length/width, volume)

The comparison of the two groups (Pavlovce 1 and Pavlovce 2) with the other *Nosema*-isolates showed that Pavlovce 2 is grouped together with the *Nosema* from Germany, both are practically identical. Pavlovce 1 is grouped with the *Nosema* from Poland, but with less similarity (fig. 3).

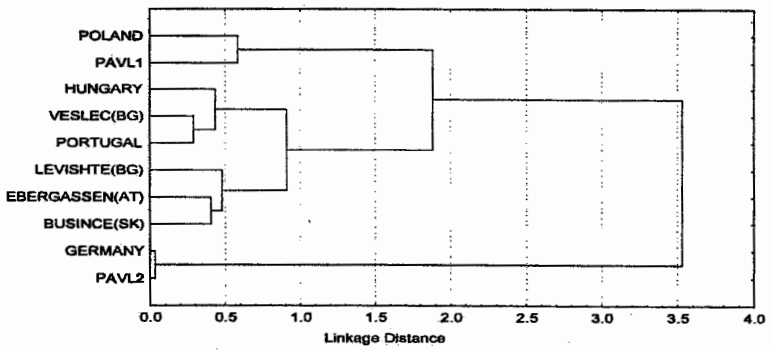

Fig 3. The place of *Nosema* Pavlovce clusters in the tree diagram for all European *Nosema* isolates

Endoreticulatus -*isolates*

The two *Endoreticulatus* - isolates from Gypsy Moth populations in Portugal and Bulgaria (Asenovgrad) were compared with one *Endoreticulatus* - isolate from a Browntail Moth (*Eurproctis chrysorrhea)* population in Bulgaria (Dolni Lozen). This isolate infects Gypsy Moth larvae under laboratory conditions. Cluster analysis based on the morphometrical characteristics (table 3) of their spores revealed that the *Endoreticulatus* from Asenovgrad is most similar to the one from Browntail Moth and both form one cluster (fig. 4). The *Endoreticulatus* from Portugal forms the second cluster. Based on these data and the results on the development in the Gypsy Moth we can suppose that both *Endoreticulatus* from Bulgaria belong to one species which is different from the *Endoreticulatus* from Portugal.

Table 3. Metrical characteristics of *Endoreticulatus* isolates

	Portugal	**Asenovgrad**	**Dolni Lozen**
Length	2,32	2,44	2,48
Width	1,21	1,24	1,23
L/W	1,95	2	2,06
Volume	1,82	2,01	2,01

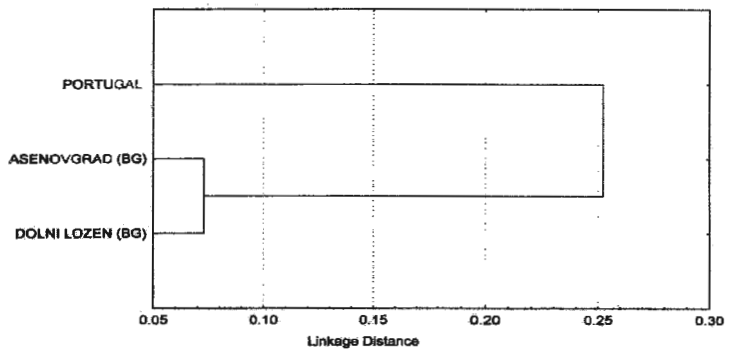

Fig. 4. Tree diagram for three *Endoreticulatus* isolates based on their metrical indexes (length, width, lenght/width, volume)

Our studies of eleven isolates from Gypsy Moth populations in Europe provide information for the similarities and differences among the *Nosema* and *Endoreticulatus* isolates. This information allows a better interpretation of the results obtained on the development, ultrastructure of developmental stages, and tissue specificity of the isolates. We believe that additional investigations are needed to clarify why Gypsy Moth is host for such a variety of different microsporidian isolates or even species.

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Workshop II a / Part 1:

"Identification of Pathogens in Locusts"

Insect Pathogens and Insect Parasitic Nematodes
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Diagnosis of insect pathogens from locusts and other orthopterans

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Introduction

The possible use of entomopathogenic microorganisms and viruses in biological pest control is studied since more than 100 years, e.g. Metschnikoff (1879) used successfully the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin against coleopteran larvae. Also the ever aggravating problems with locusts were and are tried to be solved with the use of entomopathogens. The microorganisms and viruses found in locusts are summarized e.g. by Uvarov (1966, 1977) and amplified by Streett & McGuire (1990). The possibilities of a broad application of locust pathogens are discussed e.g. by Henry (1977), Streett (1986), Farrow (1989, 1990), Greathead & Prior (1990), Streett & Henry (1990) as well as by Bidochka & Khachatourians (1991).

Yet it must be underlined that intensive diagnostic studies are a crucial prerequisite for any practical use of an entomopathogen in biological or in integrated control. In a broad sense, diagnosis is not only the detection and identification of a pathogen, rather it comprises thorough studies of all aspects of the inherent disease process (e. g. Steinhaus, 1963, 1964; Huger, 1974). Here in our introductory diagnostic course we can only focus on the light microscopic identification and recognition of important pathogens of selected topical pests, i. e. locusts and bark beetles.

Table 1 presents a compilation of important locust pathogens known so far. In the following, basic informations on the most important locust pathogens and some elementary diagnostic procedures are given.

FUNGI

Already in the last century fungal epizooties were found in locust populations (summaries by Bruner, 1902 and Benois, 1929). Most intensive studies have been carried out with *Entomophaga* (= *Entomphthora*) *grylli* (Fresenius, 1856) (Table 1) since the experiments by Howard (1901). The fungus often results in considerable mortalities of locusts all over the world, e. g. North America (Treherne & Buckel, 1949; Soper *et al.*, 1983), Europe (Fresenius, 1856), Africa (Chapman & Page, 1979), Asia (Roffey, 1968), and Australia (Milner, 1978). But so far this fungus cannot be produced on artificial media. Therefore, a broad practical application is not possible.

Table 1. Entomopathogenic microorganisms and viruses of locusts and other orthopterans.

Species	Original host	Place of detection	Infection site	Reference
FUNGI				
Aspergillus flavus	*Schistocerca gregaria*	India	Whole insect body	RAE (1977)
Beauveria bassiana	*Locusta migratoria*	?	"	Trabut (1891)
Beauveria brongniartii	*Locusta migratoria*	?	"	Brady (1979)
Conidiobolus thromboides	Not determined locust	USA	"	Anon. (1986)
Entomophaga asiatica	*Parapodisma* sp.	?	"	Anon. (1986)
***Entomophaga grylli*-Complex:**			"	Chapman *et al.* (1986)
***E. calopteni* (*E. grylli* Pathotype 2)**	*Melanoplus* spp.	Canada		Anon. (1986)
***E. macleoidii* (*E. grylli* Pathotype1)**	*Melanoplus* spp.	Canada		Anon. (1986)
***E. praxibuli* (probably also ordered to the *E. grylli*-Complex)**	*Praxibulus* sp.	?		Anon. (1986) Balfour-Browne (1960)
Metarhizium anisopliae	*Schistocerca gregaria*	Erythrea	"	Anon. (1986)
Metarhizium flavoviride* var. *minus	*Zonocerus elegans*	Tanzania	"	Evans & Samson (1982)
***Nomourea* sp.**	Not determined locust	Java	"	Evans (unpubl.)
Paecilomyces guanensis	Not determinded cricket	Sulawesi	"	Anon. (1986)
Paecilomyces reniformis	Not determined Tettigonide	Ghana, Tafo	"	Samson & Evans (1974)
Paecilomyces lilacinus	*Segestidae* sp.	Papua Neuguinea	"	Anon. (1988)
***Sorosporella* sp.**	*Melanoplus* sp.	?	"	Anon. (1986)
	Schistocerca gregaria	Madagascar		Welling *et al.* (1995)
Verticillium lecanii	*Melanoplus* sp.	Laboratory, field tests	"	Johnson *et al.* (1988)

Table 1 continued

Species	Original host	Place of detection	Infection site	Reference
PROTOZOA				
Nosema locustae	*Locusta migratoria migratorioides*	Laboratory, U.K.	Fat body, pericard, nervous tissues	Canning (1953)
Nosema acridophagus	*Melanoplus* spp., *Schistocerca gregaria*	USA	Fat body	Henry (1967)
Nosema cuneatum	*Melanoplus confusus*	USA	Fat body	Henry (1971 a)
Nosema montanae	*Melanoplus packardii*	Montana, USA	Fat body	Wang *et al.* (1991)
Perezia dichroplusiae	*Dichroplus elongatus*	?	Fat body	Lange (1987)
Johenrea locustae	*Locusta migratoria capito*	Madagascar	Fat body	Lange *et al.* (1996)
Malamoeba locustae	*Melanoplus* spp.	Laboratory, USA	Malpighian tubules	King & Taylor (1936)
***Schizogregarine* sp.**	*Melanoplus bivittatus, M. sanguinipes, M. bilituratus*	Alberta, USA	Fat body	Bucher (1966)
Gregarina garnhami	*Schistocerca gregaria*	U.K.	Midgut-lumen	Canning (1956)

Table 1 continued

Species	Original host	Place of detection	Infection site	Reference
VIRUSES				
Entomopoxvirus (Fam. Poxviridae)	*Melanoplus sanguinipes*	Arizona, USA	Cytoplasm of fat body cells	Henry & Jutila (1966)
Entomopoxvirus (")	*Locusta migratoria*	Tanzania, Africa	"	Purrini *et al.* (1988)
Entomopoxvirus (")	*Schistocerca gregaria*	Yemen, Arab. Republic	"	Purrini & Rhode (1988)
Entomopoxvirus (")	*Anacridium aegyptium*	France	"	Meynadier *et al.* (1992)
Crystalline Array Virus (Fam. Picornaviridae)	*Melanoplus bivittatus*	USA	Cytoplasm of pericard, muscle and tracheal matrix	Jutila *et al.* (1970)
Cytoplasmic Polyhedrosis Virus (Fam. Reoviridae)	*Caleda captiva*	?	Cytoplasm of midgut epithelial cells	Colgan (1986)
Cricket Iridovirus (CrIV) (Fam. Iridoviridae)	*Gryllus campestris, Acheta domestica*	The Netherlands	Cytoplasm of the fat body, hypo-dermis, tracheal matrix, muscula-ris of the gut, and others	Kleespies *et al.* (1999)
BACTERIA				
Coccobacillus acridiorum ***= Aerobacter aerogenes***	*Schistocerca pallens*	Mexico	Gut-lumen, fat body	D'Herelle (1910) Steinhaus (1949)
Serratia marcescens	*Schistocerca gregaria*	Laboratory, USA	"	Stevenson (1959)
RICKETTSIA				
Rickettsiella grylli	*Gryllus bimaculatus, G. capitatus*	France	Fat body, hemo-ytes, salivary glands, & others	Vago & Martoja (1963)

Adapted from RAE = Review of Applied Entomology and PRIOR & GREATHEAD (1989)

Metarhizium anisopliae (Metschnikoff) Sorokin, *Beauveria bassiana* (Balsamo) Vuillemin and *Verticillium lecanii* Nees. (all Deuteromycetes: Moniliales) have a broad host range including also Orthoptera. Balfour-Browne (1969) reported a remarkable reduction of a swarm of *Schistocerca gregaria* due to *M. anisopliae.* Veen (1966, 1968) received high mortality rates by feeding of conidia and blastospores of this fungus to first and second nymphs of *S. gregaria.* First positive results in the control of *S. gregaria* were reported by Prior *et al.* (1988) who used another species of *Metarhizium, M. flavoviride* Gams & Rozsypal (1973). This fungus has been originally isolated first from *Zonocerus elegans* (Evans & Samson, 1982).

The development of an oil formulation and an ultra-low-volume (ULV)-application technique delivered further promising results for biological control of locusts with *M. flavoviride* (e.g. Prior, 1989; Bateman *et al.*, 1991). Further investigations on the production and formulation of *Metarhizium* spp. were carried out in our institute (e. g. Kleespies & Zimmermann, 1992, 1994, 1998; Stephan & Zimmermann, 1998). *M. flavoviride* (International Mycological Institute (IMI) Strain No. 330189), recently described as *M. anisopliae* var. *acridum* (Milner, 1997), isolated from *Ornithacris cavroisi* (Acrididae), will be presented in this course.

Systematics:

Today, about 35 genera with more than 400 species of entomopathogenic fungi are known. The systematic position of the most important locust pathogenic species is presented below:

Microbial Group:	Fungi
First section:	Oomycota
Second section:	Eumycota
First class:	Chytridiomycetes
Second class:	Zygomycetes
Species:	***Entomophaga grylli*** Fresenius
	Entomophaga praxibuli
Third class:	Ascomycetes
Fourth class:	Basidiomcetes
Fifth class:	Deuteromycetes (Fungi imperfecti)
Order:	Moniliales
Species:	***Beauveria bassiana*** (Bals.) Vuill.
	Beauveria brongniartii (Sacc.) Petch.
	Metarhizium anisopliae (Metsch.) Sorokin
	Metarhizium flavoviride (Gams) Rozsypal
	Metarhizium album Petch
	Sorosporella sp. Sorokin
	Verticillium lecanii (Zimm.) Viégas

(Adapted from Roberts & Yendol, 1981; Mueller & Loeffler, 1982; Strasburger, 1983).

Metarhizium flavoviride (Evans & Samson, 1982)

M. flavoviride is a facultative pathogen parasitizing insects under natural conditions. It can easily be mass-produced on artificial media. Fungal development terminates with the formation of conidia as a resting stage.

Data for diagnosis:

Transmission: Conidia are transported passively by wind, rain or animals. In this way, insects contact condia with their legs and cuticle or with their mouthparts by feeding fungus contaminated material.

Infection site: The whole insect body is infected.

Infection and fungal growth:
Germination of conidia takes place on the surface of the cuticle under suitable humidity and temperature conditions. Following germination, the fungus actively penetrates the cuticle by generating enzymatic processes. Subsequently the fungus propagates by mycelial growth and production of blastospores all over the insect body. Blastospores are transported by the hemolymph to all tissues and organs. In a later state of development, the insect is densely packed with fungal mycelium and blastospores. Finally, under suitable temperature and humidity conditions sporulation takes place on the surface of the insect, thus producing masses of conidia (resting stage) accumulated in dense cushions.

Morphology: Conidia are yellow-green in colour and have a size of 7.0–9.0 µm in length and 4.5–5.5 in width (Barnett & Hunter, 1972, Gams & Rozsypal, 1973).
Blastospores are of variable size and shape.

Signs and Symptoms:
Often, melanotic areas can be observed on the insect cuticle where fungal entrance occurred and the cuticle colouration turns dark red prior to death. In addition infected locusts reduce feeding, display difficulties in moulting, lethargy and have a reduced lifespan. After death, locusts are hard and mummified, and develop cushions of conidia on the integument.

Initial diagnosis: 1. Exterior appearance:
Recently infected locusts display melanotic areas on the cuticle. Later the locusts turn reddish in colour. At the postmortal stage of disease cushions of yellow-green condia are covering the cuticle.
2. Light microscopy
a) For cadavers with conidial cushions preparations with Tesafilm are recommended: The film is touched slightly onto the conidia and then fixed on a slide together with a drop of lactophenol-cotton blue. In the light microscope (phase contrast, magnification 400x) hyphae, phialides (these cells carry the conidia) and conidia can be observed in a specific manner.

b) Squash preparations of various infected tissues are viewed in phase contrast at a magnification of 400x; blastospores and hyphae of different sizes as well as conidia can be observed. The same procedure can also be employed with pieces of the conidial cushions outside the host.

Quite often insects show no external signs of disease, but in tissue preparations fungal hyphae or hyphae and blastospores are disclosed. In these cases it is recommendable to keep such specimens in a moist chamber to favour final development of conidia / spores for a closer diagnosis.

Composition of Lactophenol-cotton blue

20 g Phenol
20 g Lactic acid
40 g Glycerol
20 g Aqua dest.
0.1 g Cotton blue

PROTOZOA

Protozoa are definded as single cell, eukaryotic protists. For classification, Undeen & Vavra (1997) referred to two publications: Corliss (1994) who divided the Protozoa in two kingdoms and 34 phyla, and Levine *et al.* (1980) who used a modified classification considering the Protozoa as a subkingdom of the kingdom Protista and dividing the Protozoa into seven phyla:

1. Phylum: Sarcomastigophora
   Including: Flagellates
   Amoebae

2. Phylum: Labyrinthomorpha

3. Phylum: Apicomplexa
   Including: Gregarines
   (Eugregarines and
   Neogregarines)
   Coccidia

4. Phylum: Microspora
   Including: Microsporidia

5. Phylum: Ascetospora

6. Phylum: Myxozoa

7. Phylum: Cilophora
   Including: Ciliates

The most important pathogenic Protozoa of locusts belong to amoebae, gregarines (neogregarines, eugregarines), and microsporidia.

Amoeba

Malamoeba locustae (King & Taylor, 1936)

In view of natural limitation and biological control of insects with entomopathogenic amoeba, *Malamoeba locustae* King & Taylor is the most important one (McLaughlin, 1971). King & Taylor (1936) discovered *M. locustae* in the Malpighian tubules of different *Melanoplus*-species. This amoeba-species has a relatively broad host range which was studied e.g. by Ernst & Baker (1982) and Henry *et al.* (1985). Horizontal transmission by ingestion of *M. locustae*-cysts is the only known mode of infection. It is a chronic disease which gradually reduces populations, especially locust cultures in the laboratory. In this course specimens of *Locusta migratoria* infected with *M. locustae* are offered for preparation and diagnosis.

Data for diagnosis:

Transmission: Per os	(ingestion of oval cysts).
Infection site:	Malpighian tubules.
Life cycle:	Upon ingestion of cysts (Fig. 1A), primary trophozoites (Figs. 1A-B) are hatching from the cysts and infect epithelial cells of the midgut. The primary trophozoites divide into secondary trophozoites (Fig. C) which infect the Malpighian tubules, were they begin to multiply 12-16 days after infection. Finally mature cysts are formed (Fig. 1A) and are completely occupying the lumen of the Malpighian tubules (Evans & Elias, 1970).
Morphology:	Mature cysts are oval in shape and measuring ca. 12 x 8 µm, trophozoites are of variable size (diameter 4-14 µm, mostly about 8 µm).
Signs and Symptoms:	Change of colour (not obligatory), reduced feeding, difficulties in moulting, lethargy, secondary septicaemia, reduced fecundity and fertility, reduced lifespan.
Diagnosis:	Light microscopy: Investigation of native preparations of Malpighian tubules (squash preparations) in phase contrast at a magnification of 400x; amoeba-infected Malpighian tubules are swollen and whitish coloured. In phase contrast especially light refracting cysts are characteristic diagnostic stages of this pathogen.

Microsporidia

These organisms are obligatory intracellular parasites. Besides the common peroral mode of infection (cannibalism, contact with faeces, contaminated food) quite often also germinative transmission of microsporidia via the eggs takes place. Spores can be transmitted on the outside of the egg (= trans-ovum transmission) or within the egg (= transovarial transmission). Table 1 shows that there are 6 important species of microsporidia pathogenic against locusts. 4 of them belong to the genus *Nosema*. Most of the studies on microsporidia of locusts were done with *Nosema locustae*. Overviews are offered by Henry & Oma (1981) and by Ignoffo (1988).

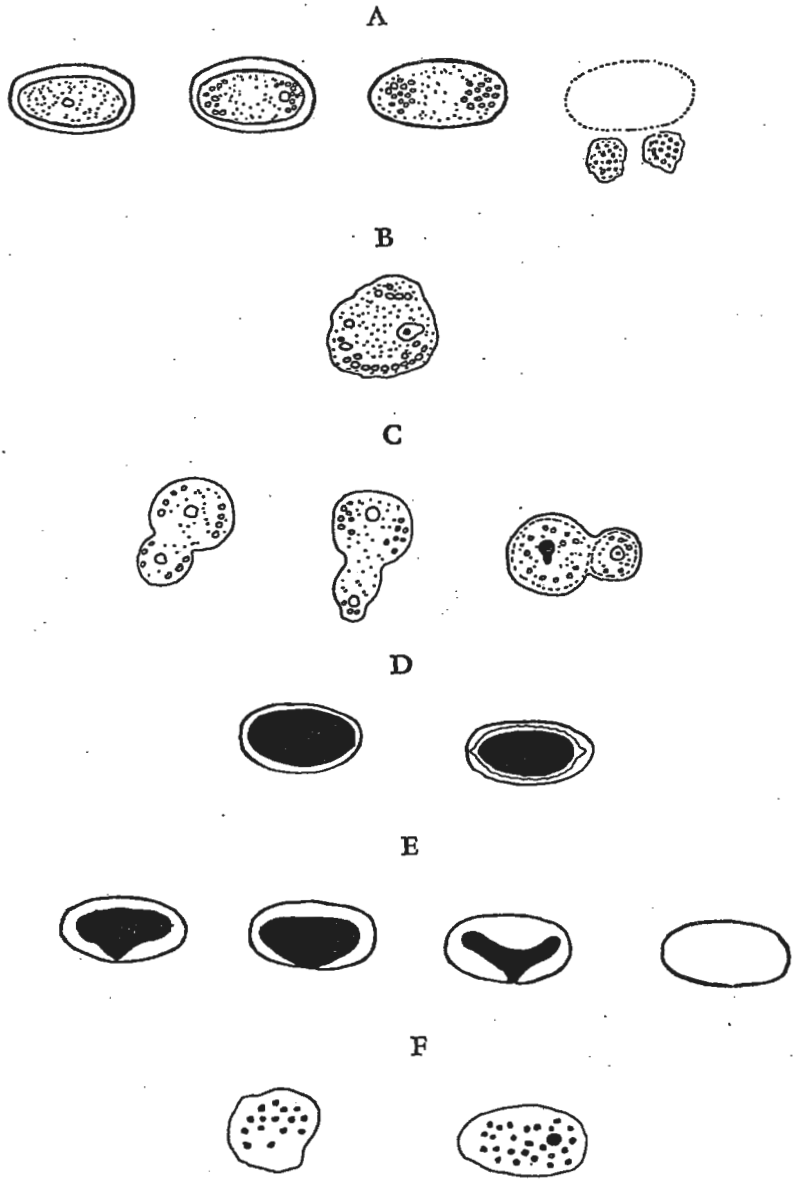

Figure 1. Cysts and trophozoites of *Malamoeba locustae.* **A** Live cysts; **B** Stained trophozoite; **C** Binary fission of trophozoites: **D** Stained cysts with or without a differentiated wall; **E** Germination of cysts (stained material); **F** Trophozoite and cyst stained with osmium tetroxyde (from Prinsloo, 1960).

Nosema locustae (Canning, 1953)

Nosema locustae, first dicovered in *L. migratoria* (Canning, 1953), is infectious for most of the locust species belonging to the family Acrididae (e. g. Canning, 1962 a) and different crickets belonging to the family Gryllidae. Field tests done by Henry *et al.* (1973) delivered the highest mortality rates of *Melanoplus sanguinipes* when third instar nymphs were treated. 1.6×10^9 to 2.3×10^9 spores admixed to 1.12 to 1.68 kg wheat bran and spread per ha resulted in a mortality of 50-60% within 4-6 weeks. Older nymphs are less susceptible. Commercial preparations of *Nosema locustae* under the trade name „Noloc".

Data for diagnosis:

Transmission: Per os (ingestion of spores).

Infection sites: Fat body, pericard, nervous tissues.

Life cycle: The developmental cycle is shown in Fig. 2/1-16. Upon peroral ingestion, the spores extrude their polar filament into epithelial cells of the midgut. In this way the infective planont (sporoplasm with nuclei) is injected through the polar filament into a cell (Figs. 2/15-16). Massive multiplication of meronts (schizonts) (merogony/schizogony) especially takes place in the fat body. Meronts transform to sporonts, sporoblasts and finally spores (Figs. 2/9-14).

Morphology:

Uninucleate meronts (=schizonts) measure	2.5 to 3.5 µm,
binucleate meronts (=schizonts) measure	3.0 to 4.0 µm,
tetranucleate meronts (=schizonts) measure	5.0 to 6.5 µm,
oval shaped spores measure	3.5 x 5.5 µm,
length of polar filament	about 165 µm.

Signs and Symptoms:

The development of infected locusts is much slower as compared to healthy ones; they are more cannibalistic (Lockwood, 1989), feeding, fertility, fecundity and lifespan are reduced; secondary septicaemia may occur. (Canning, 1962 b; Ewen & Mukerji, 1980; Henry & Oma, 1981; Erlandson *et al.*, 1986).

Diagnosis: Light microscopy:

Investigation of native preparations of the fat body and other infected tissues (squash preparations) in phase contrast at a magnification of 400 x;

Infected organs and tissues often appear white like lime. The presence of spores is the most reliable criterion for diagnosis. Spores are oval in shape and severely light-refracting in phase contrast.

For the study of the life cycle, Giemsa stained smears have to be prepared.

Most characteristic for microsporidian spores is the polar filament spirally coiled inside the spore. Because of the thick spore wall, it cannot be recognized in the light microscope. Therefore, attempts to extrude the polar filament were done with many solutions, such as diluted acetic acid (CH_3COOH), hydrochloric acid (HCl), sodium hydroxide (NaOH) or hydrogene peroxide (H_2O_2). Not infrequently, polar filaments can be extruded just by exerting pressure onto the coverglass of an aqueous spore preparation. Filaments extruded in this way can best be observed in phase contrast after drying out of the preparation.

The ultrastructure of these spores was described by Huger (1960). In this connection he also designed the first model of the internal architecture of a microsporidian spore (see Fig. 3) which in its general features also proved true for microsporidian spores in common.

Giemsa staining (adapted from Romeis, 1989)
For the study of life cycles, the preparation of Giemsa stained smears is a most useful tool.
- For smears use only very clean microscope slides (last step with alcohol).
- The smears are briefly dried on air.
- Fixed in methanol for 10 min., again air drying.
- Stain with Giemsa's solution for 30-60 min.
- Wash the slides briefly in fresh tap-water
- Air drying for one hour
- Embedding

Preparation of Giemsa's solution
Concentrated solutions of Giemsa's stain are produced by different companies. It has to be diluted with buffered aqua dest.

A phosphate buffer (Sœrensen) is used:

Phosphate buffer	0.245 g	KPO_4
	0.570 g	$NaPO_4$
	in 500 ml	aqua dest.

For staining use	10 ml	Phosphate buffer +
	0.3 ml	Giemsa's solution (about 15 drops)

Gregarines

In the gregarine group, schizogregarines (neogregarines) and eugregarines are differenciated. Schizogonic divisions are typical for schizogregarines, while in the development of eugregarines a schizogony is lacking.

Canning (1956) discovered *Gregarina garnhami* (eugregarine) in *Schistocerca gregaria* (Table 1). Harry (1965, 1970, 1971) investigated locust infections with this gregarine. He observed a reduction in weight only of the males. Eugregarines mainly occur in the lumen of the host gut, while schizogregarines develop in the cells of the fat body and other tissues. Bucher (1966) found a schizogregarine infection in *Melanoplus bivittatus, M. bilituratus* and *M. sanguinipes,* but it was not the only reason for disease, as at the same time an infection with the bacterium *Serratia marcescens* was observed.

In this pathogen group only few investigations on host-parasitic relationship in locusts and on epizootics were done. Therefore, this group is neglected in this course.

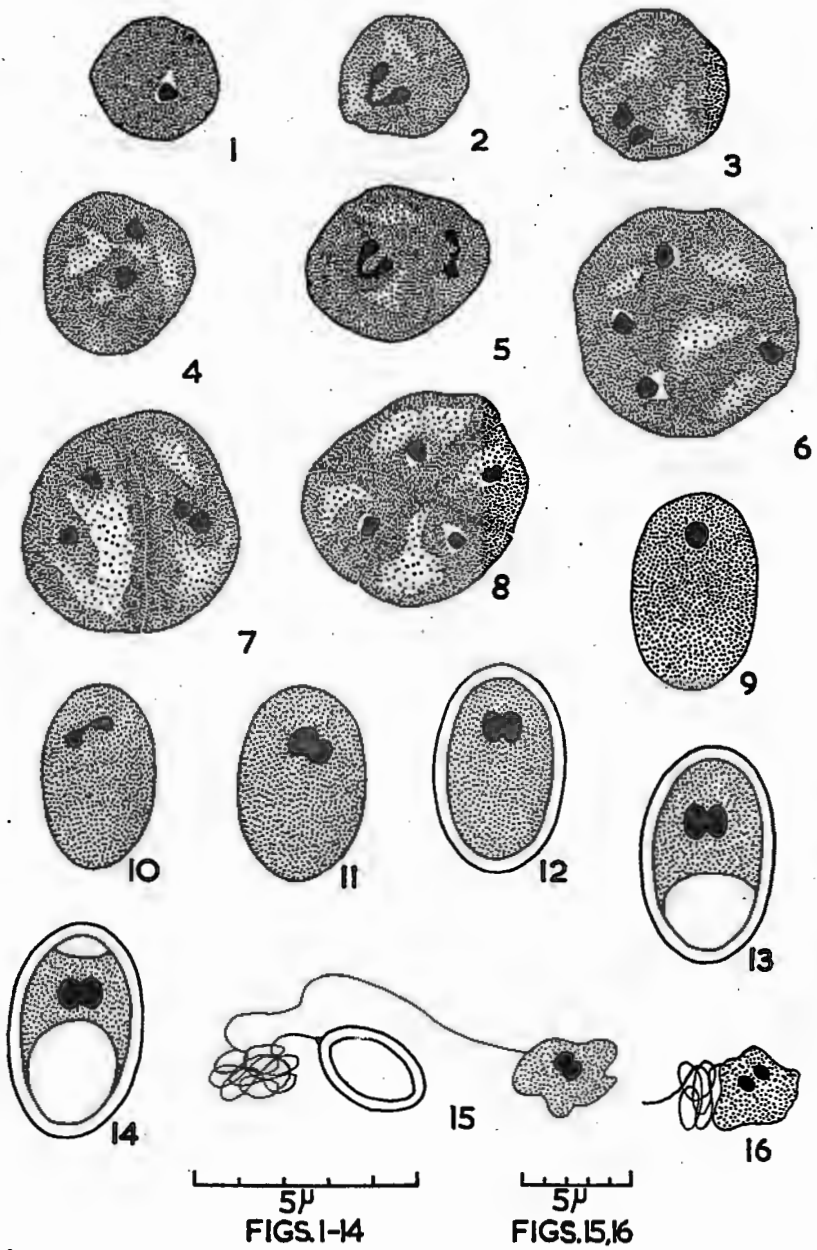

Figure 2. Life cycle of *Nosema locustae*. **1-8** mergony (=schizogony), **9-14** sporogony, **15, 16** hatched spores to show emerged sporoplasm (Canning, 1962 a).

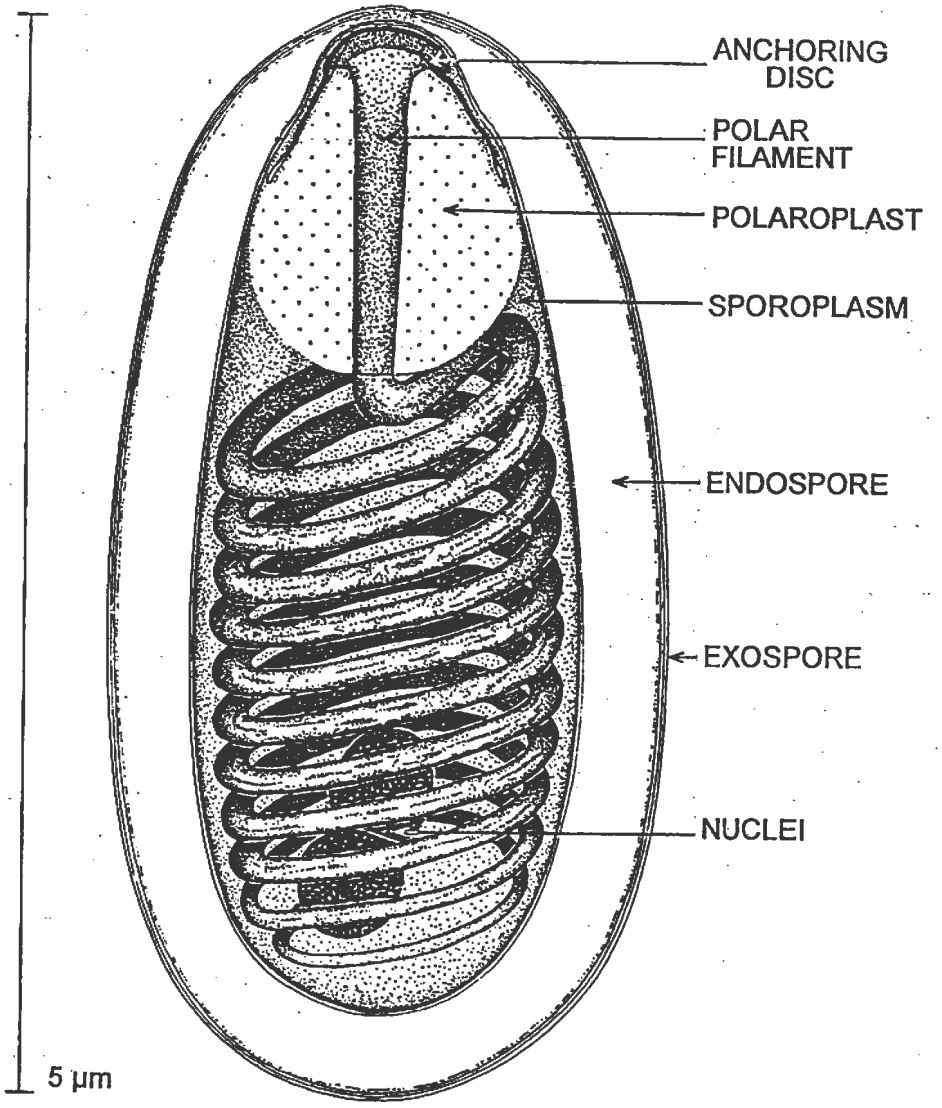

Figure 3. Scheme of the internal architecture of the spores of *Nosema locustae* (adapted from Huger, 1960).

VIRUSES

Insect viruses have been studied intensively since many years. Adams & Bonami (1991) (eds.) presented a monograph on the virus families infecting invertebrates as summarized in Figures 4 and 5. From locusts, viruses from three families were isolated (Table 1). Moreover, our iridovirus found in crickets is very infective against the most important locusts *Locusta migratoria* (Kleespies *et al.*, 1998; Kleespies *et al.*, 1999) and *Schistocerca gregaria.* Therefore, it is presented in this course.

Cricket Iridovirus **(CrIV)** (Kleespies *et al.*, 1999)

Data for diagnosis:

Transmission:	Per os.
Infection site:	Primarily fat body. At later stages of disease: hypodermis, tracheal matrix, muscularis of the gut, envelope of the gonads, hemocytes.
Virus reproduction:	Takes place in the cytoplasm of infected cells.
Morphology:	Icosahedral shaped virus particles with a size range between 151 nm (side-side) to 167 nm (apex-apex).
Signs and Symptoms:	14 days post exposure to CrIV locust nymphs are showing swollen abdomens and a striking sluggishnes; lifespan is much reduced.
Diagnosis:	On dissection of CrIV-infected locusts, a striking bluish iridescence of the affected hypertrophied fat body cells is obvious. Light microscopy: Investigation of native preparations of fat body cells (squash preparations) in phase contrast at a magnification of 400 x; CrIV is observed as crowds of very tiny particles dancing in vigorous Brownian movement. Definite proof on the nature of such tiny particles has to be obtained by electron microscope studies, e.g. negatively stained preparations and studies of ultrathin sections.

BACTERIA

From all known insect pathogens, spore-forming bacteria from the *Bacillus thuringiensis* (B.t.) group play the most important role in biological control.

Many attempts have been made to isolate locust specific B.t.-strains, e.g. Zelazny *et al.* (1990, 1991, 1997), yet without success. *Coccobacillus acridiorum* d'Herelle (1910) was isolated from *Schistocerca pallens* in Mexico and reclassified by Steinhaus (1949) as *Aerobacter aerogenes.* It causes mortalities on *M. bivittatus, M. bilituratus* (Bucher, 1959 a), and *S. gregaria* (Stevenson, 1954). However, this bacterial species is closely related to *Enterobacter aerogenes* known to cause suppurations of mammals. Thus, from hygienic reasons, it cannot be considered as a candidate in biological control.

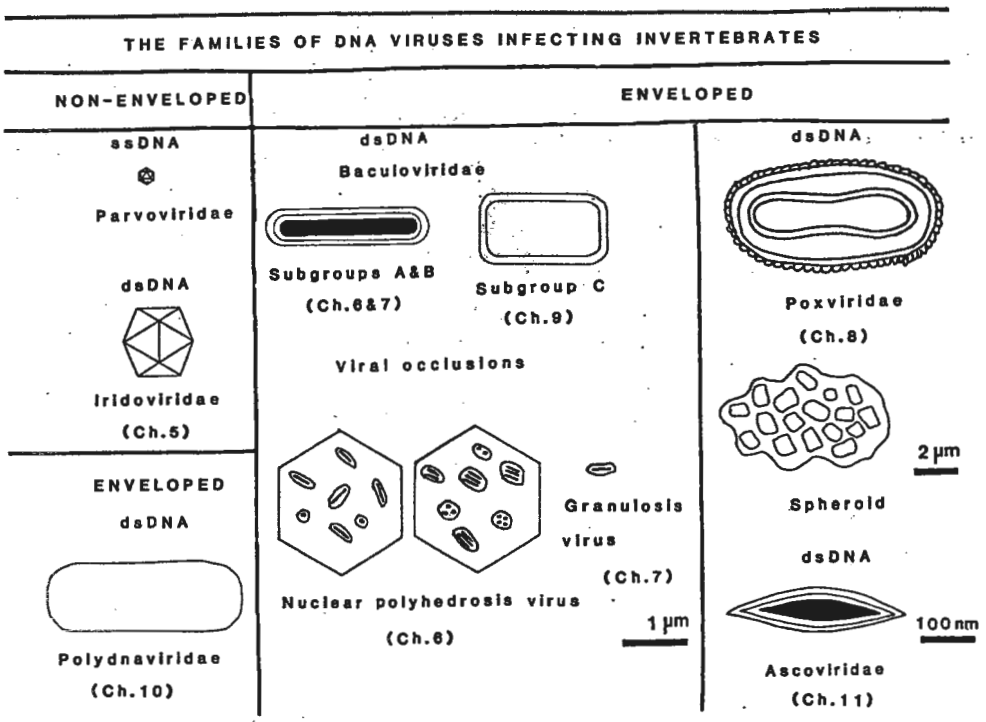

Figure 4. The families of DNA viruses infecting invertebrates. The viral structures are drawn to the following scale: (1) virions, bar = 100 nm (shown by the Ascoviridae); (2) the viral occlusions – nuclear polyhedrosis virus (NPV) and granulosis virus (GV), and the spheroid are shown at the scales indicated. Spheroids may vary greatly in size. The spheroid shown is of comparable dimensions to the virus reported from *Choristoneura conflicta*. The nonoccluded baculovirus shown is of comparable dimensions to the virus reported from *Oryctes rhinoceros*. The tail-like appendage (not shown) measures 10 x 270 nm. The polydnavirus shown is of comparable dimensions to those found in Ichneumonidae (from Adams & Bonami, 1991).

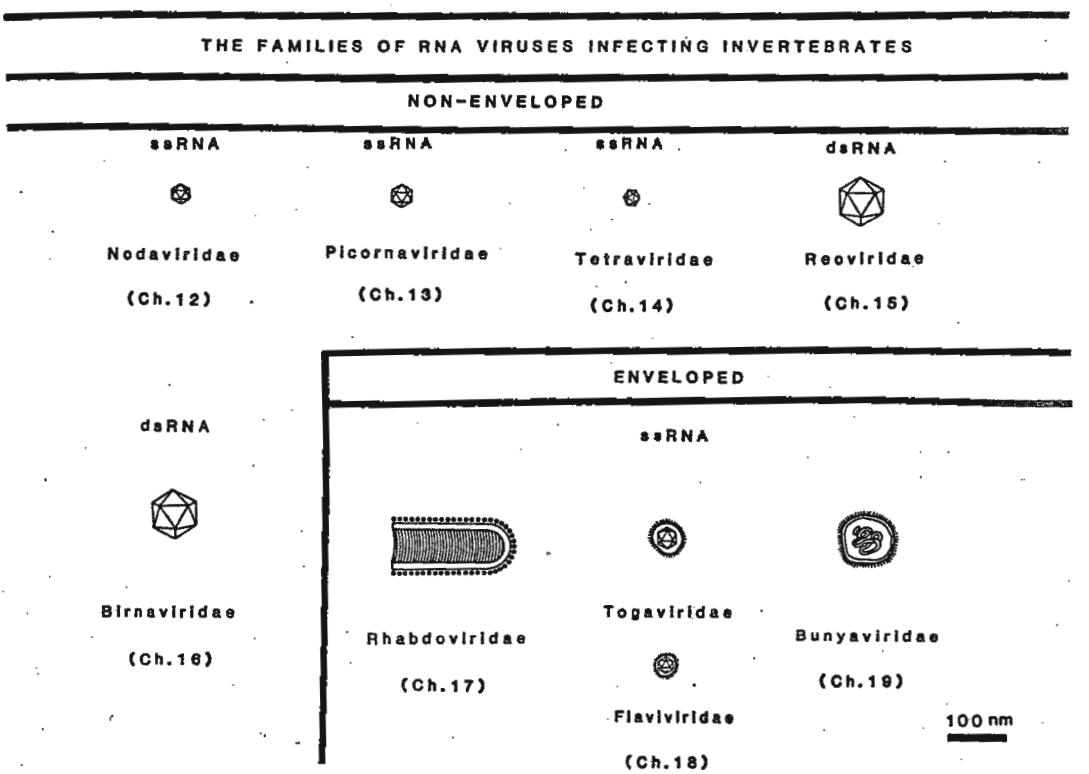

Figure 5. The families of RNA viruses infecting invertebrates. The Reoviridae contains a virus occlusion, cytoplasmic polyhedrosis (CPV), which is not shown due to the space limitations. CPVs may range from 1 to 15 µm in diameter. The virions are drawn to scale, bar = 100 nm (from Adams & Bonami, 1991).

Serratia marcescens is common, and for insects it is usually facultatively pathogenic. The colonies are generally characteristically pink or red from a water insoluble pigment, prodigiosin, but some strains produce white colonies. It is a facultatively anaerobic, gram negative bacterium (Figure 6) building straight rods. For a definitive determination detailed morphological and biochemical characterizations are essential.

S. marcescens was found in *S. gregaria* (Stevenson, 1959) were it resulted in an epizootic. The bacterial infection was transmitted by cannibalism. As *S. marcescens* can also be pathogenic for human beings (Burges, 1981), a practical use could only be considered if a locust specific *Serratia* strain or a new, absolutely safe *Serratia* species could be found. An overview about bacteria of locusts was presented by Bucher & Stephens (1959 a, b), Hunt & Charnley (1981), Bucher (1959 b) and by Sezginman (1973).
Petri dishes with colonies of *S. marcescens* on artificial medium are available for microscope studies in this course.

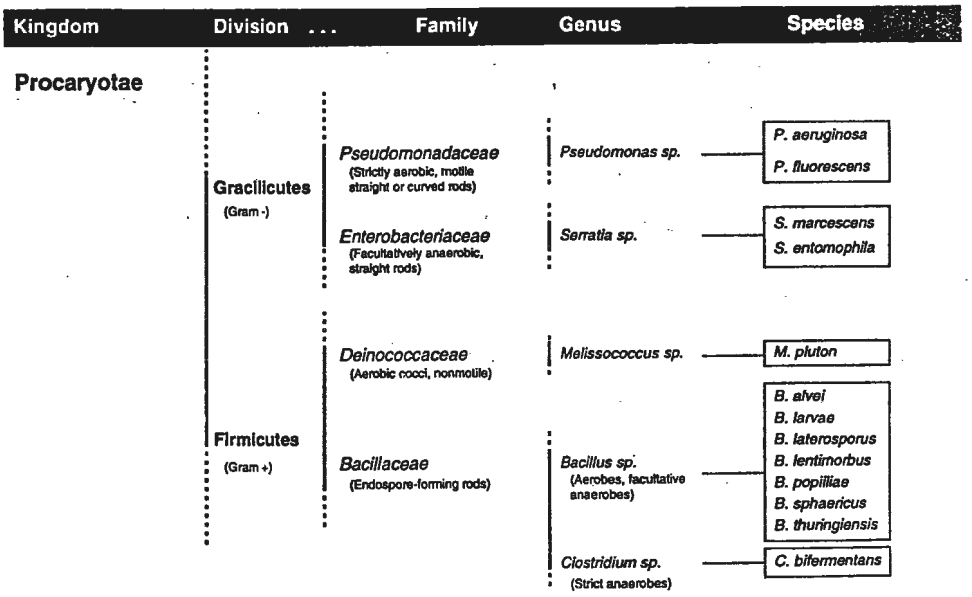

Figure 6. Classification of the most well-known entomopathogenic bacteria. After Krieg (1981), Sneath *et al.* (1986) and Wistreich & Lechtman (1988) (from Lacey, 1997).

RICKETTSIAE

Rickettsiae are microorganisms close to bacteria, gram negative, have a size range of 200-400 nm and reproduce intracellularly. Rickettsiae of locusts were assigned to the genus *Rickettsiella.* VAGO & MARTOJA (1963) isolated *Rickettsiella grylli* from crickets, which was also pathogenic for *S. gregaria* and *L. migratoria.* HENRY (1986) also isolated this species from *Zonocerus variegatus* L.

As *Rickettsiella grylli* is not available, *Rickettsiella blattae* in *Blatta orientalis* is offered for preparation and diagnosis. The diagnostic features are similar to that of *R. grylli.*

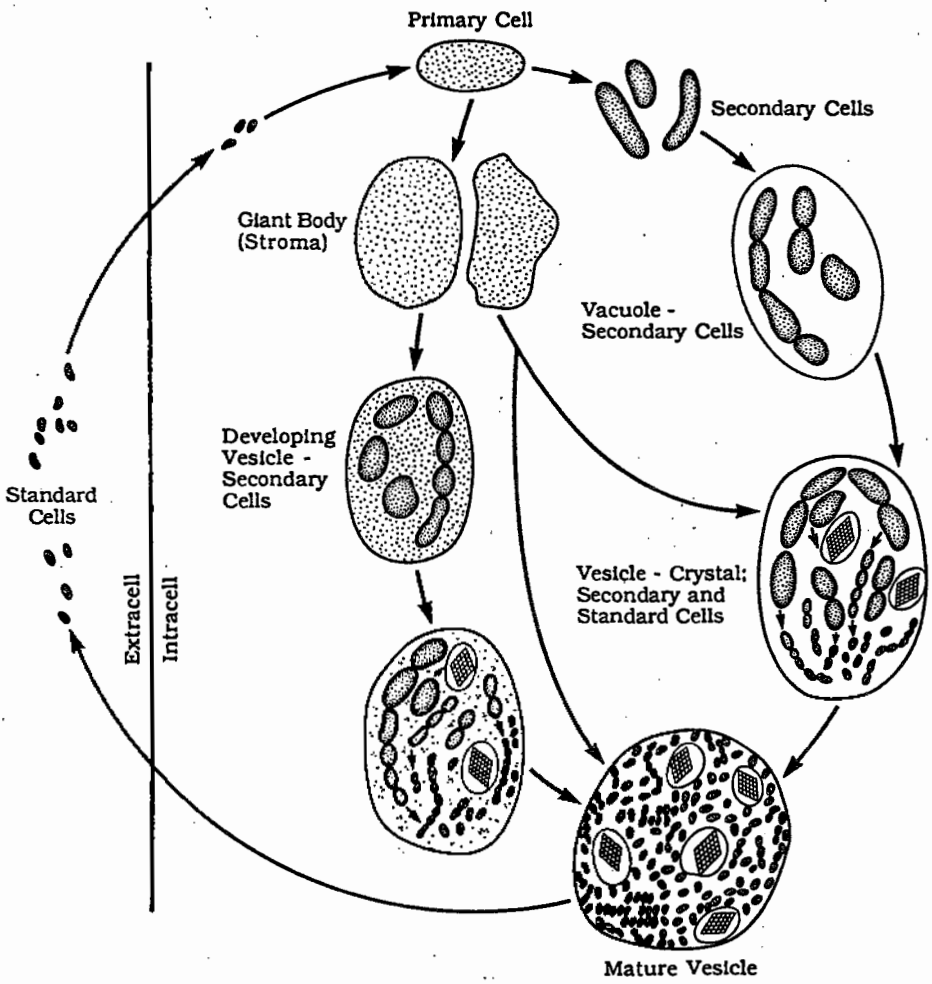

Figure 7. Life cycle of an insect pathogenic rickettsia as proposed by Huger & Krieg (1967) (from Tanada & Kaya, 1993).

Data for diagnosis:

Transmission: Per os.

Infection site: Fat body, in a late state of infection also in the hemocoel, hemocytes, oenocytes, and salivary glands.

Reproduction: After peroral ingestion, rickettsiae multiply in the cytoplasm of fat body cells by binary fission. There, often numerous small vacuoles filled with rickettsiae can be observed in squash preparations.

Morphology: Oval to longitudinal-oval particles with a size of 200-400 nm.

Signs and Symptoms:

25-28 days after infection the host is becoming lethargic and the abdomen is swollen.

Diagnosis: Light microscopy:

Investigation of native preparations of fat body cells (squash preparations) in phase contrast at a magnification of 400x; Rickettsiae are observed as tiny black particles in Brownian movement. Moreover, little bipyramidal crystals being typical for *Rickettsiella* species can be observed. Dark field microscopy is also a useful tool for this kind of investigations.

Acknowledgement

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