IOBC/WPRS

Working Group

"Insect Pathogens and Insect Parasitic Nematodes"

OILB/SROP

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

4th EUROPEAN MEETING "MICROBIAL CONTROL OF PESTS"

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Introduction

This Bulletin contains the proceedings of the fourth general meeting of the IOBC/WPRS Working Group "Insect Pathogens and Insect Parasitic Nematodes". The meeting, organised by and in honour of Professor Georg Benz, was held from 5-9 September 1993 in Zürich, Switzerland and was attended by 117 persons. The meeting was directly followed by a 1-day workshop on Entomophthorales organised by Siegfried Keller. At the meeting 78 papers or posters were presented on insect pathogenic bacteria, viruses, fungi, nematodes and protozoa. Special themes at the meeting were:

a) insect pests difficult to control with microbials

b) interaction between pathogens and host defense mechanisms

The meeting was held in honour of Georg Benz who retired in September 1993, just after the meeting, from his position of Professor of Entomology at the ETH in Zürich. For 33 years, of which 25 years as Professor, he worked at the ETH in the fields of entomology en insect pathology. In that fruitful period many papers and dissertations were published. He also trained and educated many nowadays renowned researchers in this field of research. We can certainly call him one of the "fathers" or "grand old men" of Insect Pathology in Europe.

On behalf of the Working Group I would like to thank Georg Benz and his staff for the excellent manner in which they organised the meeting at the very suitable facilities of the ETH in Zürich.

The next general meeting of the Working Group will be held in Poznan, Poland, in 1995. It will be a joint meeting with the Working Group on insect pathogens of the Eastern Palaearctic Regional Section (IOBC/EPRS). I hope to see many of you there again.

Peter Smits
Convener

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INSECT PATHOLOGY FROM 1960 TO 1993 AT THE ETH-INSTITUTE OF ENTOMOLOGY. SOME REMINISCENCES AND UNPUBLISHED DATA

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Introduction

Dear Colleagues, I am glad the working group gave me the opportunity to organize this 4th meeting at the Swiss Federal Institute of Technology, Zurich (ETH, as we call it) and am very much pleased that so many of you gathered here. Scales change! I remember quite well my first participation at an international meeting in 1962. It was the "Colloque International sur la Pathologie des Insectes" in Paris and I had the impression of participating at a very large gathering. However, the number of participants then was almost exactly the same as in our working group here. On the other hand, it is a melancholy pleasure to speak to you on insect pathology at the Entomological Institute of the ETH, because I am retiring at the end of this month, and henceforth the Entomological Institute will not exist any longer. This meeting is our "swan-song".

As I am addressing you as an insect pathologist, I should perhaps first make a statement concerning my position in insect pathology. In the course of 25 years as a Professor at the ETH, I supervised 43 Doctoral Theses. However, only 18 of them (i.e. some 40%) deal with insect pathological themes. The same is true for the papers I published. Since one of my former teachers was Professor V.B. WIGGLESWORTH of Cambridge (England) and since I had published on physiology and pathology of insects, I received my *venia legendi* at the ETH for both areas and worked with my students and co-workers in both areas, often combining the two and including ecology. As a matter of fact, I already got a lecturership for insect physiology in 1963, but was only entitled to offer courses in insect pathology after I had the *venia legendi* in 1967. However, I do hope you will forgive me for not having been a pure-bred pathologist.

The inheritage

When I wrote the title "Insect pathology from 1960 to 1993 at the ETH Institute of Entomology" I thought of the time I myself was engaged in pathological research. I came to Zurich in July 1960, after studies with Professor E.A. STEINHAUS, MAURO MARTIGNONI and Y. TANADA at the University of Berkeley, California, and work with Dr. T. BIRD at the Insect Pathology Research Institute in Sault Ste. Marie, Ontario (where I also met Drs. G. BERGOLD, T. ANGUS and A. HEIMPEL). However, I was not the first insect pathologist at the ETH. HANS WILLE made a thesis on the milky disease of the European cockchafer (a *Bacillus* that later on also was the subject of the thesis of Professor PETER LÜTHY), and MAURO MARTIGNONI after describing a rickettsial disease

of the cockchafer and two viral dieases of forest insects, published his doctoral thesis on the granulosis of the grey larch budmoth, *Zeiraphera diniana* (GN.), in 1957. At the height of the budmoth gradation 1953-55 which he was investigating he found that the granulosis killed locally up to 87% (on the average 40%) of the larch budmoth populations (results basing on 9 and 6 samples respectively, each comprising 15 larvae only). And because 60 years earlier the Swiss Federal Inspector of Forests, COAZ, had described the epizootic breakdown of a larch budmoth outbreak in the Engadine, MARTIGNONI thought he had found the key to the cyclic gradations of the larch budmoth in the subalpine larch forests - an "immortal" error (see below) that unfortunately was revived by ANDERSON & MAY (1980).

By the way, another mistake of MARTIGNONI (1957) is also "perpetuated" in the literature. He thought he had found a 40fold increase in GV resistance of the budmoth larvae from 1954 to 1955. However, as HUBER (1973) in his unpublished thesis pointed out, the 40fold increase in the LD_{50} values was not significant, because the dose-mortality-curve of 1954 based on a much biased probit line determined by 3 points only, all of them situated above 90% mortality. Since similar doses gave mortality values above 90% in the following year as well, increased resistance is not proved.

Professor PAUL BOVEY - head of the Entomological Institute at that time and engaged in a large research project on the cyclic gradations of the grey larch budmoth in the Upper Engadine - wanted MARTIGNONI to continue research in the budmoth team and develop the microbial control of the budmoth. But MARTIGNONI preferred working with Professor STEINHAUS at Berkeley. Therefore, I was asked to replace him and tackle the problem of the larch bud-moth gradations, an insect pest difficult to control with biopesticides. That was my start with insect pathology at the Entomological Institute of the ETH. I do not want to bore you with too many details of our doings during the last 33 years; I shall concentrate on a few interesting points and results that for some reason or another have never been published.

Development and loss of resistance to a granulosis virus

Bevore dealing with the grey larch budmoth I want to speak on a strange intermezzo with the GV of *Pieris brassicae*. Some Russian papers reported synergistic effects of sublethal doses of DDT on insect viruses and other pathogens. Therefore, in 1961 I gave my first PhD student, ULI SCHNYDER, the task of investigating whether or not the GV of the larch budmoth could be synergized by sublethal doses of insecticides. However, as the larch budmoth is monovoltine, it was impossible to acquire larvae for experiments throughout the year and to get significant budmoth results. On the other hand, I had a healthy laboratory stock of *P. brassicae* that could be reared the year round. Consequently I told SCHNYDER to change and start experiments with third instar *Pieris* larvae, giving him a suspension of a *Pieris*. GV which I had recently isolated from wild larvae in our garden and which I had found to be active in a test with wild *Pieris.* A few days later SCHNYDER reported that the larvae would not die of granulosis. I suggested that the larvae he had chosen were perhaps too old. SCHNYDER then started another experiment with younger larvae, but failed again. So I decided to make an experiment of my own. The result was the same, no granulosis, even when the cabbage leaves were sprayed white with GV. Evidently, my lab stock of *Pieris* was 100% resistant to granulosis.

I received my *Pieris* lab strain from Dr. J. WEISER of Prague for experiments with *Bacillus thuringiensis* in 1960. Dr. WEISER told me that he had the strain from Dr. DAVID of Cambridge, where it was selected from the survivors of a GV epizootic (DAVID and GARDINER, 1960), and that in Prague the strain had recovered from a second epizootic of granulosis. Apparently, only fully resistant *Pieris* larvae had survived the second epizootic.

In 1963, I gave the strain to Dr. D. MARTOURET of La Minière for experiments with *Bacillus thuringiensis*. Three years later, a Canadian postdoc, Dr. J.-M. PERRON of Quebec, wanted to do research in my lab. I suggested that he investigate the problem of viral resistance in *Pieris*. We therefore collected a wild strain of *Pieris* in the field and asked Dr. MARTOURET to send us the resistant strain I had given him. Surprisingly, the formerly resistant strain was now just as susceptible to the GV as the new wild strain, i.e. within three years total resistance was lost. On inquiry in La Minière genetic contamination with any other *Pieris* strains was firmly excluded. The riddle is still unsolved.

It may be noteworthy that the problem was taken up in the opposite sense in 1968, when I asked my assistant JÜRG HUBER to prepare a thesis on "Selection of resistance in a laboratory strain of the codling moth, *Laspeyresia pomonella* (L.), against peroral infection with a granulosis virus". Selection over 7 generations did not result in increased median resistance of *C. pomonella* to its GV, but a flatter dose-mortality-curve was found, indicating increased heterogeneity of the selected population. HUBER (1973) concluded that one part of the insects were more resistant whereas another part harbored the virus in an inapparent form and, therefore, were more sensitive to infection.

Epizootiology of budmoth GV or: mathematical models must be verified

As the gradation in the Western Alps precedes the gradation in the Engadine by 1-2 years, I had the chance to observe the budmoth population dynamics in the Italian Piedmont and the French Briançonnais already in 1961-62. There the gradation had locally transgressed its climax as early as 1962. But no GV was found[1], though it existed in the Engadine where the budmoth populations were much smaller (Tab. 1). This led me to understand that in the Western Alps neither granulosis nor parasitoid incidence was important enough to account for the break-down in the budmoth populations. Instead of disease I found budmoth induced resistance of the larch trees[2] to be the decisive factor of budmoth population dynamics in the Western Alps, as described in my report to the budmoth team in February 1963. I was astonished all

[1]The budmoth GV existed, though. I found it in 1963 and later on at low incidence all over the Alpine Arc: in the Briançonnais, the Piedmont, the Valle d'Aosta and the Swiss Valais in the west, as well as in the Austrian Tyrol and Styria in the east. Never ever did GV incidence reach epizootic intensity.

[2] In an attempt to find out whether or not latent granulosis of budmoth larvae might be activated by food stress, i.e. by food from larch trees which were defoliated in the previous year, I artificially stripped the needles from the lower two thirds of a 4 m high young larch tree in Sils in 1961 and compared the flushing of the nedles in spring 1962. In the stripped part flushing was delayed, the needles did not grow to normal length nor were they readily consumed by the larvae. This resulted in retarded development. I found the same conditions in the Piedmont on larch trees defoliated by budmoth larvae in 1961.

the same when in 1963 granulosis incidence in the Upper Engadine reached an average of not more than 0.3% (258 samples, each 1.5 kg of small branches from 258 larch trees with contingency distribution in the 2000 ha of forests of the Upper Engadine, yielding a total of 65,849 larvae that were reared and diagnosed)(Tab. 1). Moreover, diseased larvae were not found on all the trees but merely on 20.5% of them. However, still believing in MARTIGNONI's theory that the GV was an important budmoth mortality factor in the Engadine, I expected an epizootic for 1964. Therefore, another 32,891 larvae collected in 1964 from 200 trees were examined, but only poor 2.5% GV infected larvae were found on 46% of the trees (Tab. 1). Fig. 1 shows that the disease was not evenly distributed in the larch forests of the Upper Engadine.

Tab. 1: Population census and GV incidence in the larch forests of the Upper Engadine in 1963 and 1964 (large scale random sampling by tree climbers fulfilling contingency conditions). In 1962 larvae were collected within reach of persons standing under trees (no contingency conditions). N_T = number of trees and of samples (1.5 kg larch twigs per tree), N_{BL} = number of budmoth larvae examined, Density = number of larvae per 1.5 kg of larch twigs, N_V = number (and percentage) of GV infected larvae and pupae, T_V = number (and percentage) of trees with GV infected insects.

Year	N_T	N_{BL}	Density	N_V	T_V
1962[a]	-	2,006	53.4[b]	246 (12.2%)	-
1963	258	65,849	255.2	213 (0.3%)	53 (20.5%)
1964	200	32,891	164.4	822 (2.5%)	93 (46.5%)

[a] Not comparable to 1963 and 1964. [b] Value of general census.

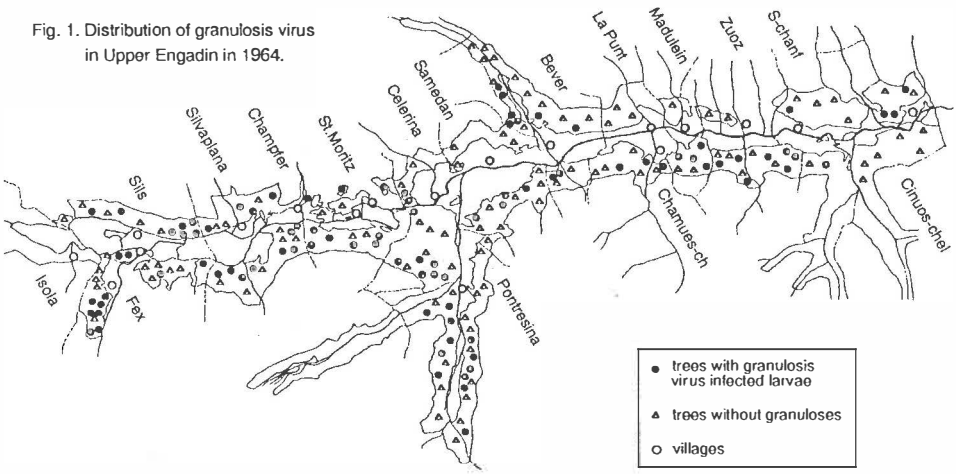

Fig. 1. Distribution of granulosis virus in Upper Engadin in 1964.

In 1965 the budmoth populations broke down, although granulosis incidence dropped to less than 1%. It follows that during the gradation 1962-65 in the Engadine, there was no true granulosis epizootic either and the reactions of the repeatedly infested larch trees were the same as in the Western Alps. The idea of insect induced resistance of plants was then so very sensational and new that I refrained from publishing my findings till 10 years later - during the next gradation - I verified that the insect induced tree reaction had developed again (BENZ, 1974). In this gradation (1972-74) my assistants A. SCHMID and X. OMLIN found even less granulosis incidence (0-1%).

As mentioned before, epizootic disease causing high mortality was reported in the Engadine only for the gradations of 1887-88 by COAZ, and 1954-55 by MARTIGNONI (35% and 48% mortality). But neither COAZ nor THOMANN mentioned disease for the gradations of 1879 and 1927-29, respectively, suggesting that in former times, too, disease was not necessarily the cause of the break-down in the larch budmoth populations in the Engadine. When comparing today the granulosis incidence of 12,2% (Tab. 1)[3] in the small scale samples collected in 1962 on the one hand, with the granulosis incidence of 0.3% in 1963 from large scale contingency collections on the other hand, I conclude that only the latter method gives epizootiologically reliable numbers for a whole region. Since my 1962 small scale values were obviously too high, I rather suspect that Martignoni's values for 1954-55 from small scale samples were also too high. All evidence I gathered after 1962 showed that in the Engadine, too, granulosis was not the driving force of the regular budmoth gradation cycles. You can imagine how very much astonished I was when, at the 3rd International Colloquium on Invertebrate Pathology in Brighton in 1982, R.M. ANDERSON presented a mathematical model of insect-virus population interactions which, basing on our data of the larch budmoth, showed that the budmoth cycles were driven by granulosis. I was even more astonished when I, having dared to critizise the unrealistic model, was told by ANDERSON that I obviously did not understand the population dynamics of the larch budmoth. I countered that my ignorance must have to do with the fact that I had done practical work with the insect in question and studied its diseases for many years whereas he had not done so at all.

The Entomopoxvirus or, Dr. Else Jahn was not completely wrong, after all

Speaking on population dynamics of the larch budmoth and the influence of viral disease, I remember an incidence that showed me that one should never stick too much to an idea. There was a dispute between MARTIGNONI of Zurich and Dr. ELSE JAHN of Vienna. MARTIGNONI said that the GV was the regulating factor of larch budmoth population dynamics, whereas JAHN maintained that - at least in Austria - it was a polyhedrosis. When I was in Berkeley in 1959-60, MARTIGNONI showed me some slides of the fatbody of larch budmoth larvae that were sent to him by Dr. JAHN. He was quite amused at the idea that this should be a polyhedrosis and wrote in that sense to Dr. JAHN. When in 1963 I found the GV also in Austria, I was equally convinced that JAHN was wrong and MARTIGNONI right. However, meanwhile VAGO and HURPIN (1963) had published on a new virus of cockchafer white grubs - the first *Entomopoxvirus*. In

[3] GV incidence in samples of individual trees varied from 0 to 67%, giving the empression of epizootic disease locally. However, no granulosis was found in much larger contingency samples of Dr. W. BALTENSWEILER that were collected in the Engadine at the same time.

a routine check of dead larch budmoth larvae in 1964 I found such a "spindle disease" which obviously was related to VAGO's disease (though the spindles were much smaller than those described in *Melolontha*). Since entomopoxviruses produce spherical occlusion bodies resembling polyhedra, the disease also resembled the disease I had seen on Dr. ELSE JAHN's slides in Berkeley. It was quite by accident that I came across the disease in a squash preparation, because later on I found out that the spindles of the larch budmoth dissolve quickly when fresh tissues are squashed in water. The cadaver in which I found the spindles had been stored dry for almost a year, which rendered the spindles less soluble. Henceforth, we used alcohol instead of water for making squash preparations of fresh cadavers and so we found more spindle diseases. I realized that we had probably overlooked the disease before and that it might be more important than we thought. Therefore, Dr. JAHN was not completely wrong after all, although the incidence of the budmoth *Entomopoxvirus* in the Engadine as well as in the Tyrol was even lower than the GV incidence.

An insect difficult to control

In the years 1961-62 I made several small scaled trials for budmoth control with *Bacillus thuringiensis* in the Briançonnais and with GV in the Piedmont (the latter in cooperation with Professor GOIDANICH of the University of Torino) as well as in the Engadine. The virus trials gave unsatisfactory results. Beginning in 1962 we closely cooperated with the French group of Dr. GRISON in La Minière, who sent two technicians to the Engadine to help me to mass produce GV. In 1963 we carried out large scale field trials on 18 plots of 3 or 4 ha in the Engadine. A French, a German, and an American preparation of *B. thuringiensis* (*B.t.*) and GV were spread by helicopter. The results with GV were utterly disappointing. The highest dose of 1760 larval equivalents per ha infected not more than 33% of the budmoth larvae. In 1964, therefore, only *B.t.* was tested again. Whoever thinks our French friends would have refrained from further GV experiments is mistaken. The idea was that perhaps the virus would be more effective when applied earlier in a gradation cycle. Therefore in 1970 we treated again two plots of 8.5 ha each with a French GV preparation at a dose of 1000 LE/ha, the first plot when 50% of the larvae were in the 2nd instar, the second plot with mainly 3rd instar larvae. The immediate effect of the GV application was no better than in 1963, and no epizootic could be detected on the plots in 1971-72. Evidently, the grey larch budmoth cannot be controlled with its GV.

Agriculture is closer to the stomach than forestry

As an appointed Professor of Entomology my philosophy in insect pest control changed as I felt that forests should be manageable without pest control, whereas agriculture cannot do without. I directed my research efforts in forest entomology more towards insect induced resistance and ecophysiology and reserved microbiological control for agricultural pests. At the beginning experiments with GV, *B.t.*, especially the ß-exotoxin (now almost forgotten) and *Beauveria bassiana* had similar importance. However, when Dr. PETER LÜTHY after three years of insect pathological research in Sault Ste. Marie returned to the ETH Institute of Microbiology, where he wanted to concentrate research on *B.t.*, I left *B.t.* studies to him and concentrated on baculoviruses, especially the CpGV of the codling moth, *Cydia pomonella*. The PhD theses of SIEGFRIED KELLER, JÜRG HUBER, RUDI WAEGER, PIA KÜNG, MARIE-FRANÇOISE

MAIGNAN, JACOB BRASSEL, FRANCESCO CAMPONOVO, and ANNEMARIE BLUMER deal with this virus (and many more with the insect and its physiology), not mentioning diploma-theses and semester papers.

With the introduction of modern cultural methods in apple and pear production the summer fruit tortrix, *Adoxophyes orana*, became important in some regions of Switzerland while *C. pomonella* became of secondary importance. Therefore I began research on an AoNPV and a Japanese as well as a Swiss isolate of an AoGV. Whereas the NPV quickly killed the larvae of *A. orana* at any stage, dependent on the time of infection, the GV fascinated, because it produced abnormally large last instar larvae that never pupated, independent of the stage in which they were infected. M.-F. MAIGNAN, whom I had asked to have a look at the infected fatbody with the EM, found already in 1974 that the virus developed either in the cell nucleus with rupture of the nuclear envelope (as in other known GVs), or in the cytoplasm of a cell, the nucleus apparently remaining untouched by the virus. The PhD theses of CLAUDE FLÜCKIGER, MARTIN ANDERMATT, JACQUES DROLET, XING LI, JÜRGEN ADAMEK, and NICOLAS LUISIER as well as number of diploma theses and semester papers deal with *A. orana* and its viruses.

With *Cydia* and the CpGV as well as *Adoxophyes* and its viruses, especially the GV, basic as well as applied research was carried out. Our applied research was quite successful and most problems of the use of CpGV have been solved, including the elimination of bacterial contaminations of GV and NPV suspensions by sonification. Not fully solved are the problems concerning the AoGV, as N. LUISIER will inform you in the course of this meeting. Many, but not all of our findings have been published. However, I want to leave agriculture and the stomach for a while and refer to a few interesting results of basic research only.

On the granuloses of *C. pomonella* and *A. orana*

It seems to me noteworthy that HEIDI ALLENSPACH (now Professor of Biopharmacology at the ETH) was the first who (in 1970) observed that the enveloped nucleocapsids of the CpGV develop in the cell nuclei when the nuclear envelopes are still intact, whereas the formation of the occlusion bodies does not begin before the nuclear envelopes rupture. These and many more ultrastructural details of CpGV development in fatbody and epidermal cells have been confirmed and refined by M.-F. MAIGNAN.

Noteworthy are also the findings of PIA KÜNG (1974) who showed that the development of the CpGV can be suppressed with mitomycin C and actinomycin D when applied at the time of infection ± 1 hr, i.e. by suppressing DNA synthesis and DNA dependent RNA synthesis, respectively. The antibiotics lose their granulosis suppressing power successively when applied more than 1-2 hr after the infection. Obviously, DNA and RNA synthesis are required in the process of viral infection.

F. CAMPONOVO compared our wild lab strain of the CpGV with the $CpGV_S$ (selected for UV resistance by J. BRASSEL) and found a quantitative difference in one of 11 structural proteins. The 21 kDa protein was much reduced or almost absent in the $CpGV_S$. He also found that infection with an optimal dose of the CpGV leads to abnormally large larvae that produce more CpGV units.

ANNEMARIE BLUMER tried to cultivate the CpGV *in vitro*. She found that the haemolymph of last instar larvae fed with CpGV became infectious within 60-90 min as demonstrated by injecting joung L_5 with such haemolymph. Her *in vitro* studies showed that virogenesis in fatbody organ cultures was possible when the tissue was explanted 2 or more hr after infection of the donor with the GV, but not when explanted earlier. Juvenile hormone delayed virogenesis *in vitro*. Attempts to infect *in vitro* cultivated fatbody with infective haemolymph and/or infective fatbody extract failed with one exception. Even when fatbody from a healthy larva was cultivated in contact with an infected fatbody *in vitro*, no infection resulted. Since the media of such cultures proved infective when tested *in vivo*, it must be concluded that infection *in vitro* was not possible under our culture conditions, possibly because a vital factor was missing. This is astonishing since the media she used included that of NASER *et al.* (1984), the only one that ever supported growth of the CpGV in a cell line. Infection of embryonic primary cell cultures with infective haemolymph, too, only succeeded in about 1% of the experiments. Thus, CpGV infection *in vitro* is possible, but as yet with poor results. Nobody seems to have been able yet to repeat NASER's positive results.

The success of separating the nuclear and the cytoplasmic replication types of the AoGV in two isolates shows that we deal with different AoGVs, though very closely related as XING LI will demonstrate you in the course of this meeting. Unpublished research of my own concerning the suppression of the pupal molt of AoGV infected *A. orana* larvae led to the conclusion that this suppression is not the result of missing ecdysterone but is caused by too high levels of juvenile hormone.

All's well that ends well?

Dear Colleagues I conclude my lecture. Thirtythree years of research and 30 years of teaching in insect pathology and physiology come to an end. I would be happy if I could merrily say: All's well that ends well. As you know this is not so. The institute is gone and insect pathology at the ETH is at its end. Nevertheless there is light around me. The thought that I inspired a few of my former students so much for insect pathology that they made it their job and became recognized members of the scientific community consoles me, and the knowledge that one of my students conquered all the adversities of bureaucracy and founded a company for the production and promotion of biological control products gives me great satisfaction.

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INSECT PESTS DIFFICULT TO CONTROL WITH BIOPESTICIDES

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Summary

Difficult insects are in four main groups: Mining insects hidden in sterile plant tissues, sucking insects living all their life on sterile saps of plants and insects of the root system,with poor access of biopesticides from the surface.The fourth group are insects which are abandoned or overlooked in general effort of application of bioinsecticides. Solution in preparing efficient treatments is the improved use of contact biopesticides,especially of fungi, perfected use of nematodes, development of genemanipulated plants with entomotoxic groups in their tissues or plants with pathogens circulating in their saps. Improved applications must include new formulations of biopesticides and new ways of application.It is important to avoid formation of resistance and use combined treatments for postponing formation of resistance. It is very important to gain cooperation with the big industry of insecticides, and environment protecting agencies. The registration procedures must be adapted to biopesticides and relations of the basic research in insect pathology and the industry research must be closer and continuous.

1. Introduction

The IOBC was the first international organization in Europe dedicated to research of biological methods of control of important pests and the ETH Institute of Entomology was its very important partner from the beginning. Effort of the IOBC concentrated especially on valuable crops: glasshouse cultures, pests of orchards and ornamentals and on special agricultural cultures, in protection against quarantine pests introduced from other zoogeographic regions. It was evident very soon that there are some groups of pests where the effort only in using available materials is not rewarded with adequate results and that some groups are very difficult to attack. A general rule in nature, that every group of organisms must have some natural controlling factor which can be developed to an efficient pesticide, did not work precisely, as the existing limiting factors were difficult to produce in mass and apply in a critical situation. There are three groups of pests which are difficult to attack with biopesticides: 1. The mining insects ,2.Sucking insects and 3. Root attacking insects deep in the soil.

The mining insects are recruited from different groups, we may mention the bark beetles,seedstalc curculio,mining lar-

vae of Diptera,stem sawflies, or Lepidoptera as European corn borer, codling moth or potato tuberworm. Their relatively fixed position in plant tissues attaracts a specific range of antagonists: Hymenopteran parasites which lay eggs into larvae as they are under cover of the bark or surface of stalks and leaves. A second group are different Nematodes and mites which enter their galleries and live in a symbiosis and antagonism with the inhabitants. Other types of diseases should be excluded. But in analysing such populations we find also two other types of reducing factors: viruses which are transmited with eggs or are injected by attacking Hymenoptera and fungi of the group of Entomophthoraceae and Deuteromycetes which are brought in by different mechanisms in the final period of development of the pest. Among the infections transmited via the egg belong also the microsporidia.The late period of development of mining insects opens the sterile conditions of the galleries and often individual colonies are crossing over and share eventual pathogens.In mining insects such as bark beetles it is the mating come-together which is the best period of transfer of specific pathogens.

Sucking insects, aphids, leafhoppers, scale insects, thrips,are sitting on the surface of attacked plants, but they accept only the sterile sap from attacked plants and the usual peroral administration of pathogens, as it is the rule with bioinsecticides, does not bring the effect. The role of entomophagous insects remains the same, important is the role of predators, Reduced is the activity of nematodes. Typical is the increased role of fungi and their appearance in broader or local epizooties,usually only in damaged populations at the end of their development. It could be a transmission of diseases on the ovipositors of parasites,but this type of transmission was not observed and definitely is not any prominent way of distribution of a disease.

The third group, pests which are hidden in deep soil,includes representatives of very different categories: curculionids feeding on deep roots of plants such as the alfalfa weevil and snouth beetle,the pine weevil,but to some extent also the vine phylloxera or the spruce sawfly and the different grubs. They all are living in a contaminated environment,exposed to attacks of all groups of pathogens introduced by different mechanisms from infections via-the-egg to normal peroral infections. But their localization on roots of plants and only short period of migration on the surface of soil,makes any manipulation and treatment rather difficult. In soil they usually migrate into rather cold and sterile parts where contacts with pathogens are avoided and where it is inposssible to bring bioinsecticides in doses necessary to produce infections and mortality.

Besides these "difficult" insects there is a vide group of pests which were not tried. Outside of the evident group of Lepidoptera where Bac. thuringiensis is a general solution, there are other accessible pests of field crops among beetles, Hemiptera and Diptera where methods of treatment by biologicals were not yet tried and where we still have a chance.

2. Management of pathogens

Conditions in agriculture are changing with the requirements of the market and new types of seeds used for higher yields. This means that all conclusions made early in the time of first applications of biological means must be revised and repeatedly confirmed. Field crops are planted much denser than before some 30 years and conditions of moisture and contact typical for glasshouses are often provided in grain and alfalfa. It is not very good for biological control that the use of toxin producing sequences of Bacillus thuringiensis were tested in transfers into several crops includind tobaco, tomatoes or potatoes. There is no other group of topics where were prepared so many experimental transfers of active groups as it was recently inside the B.thuringiensis group and in eventual targets, the food plants exposed to attacks of Lepidoptera or Coleoptera. Studies of genetists / McGaughey and Whalon,1962/stressed the impact of transgenic plants protucing the B.thuringiensis delta endotoxin on development of resistance to this toxin. For us it would be better to get into this very modern but dangerous contact with new constructed plants resistant to attacks of pests much later, when consequences of release of reconstructed plants in nature will be known on other exemples.

Another very progressive idea is to colonize plants with entomopathogenic microorganisms and mediate in this way the infection in insects living in the sterile tissue of plants. There are anouncements that Agrigenetics tries with Mycogen Co. and ARS applications of Beauveria bassiana within hybrid field corn,colonization of circulating saps may contact the mining larva of the European corn borer with the fungus and initiate infections which provide season-long suppression of the borer. Again, this early experience does not say what will be the distribution of hyphal bodies in the plant. Observations on populations of the corn borer must be continued with observations of the effect of the fungus on aphids attacking corn and with replacements of Beauveria with Paecilomyces,Verticillium or Metarhizium.This method may eventually be useful also for control of aphids and other sucking insects given that the fungi will be able to infect the insect from the intestine.

The individual pathogens aplicable to different host or host groups had also some positive development during the last years.

Viruses are insignificant as reducing factors in sucking insects. This seems to be strange, compared with their impact in other insects and more effort may be dedicated to possible introduction of infections from other groups to initiate a via-the-egg transmission or transmissions with the ovipositor of hymenopteran parasites. In the practice man-induced, artificial links of disease and host lead to mortalities which are not recorded from nature. In other groups, especially in mining insects, viruses play some role , at least in Lepidoptera: for the corn borer and the codling moth they represent a chance which may be more valuable with development of more efficient culture of viruses in cell cultures.

Strange is the absence of virus infections in mining beetles. There is no analogy of the baculovirus of the rhinoceros beetle in the bark beetles.Nevertheless, Scolytus scolytus is listed among hosts with a poxvirus infection in the catalogue of Martignoni and Iwai 1981, but there is no further evidence of the effect of virus infections reported from curculionid, lucanid or tenebrionid beetles. Only studies of grubs of scarabaeid beetles brought larger evidence of possible interactions of virus with beetles. Virus infections of some kind are reported in the cited list from several aphids, some Delphacidae and Cicadellidae and the recorded cases should be reinvestigated for eventual cross transmission .If we consider the baculovirus of rhinoceros beetles as one of the perspective pathogen for control of beetles, further possibilities of use of analogous viruses should be investigated.

Bacteria are entering galleries only exceptionally, therefore is the use of B.thuringiensis tenebrionis for control of bark beetles without result /Wegensteiner,1992/ and so are applications of B.thuringiensis kurstaki against codling moth and the corn borer. It is expected that the amber disease of grass grubs in New Zealand caused by Serratia entomophila may find some use in control of some gallery forming insects and insects of the third group feeding on roots in soil, such as the alfalfa snouth beetle or the japaneese beetle. A complicated affair is any testing of bacteria for control of aphids.They are not susceptible to the B.thuringiensis in general and in tests of application we must remember the risk of development of bacteria in the honeydew remaining on leaves of host plants where it will be collected by honeybees.

The fungi are close to ideal for application against all three groups of difficult pests. They are also rather common in nature and some are very easy to maintain in culture and produce in mass. Absent in Europe is evidence of the Chytrid fungus Myiophagus ucrainicus in citrus scales and there is no evidence of its introduction from Florida where the infection in Lepidosaphes is common and rather efficient. This fungus is recorded from infected Bothynoderes and Anisoplia from the Ukraine by Wize /1904/. Studies of entomophthoracean fungi have shown during the years that they are very specific for the second, sap sucking group of "difficult" pests. During the years the isolation and cultivation on egg yolk slants /Müller-Kögler,1965/ was no more a problem and some species with broad host range were produced in submerse liquid cultures /Weiser, 1966/. The application of produced material in large applications did not bring good results. There are epizooties in nature caused by one species and attacking one host in an area of several thousend hectars, but initiation of such epizooties by seeding or large scale application of produced fungus stages was not reached.

More plastic and aplicable are the Deuteromycete fungi. In all three categories Beauveria bassiana, Paecilomyces farinosus, Verticillium lecanii, Metarhizium anisopliae and several other species appear as chronic infections and applications of conidia or blastospores cause infections of different intensity. Methods of production of blastospores in deepfermentation

batteries /Samšináková, 1961/ or methods of production of conidia on solid media /review see Samson et al.1988/ provided to everybody an opportunity to prepare large quantities of infectious germs of fungi which were common on pests of all three groups. They are "contact insecticides" and infect from the surface and in this sense are ideal for applications.In the group of mining insects, the problem always is to provide a contact with a critical level of conidia or use any secondary stress which may initiate infections also when contacts are lower than threshold. In this direction methods have changed: Pregermination of conidial or blastospore materials with use of water, peptone water or just citrus juice will shorten the period of incubation in aqueous sprays. In dustings, the addition of dry surfactants such as silicagel , remove fatty acids from the surface of insect bodies and the suppression of germination of conidia caused by lower fatty acids. A recent progress in applications of Beauveria and Metarhizium is the use of oily suspensions as presented by ARS laboratories of the USDA and some firms such as Mycotech. Large actions with oily Beauveria for control of migratory locusts and grasshoppers will bring necessary informations for further arrangement of control actions and evaluations of results. With this experience, we expect important changes in applications in the second group of "difficult pests", with analogous applications of Verticillium. In glasshouse applications it must be decided if,under application of active Verticillium, the use of Encarsia will be necessary. In the third group, the soil inhabiting pests, oily suspensions may play only a limited improvement: applications on adults when they appear on the surface for matting and egg laying. Oily suspensions applied in ULV apparatus may provide a much better adhesion of the conidia on the surface of target insects and the oil, mineral or organic, pure or with addition of kerosene may act as a stressor supporting the invasion by germinating hyphae. Experiences with commercial oily suspensions of Beauveria /Jaronski,1993/ show a very good shelf life of conidia and excellent survival at normal and rather high temperatures /90% viability after 2 years at 25°C, 4 weeks at 40°/ enabling the use of oily materials under conditions of subtropics and tropics. Tests of nonpathogenity of Deuteromycetes for vertebrates when in food and nontoxicity of plant oils on vegetables together give a good chance of application of oily Beauveria in glasshouse if Verticillium will not meet the same requirements.

Protozoa, especially Microspora,play as bioinsecticides only a secondary role,without a fast effect, with long lasting endemies. They are, in contrary, detrimental to mass rearings of parasites or predators. During the last period microsporidia complicate the mass rearings of Amblyseius when this predator is produced on Tyrophagus mites./Beerling et al.,1992/.

The progress in mass production, adjustation and distribution of entomophilic nematodes of the Steinernematidae and Heterorhabditidae groups is most favorable for their application for control of soil inhabiting pests of the third group. Results in galleries building insects and in sucking pests are not very encouraging, but this does not mean that further

research may not improve the situation. Actually the main task seems to be the improvement of migration and searching ability of strains and their selection for activity under high and low temperatures. A perspective for the "difficult" pests is the increasing study of Tetradonematid nematodes as specific agens for control of flies including these attacking cultures of mushrooms, where the use of special fungi was not efficient enough.

3. The menace of resistance

Resistance of pests to chemical insecticides is the night-/and day-/ mare of the producers and the users of insecticides. Microbial insecticides where the active substance is an insecticidal toxin produce under genetic pressure development of a resistance. This resistance is to one serotype delta endotoxin or,eventually, to different endotoxins active in the same way. A third category is the resistance to the beta exotoxin and its chemical analogues. Bacteria as same as fungi produce during their development toxic metabolites of peptidic or nucleotidic nature. In the first group is a cluster of delta endotoxins of B.thuringiensis and an analogous system in B. sphaericus. The three known exotoxins of B.thuringiensis are belonging to the nucleotides.Entomopathogenic fungi produce analogous peptidic metabolites and substances with fungistatic and bacteriostatic properties. They protect the fungus growing on his insect "prey" against attacks of competting microorganisms:bacteria,other fungi and also saprophagous insects. Therefore peptidic metabolites such as beauvericin, destruxins or tolypin /Roberts,1981, Weiser and Matha,1988/ are also bioinsecticides of different quality and orientation. For formation of resistance are most dangerous opportunities with steady contact of the insects with subtoxic doses of the toxin /genetic pressure/. In this sense the genetically engineered crops are most dangerous.

In this connection appears another priority: by alternation of biological and chemical methods of control of a series of pests in subsequent seasons it is possible to postpone the development of resistance to chemicals as same as to biologicals in a not yet well estimated manner. I am sure that producers of chemical insecticides should be the first who may propose to alternate chemicals with biologicals because this will save monay for development and testing of new preparations. I must openly denounce the commercial competition of producers of chemical and biological preparations as a stupid job, detrimental for both.

4. Toxins and metabolites

As mentioned, metabolites,of which some are entomotoxic, are a part of the product in fermentations of entomopathogenic bacteria and fungi. In products which were centrifuged, the water soluble substances were removed / so the beta exotoxin of B. thuringiensis/. Some metabolites, such as tolypin in Tolypocladium niveum, are produced only when the fungus grows on stationary substrate, not in moved fermentation. Another insecticide in the same fungus, cyclosporin, is an endotoxin and does not leave the fungal cells, in the same way as other peptidic endotoxins. These materials are formed or during sporulation in bacteria, or during conidiation in fungi, that is rather late in their development and they play only a minor role during the in-

vasion. Open is their eventual role as insecticidal substance in different applications including an assy of use against the "difficult" pests. As it is the habit in all such substances including producers of antibiotics, the production and type of metabolite is influenced by all details of the production, including nutrition and use of precursors to switch over the metabolism to a requested direction. In the group of B. thuringiensis the existing active metabolites are identified only as materials with insecticidal activity. With use of other test organisms there will be identified other important active materials for a very large range of applications. The first open door into this world is the molluscicidal analogue of beta exotoxin isolated from B.thuringiensis H-14 /Weiser et al.,1992/.

5. Overseen possibilities

In many instances applications go the mule's way, repeating again and again the negative effort. Every experiment,even the negative, may bring some information which has to be used the next time. Applications which were performed in the first years of use of bioinsecticides may have other results with new formulations on new seeds and new experiences in evaluation. Repeated low effect applications of fungi change in three years to positive because the living environment of the pests is filled with living germs ranging over the threshold in critical niches.New pests are treated with good result by new formulations and in every case it is necessary to bring adequate documentation so that nex treatment is routine, not experiment. If a highly positive treatment is achieved once,there must be conditions which when maintained enable repeated routine treatments. Minor mortality in treatment with biologicals is less important in a sandwiched plan of alternating use of chemicals and biologicals. In farming for natural food, avoiding chemicals, biopesticides may play an important role.

Besides typical "difficult" pests in agriculture there are many others which can be treated and remained unobserved till now.In the meantime before some important questions of use of entomophthoraceous fungi or specific bacteria and viruses for control of aphids,planthoppers or scales will be solved,yet untatched questions of pests should be included into research.

6. Diplomacy in biological control

Several aspects of pest control are political. To provide a clean production of foodstuffs, to protect and clean up the human environment from remains of chemicals /and masses of insects/, to protect resources of fresh water,natural reserves and immediate environments of human settlements. Here are the departments of agriculture, chemistry,environment protection meeting in conflicting decisions. Biological means,properly presented,may bring a compromise in many questions and research in microbial pest control must be supported from all three sides because it solves the posed questions and provides knowledge necessary for qualified decisions. But the political organizations do not know it and it is a necessary advertising needed. The same is in the question of peace between biologists and the insecticide industry. Biological control is less spectacular than public actions of Green peace on entrances of ato-

mic power stations,but is a positive ansver to all restriction programmes. Further development of research and practical application of microbial insecticides need a coordinated effort of all involved and I believe that the IOBS is for it the proper organization.

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Genetic improvement of *Steinernema feltiae* for integrated control of the Western Flower Thrips, *Frankliniella occidentalis*.

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Summary

Biological methods available for control of the WFT, *Frankliniella occidentalis* remain ineffective against the insects pupating in the soil. Examination of entomopathogenic nematodes infectivity to second nymphal, prepupal, and pupal stages of the pest and subsequent breeding study revealed that *Steinernema feltiae* could be a candidate for filling this gap. Over one-hundred newly developed strains of the nematode were evaluated in laboratory for their infectivity to the WFT in the soil. The parasite effectiveness measured as a proportion of infected insects in the exposed population varied greatly among nematode strains and ranged between 3.7 and 72.6 percent. Selection for high infectivity and possibly small body diameter conducted within partially inbred laboratory strains had only limited effect on improvement of the parasite performance. Contrary, in laboratory tests a number of newly selected recombinant inbred, and recombinant congenic strains showed a potential of effective biocontrol agents against the WFT in the soil.

Introduction

The Western Flower Thrips, *Frankliniella occidentalis* have recently become major pests of field-, and greenhouse-grown vegetable and ornamental crops worldwide (Yudin et al., 1986; Mantel & van de Vrie, 1988). Both, the direct injuries caused to plant organs and transmission of the Tomato Spotted Wilt Virus lead to substantial losses in horticultural industry. The insect can develop on a wide range of host plants with large populations produced within only a few generations. High resistance to chemical insecticides makes the WFT a very difficult pest to control.

Nymphs and adults of the pest feed on the host green organs and flowers. The nonfeeding prepupal and pupal stages occupying near 40 percent of the total developmental time (Lublinkhof & Foster, 1977) are found in cryptic habitats such as a soil, where during plant growing season they is virtually unaffected by any means of control.

The problem is particularly important in greenhouse cultures, such as a cucumber, tomato and red pepper, where successful programs of biological or integrated control of other pests are already implemented and selective chemical pesticides cannot provide adequate control of the WFT. A number of beneficial organisms including predatory mites and bugs and parasitic fungi have been already tested against the WFT feeding on plant foliage. Obtained results suggest, however, that no individual bioagent can provide economically satisfactory control of the pest. Therefore, integration of control measures, and particularly, supplementing existing programs with bioagents effective against the pest in the soil can substantially aid in solving this problem.

Preliminary study on infectivity of steinernematid and heterorhabditid nematodes to *F. occidentalis* revealed that insects pupating in the soil had been occasionally parasitized by juveniles of *Steinernema feltiae*. The provided pest control level could not, however, satisfy horticultural practice until being significantly improved. Among available methods up to the present only genetic selection showed some promise to entomopathogenic nematodes improvement. However, due to extremely small sizes of the target insect, methods developed earlier for *S. carpocapsae* (Gaugler et al., 1989) and *S. feltiae* (Tomalak, submitted) could not be used here.

The reported research is a part of a complex project aimed at identification of factors affecting *S. feltiae* infectivity to the WFT, and at developing a method of the nematode selective breeding that would allow to improve its biocontrol potential against soil inhabiting stages of the pest.

Materials and Methods

Up to the present over 100 new strains of *S. feltiae* were developed in three selection groups and their infectivity was examined against the WFT in the soil. .

1. Selected Inbred strains (SI) were developed through systematic selection of newly isolated populations and partially inbred laboratory SN and ScP strins for possibly small body diameter and infectivity to the WFT. Each selection round was followed by inbreeding of the most successful individuals through brother-sister mating.

Promoting only the most effective individuals to the next generation should accumulate advantageous alleles and increase frequency of desired phenotypes in the population. This method of breeding was derived from our earlier program of *S. feltiae* selection against sciarid flies.

2. Selected Recombinant Inbred strains (RI) were obtained through crossbreeding of preselected ScP and SN strains of the nematode and subsequent selection of recombinant juveniles for potentially advantageous morphology and infectivity to the WFT. That was followed by inbreeding of isolated lines through brother-sister mating.

Intraspecific hybridization of two differing strains increases available variability in the population. That should be phenotypically expressed by new recombinant forms. During the subsequent selection and inbreeding of the most successful individuals genes from parental strains should segregate, reassort and gradually be fixed in various combinations in the new strains. This and the next method of breeding were adapted from research on genetic analysis of polygenic traits in laboratory mice (Justice et al., 1992).

3. Selected Recombinant Congenic Inbred strains (CI) were constructed by crossbreeding of preselected, partially inbred ScP (background) strain and one of donor strains bearing individual, potentially advantageous traits. That was followed by selection of the resulted hybrids for their infectivity to the WFT and the characteristic traits of the donor strains. Repeated 3 rounds of backcrossing of the most effective recombinant individuals with the ScP background strain and similar selection were followed by inbreeding within isolated lines.

Crossbreeding of the ScP strain with other strains bearing individual, potentially advantageous characteristics such as a small body diameter, high movement activity etc. should increase variability in the resulted populations. Repeated backcrosses of the recombinant, most effective individuals to the background ScP strain and selection followed by inbreeding through brother-sister mating should result in construction of a series of strains, each carrying only a small, particularly advantageous fraction of the donor genome on mostly ScP strain genetic background.

Laboratory tests evaluating effectiveness of nematode strains against the WFT were conducted in 3:2 peat-moss and bark compost mixture at 20^{0}C. For direct examination of infectivity 25 late second nymphs or prepupae were exposed to soil inoculated with nematode juveniles in 5 cm Petri dishes. The nematode dose was 100 IJ per cm^2 of the soil surface and remained constant throughout all experiments. After 3 day exposure the insects were recovered from the soil and examined for the nematode infection.

The effect of nematode infection on emergence of adult WFT from the soil was evaluated in small, 7.5 cm in diameter and 5 cm high plastic containers. Fifty or 100 late second nymphs of the pest were transferred to the surface of soil inoculated with nematodes. After pupation emerging adults were trapped on sticky, transparent cards fixed over the soil surface and results obtained in all treatment variants compared with untreated control.

All experiments were performed in 6 replicates.

Results and Discussion

Preliminary laboratory tests conducted in a peat-moss and bark compost mixture revealed variable infectivity to the WFT among the examined *S. feltiae* strains and isolates. After 3 day exposure to the parasite in the soil, recorded percentage of infected insects ranged between 6.4 and 15.1. Significantly different from the rest, however, was only ScP strain developed earlier for control of sciarid flies. Although the infectivity was rather low, the obtained results suggested that variability available in the nematode populations could be sufficient to respond positively to genetic selection.

Effectiveness of the newly developed nematodes measured as a proportion of infected host insects in the exposed population varied greatly among the parasite strains and ranged between 3.7 and 72.6 percent. When compared with the progenitor strains the lowest infectivity recorded among selected strains was less than a half of that observed in the least effective SN strain. The highest results showed, however, near 5-fold improvement over the most effective parental ScP strain.

All the selected inbred strains presented some degree of infectivity improvement over the parental strains. In contrast, the recombinant groups contained both, the strains with higher and lower infectivity than any of the progenitor populations.

Analysis of infectivity of the most effective strains in each breeding group provided a general information about value of the developed methods for improvement of the nematode biocontrol potential against the WFT (Fig. 1). Results obtained for all the selected strains differed significantly from those recorder for parental SN and ScP populations. Although, selection conducted within the available laboratory strain improved the parasite performance the highest infectivity recorded in this group in SI-ScP strain did not exceed 38.8 %. Such result could not be economically satisfactory for the practice. Both, the recombinant inbred (RI 0201/8) and recombinant congenic (RC 0125/1r) strains performed much better and infected 72.6 and 65,2% of exposed insects, respectively.

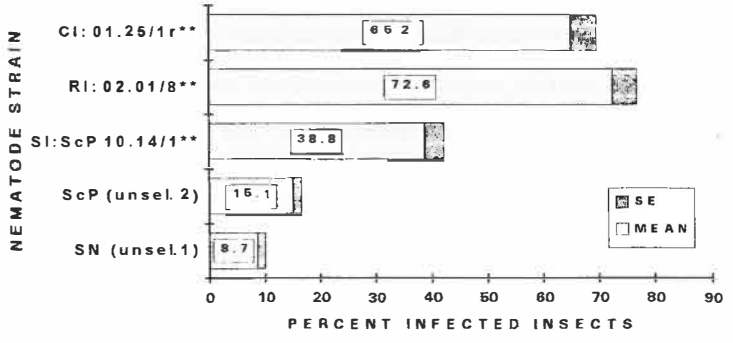

Fig. 1. Infectivity of unselected and selected strains of *Steinernema feltiae* to the WFT in the soil. (** The most effective strain in a group).

Additional experiments on influence of the most effective strains in each breeding group on emergence of adult *F. occidentalis* from the soil mixture further supported earlier findings (Fig. 2). Both recombinant strains outperformed the selected inbred-, and progenitor SN and ScP strains providing near 75% control of the pest. Overall reduction of the insect emergence attributed to the selected inbred strain was only 43.8 percent.

In spite of a strong selection pressure, so far no uniform populations of only small individuals have been obtain among the selected parasites. This is typical for characters controlled by polygenes. However, proportion of such individuals was significantly increased in several recombinant strains. In the recombinant inbred RI 0201/8 strain near 40 percent of infective juveniles was less than 22 μm wide and 700 μm long.

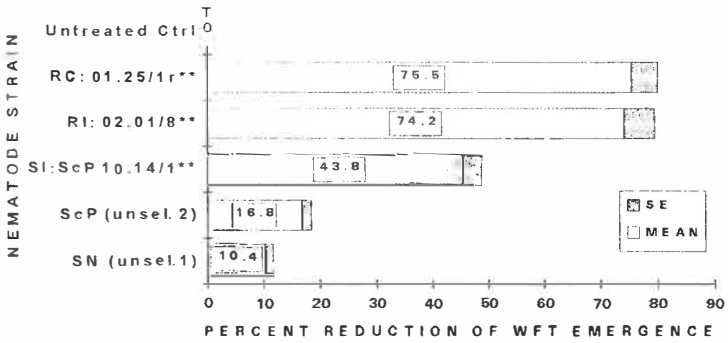

Fig. 2. Effect of unselected and selected *Steinernema feltiae* strains on emergence of adult WFT from the soil. (** The most effective strain in a group).

CONCLUSIONS

1. The reported research revealed that infectivity of *S. feltiae* to the WFT in the soil can be significantly improved through selective breeding of the nematode.

2. Selection superimposed on the process of controlled intraspecific crossbreeding of different strains followed by a series of inbreeding rounds was overall more effective in the parasite improvement than selection conducted within a single progenitor strain.

3. Although the newly obtained nematode strains showed significant variability in the level of achieved WFT control, results obtained for some of the recombinant strains, including RI 0201/8 and RC 0125/1r indicate their potential value in further development of nematode biopesticide against the pest. Implementation of nematodes into the soil may substantially contribute to the control of pupating insects and play important role in the integrated program of the WFT management.

4. The less effective among the developed strains should not be regarded as dead ends in this research. The abundance of newly constructed strains will certainly have a significant value to our further study on *S. feltiae* genome analysis directed at identification of genes affecting the parasite infectivity to insect hosts. Since the process of nematode infection consists of several steps, such as an active movement in the environment, host finding, penetration into its hemocoel and others, most of which are polygenic traits the large number of closely related, more or less inbred strains may be particularly useful for such analysis.

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TABANID SPIROPLASMAS IN FRANCE : ECOLOGY AND TAXONOMY

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SUMMARY

During 1989 and 1990, tabanid flies were collected from two different areas in Western France and assayed for spiroplasmas. From Central Britanny 62 flies of 9 different species yielded 20 spiroplasma isolates belonging to 6 different serogroups : VIII, XIV, XXIII, HYOS, TABS 1, TAAS. From Atlantic biotopes 81 flies of three different species yielded 58 isolates belonging mainly to serogroup IV, but also TABS 1, HYOS and XIV. All the 78 French isolates were able to multiply in vitro at 37°C. 1 D and 2 D proteins electrophoresis profiles of strains representaive of the different serogroups are distinct from each other and from those of mosquito spiroplasmas. Of 240 flower or plant specimens tested from "Brière" none yielded spiroplasmas. 81 animal sera from "Brière" were also examined. Using the Deformation test and 11 different spiroplasmas from honey bees, mosquitoes and tabanids, 61/76 bovine sera (or 80,3 %) were found to be positive but exclusively for the spiroplasma Ar 1357, a mosquito spiroplasma belonging to serogroup XVI_3, and previously isolated in France from Aedes mosquitoes. The fact that all the French isolates were able to multiply to high titers at 37°C (the corporeal temperature of mammals) renders highly improbable their use as biological control agent against tabanids or other dipteran pests.

1 - INTRODUCTION

Spiroplasmas (Class Mollicutes) are motile procaryotes lacking a cell wall and exhibiting a helical morphology. They are currently known from plants, insects and ticks. Recently a rich diversity of spiroplasmas has been isolated from Tabanidae in the USA (Clark et al, 1984 ; French et al, 1990 ; Whitcomb et al., 1990) and in France (Le Goff et al, 1990, 1993 ; Chastel et al, 1992). The present work summarizes taxonomic, ecological, biochemical and experimental data on tabanid spiroplasmas in France.

2 - METHODOLOGY

During summer months of 1989 and 1990, tabanid flies were collected from two areas in Western France : "Forêt de Paimpont" in central Brittany and "Parc régional de Brière" near the Loire river estuarine. Plants and flowers were also collected from "Brière". Isolation procedures and characterization of spiroplasma isolates have been previously described (Le Goff et al, 1991). Ability of isolates to multiply at 37° C and pathogenicity for suckling mice were determined as previously detailed (Le Goff et al, 1993 ; Chastel et al, 1992). Serosurvey was performed on 81 animal sera collected from "Brière" in June 1990, using Deformation test (Williamson et al, 1978). 1 D and 2 D electrophoresis profiles of total proteins of tabanid and mosquito spiroplasmas were obtained following standard procedures.

3 - RESULTS

A total of 78 spiroplasma isolates belonging to seven serogroups were isolated from the two study areas. In central Brittany, 62 flies yielded 20 isolates. Among these flies, Tabanus sudeticus proved to be the most hospitable, harboring strains of serogroups XVI, XXIII and TABS 1. In "Brière" area, 82 flies yielded a total of 58 isolates. Here, Hybomitra tropica produced 32 isolates, mainly of the serogroup IV, while Crysops pictus was the host of five different serogroups (IV, VIII, XIV, HYOS and TABS 1. These results confirm the large biodiversity of spiroplasmas circulating among Tabanidae in France and all French isolates were found belonging to serogroups already described in the USA.

All the french isolates were capable to multiply in vitro at 37° C and thus were potentialy able to infect blood-warmed animals. However, when 13 of these isolates belonging to serogroups IV, VIII, XIV, XXIII, TABS 1, TAAS and HYOS were tested in the suckling mouse model (Chastel et al., 1991) only three strains were found pathogenic ; two serogroup VIII strains from Heptatoma pellucens and Crysops pictus and one serogroup TAAS strain from Tabanus bromius.

No spiroplasma was isolated from the 240 specimens of flowers and plants from "Brière" area and no antiboy to any spiroplasma was detected in horse or sheep sera. On the contrary, 61/76 bovine sera (80%) exhibited antibody with titers ranging from 1 : 20 to 1 : 80. Howewer, these sera did not react with any tabanid spiroplasma antigen but did with a XVI-3 serogroup antigen from Aedes mosquitoes. These results appeared paradoxical since mosquito spiroplasmas are unable to multiply at 37°C and are not pathogenic for suckling mice.

At present, we have established the 1 D and 2 D gel electrophoresis profiles of total proteins from five strains of tabanid spiroplasmas. Profiles from strains belonging to different serogroups were distinct and differed from those of mosquito spiroplasmas. Reciprocally profiles from strains belonging to the same serogroup, for example VIII, were very similar. Thus, 1 D and 2 D protein electrophoresis probably represents a valuable additional tool in spiroplasmas taxonomy.

4 - COMMENTS

"Tabanidae are considered to be among the major dipteran pests of man and animals worldwide" (Foil, 1989). They are able to transmit to animals and man a great number of pathogenic agents including viruses, bacteria, protozoa and helminths (Krinskz, 1976). The study of any symbiont or potential pathogen encountered in these haematophagous diptera thus deserves attention.

Spiroplasmas are so prevalent among the Tabanidae in France than in the USA. Their prevalence may be as high as 79,5% for IV serogroup spiroplasmas in Hybomitra tropica and tabanid spiroplasmas isolated in France all belong to serogroup already known from the USA.

In spite of the absence of tabanid spiroplasma antibody in domestic animals, all the French strains were able to multiply to high titers in vitro at 37° C (the corporeal temperature of mammals) and for some of then to infect suckling mice. These properties render a priori highly improbable their use in biological control of deer flies and horse flies or other dipteras as well. This situation is quite different from that of certain mosquito spiroplasmas which appeared as good potential biological agents against medically important mosquitoes (Humphery Smith et al, 1991 a ; 1991 b).

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APPLICATION OF VECTORS FOR VIRAL INFECTION AMONG PESTS

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Summary
Possibility has been shown to transfer viral infection to insect pests by means of Bracon hebetor both, in the laboratory and in the field. Entomophage activity demonstrated in paralysing of phytophagous caterpillars is enhanced by their role as vectors of viral infection. Viral infection results in development of epizootics among insect pests and reduces pest density to the minimum. The use of predaceous insects from Bracon genus as viral infection vectors looks promising because this entomophage does not kill all pests whichhhe encounters. Some pests remain only paralyzed. Predators larvae leave their excrements which contain viral infection. The latter(together with food for phytophages) moves to the midgut where viruses begin their reproduction cycle. Thus, binary effect on insect pests takes place.

1. Introduction

Recently it has become more evident that the leading role in IPM belongs to biological control means which are safe for humans and the environment.New developments in this field improve IPM systems. One of the ways to improve IPM efficiency is binary effect of biological means, i.e. it is possible to combine the use of entomophages and viral insecticides. We developed rather effective techniques for treating adult Bracon hebetor with virus. The entomophage parasitizes pest caterpillars. We tested several methods of viral infection introduction onto entomophages. The most effective were techniques of virus introduction into the midgut and washing in viral suspension.

The above mentioned binary effect looks very promising because the technology of Bracon mass rearing and application is well developed and widely applied, especially in Middle Asia. Besides, there are viral insecticides aimed to control most important lepidopterous pests.

2. Materials and methods

We used the following phytophage species: Heliothis armigera, Mamestra brassicae, Carpocapsa pomonella. Bracon hebetor was used as viral disease vector. In one test variant larvae were given viral suspension with sugar, in another one larvae were washed in viral suspension. Larval phytophages unfected with virus were placed into cages before larval vector introduction. One of the variants consisted in introduction of larval phytophages and vectors into the jars the walls of which were treated with the viral preparation. One check of the test was without infection source and without Bracon. In another check phytophage food was treated with viral suspension of viral insecticides at standard concentration. Phytophage larvae of the 3rd instar were used in all variants.

3. Results and discussion

Data obtained demonstrate possibility to use Bracon hebetor as vector of viral infection among insect pests. It is worth noting that females can in average parasitize three caterpillars of the 3rd instar. Besides, caterpillars can spread viral infection in places of pest concentration. In this case viral infection is introduced into the places of host insect localization.

The technique proposed saves viral insecticide, i.e. the quantity applied is 1 000 times less than under complete spraying. The effectiveness of Bracon remains unchanged. It was revealed that the effectiveness made up 73.3% when the virus was introduced per os. Higher effectiveness (96.7% mortality) was reached when adult Bracon hebetor was washed in viral suspension. In the latter technique the virus penetrated both, into the insect midgut and onto their body surface.

In the test variant with introduced infected caterpillars the aim was to reveal possibilities to transfer infection from infected caterpillars to healthy ones. The effectiveness of the technique was equal to 65.8% mortality on the 10th day. The data obtained demonstrate that species from Bracon genus can serve in nature as vectors of viral infections among lepidopterous pests.

In the first test check (where caterpillars were not infected and where the entomophage was not introduced) mortality constituted 15.0%. In another check (where the food for phytophages was infected with virus) caterpillar mortality made up 93.4%.

Thus, the use of binary effect (when an entomophage can also serve as vector of viral diseases) looks very promising.

FUNGAL, BACTERIAL AND PROTOZOAN PATHOGENS OF *DELIA RADICUM* AND *DELIA FLORALIS* (DIPTERA: ANTHOMYIIDAE)

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SUMMARY

Cabbage root fly (*Delia radicum*) and turnip fly (*Delia floralis*) were studied in Denmark and Norway with respect to the natural occurrende of insect pathogens. Especially the adult stages were sampled, incubated and diagnosed for presence of pathogens. Several fungal species from Entomophthorales and Hyphomycetes were found, *Entomophthora muscae* and *Strongellsea castrans* being the most frequent. A microsporidium was found in *D. radicum*. Further, *Bacillus thuringiensis* was isolated both from adult, larvae and pupal stages of the species.

KEY WORDS

Delia radicum, Delia floralis, Entomophthorales, Hyphomycetes, Microsporidia, *Bacillus thuringiensis*.

INTRODUCTION

The cabbage root fly (*Delia radicum*) and the turnip fly (*D. floralis*) are important pest species of brassicas in temperate areas and difficult to control. In Denmark, *D. radicum* is the most important species, while in Norway, both species occur frequently.

The flies were studied in both countries in order to document the natural occurrence of pathogens. The Danish studies (restricted to *D. radicum)* include observations from 1988 to 1993, while the Norwegian observations on both species were done in 1992 and 1993. This paper presents qualitative data, while more detailed work from each country will be published elsewhere.

MATERIALS AND METHODS

Adults were sampled in cabbage fields with sweep-net and incubated separately in plastic cups, supplied with 2% water-agar and food (Eilenberg & Philipsen, 1988). During 10 days incubation, eventual fungal diseases were diagnosed (external symptoms, morphology of spores on glass slides). For the presence of microsporidia and *Bacillus thuringiensis* (Bt), flies were dissected on glass slides with water. Diagnosis were based on observations of spores in light microscope. For the diagnosis of microsporidia, flies were in addition fixed in glutaraldehyde, embedded in Epon and sectioned for light and transmission electron microscopy.

The presence of Bt in flies (larvae, pupae and adults) was also documented by subjecting the flies to a procedure for isolating Bt (Damgaard et al, 1994). The method includes incubation on petri dishes with T3 agar, selection of colonies based on morphology and final confirmation by the presence of crystals.

Finally, eggs were sampled from soil and incubated in cups with sterile water. During incubation, fungal growth was checked.

RESULTS AND DISCUSSION

The results are listed in table 1. As seen, both fly species in the adult stage are infected by *Entomophthora muscae* and *Strongwellsea castrans*. In the Danish studies, both fungal species were able to establish epizootics (Eilenberg et al., 1991, Eilenberg, unpubl.). Resting spores were found in some specimens, most often towards the end of the season.

The other fungal species from Entomopthorales were found occasionnally in a low number of specimens. This was also the case for the species from Hyphomycetes, among which *Metarhizium anisopliae* and *Verticillium lecanii* for the first time were found in 1993 (one specimen each on adult *D. radicum*).

Initial observation proved that eggs of both species were in few cases infected by fungal pathogens. Due to limited material, no final diagnosis was possible.

The presence of a microsporidia was observed in the tissue of a few adults of *D. radicum* after dissection or sectioning embedded specimens. Only mature spores were found, but we regard so far the microsporidia to be an undescribed, new species. In two cases, double infection (microsporidia and *S. castrans*) was found.

In a single case sporulating Bt was found directly after dissection of the abdomen of an adult *D. radicum*. The fly was at the same time infected with both *S. castrans* and the microsporidia, proving that triple infection may occur. The isolated starin proved to be serovar *aizawai*. This serovar has so far mainly recieved attention in relation to Lepidopterans and several products for control of Lepidopteran pests are available. Therefore, a possible natural association with Diptera is remarkable.

		Delia radicum		Delia floralis
		Denmark	Norway	Norway
Entomophthora muscae	**Con.**	**A**	**A**	**A**
	Res.	**A**		**A**
Strongwellsea castrans	**Con.**	**A**	**A**	**A**
	Res.	**A**		
Erynia spp.		**A**		**A**
Conidiobolus spp.		**A**		
Zoophthora radicans		**A**		
Beauveria bassiana		**A**		
Verticillium lecanii		**A**		
Metarhizium anisopliae		**A**		
Undetermined fungi		**E**		**E**
Bacillus thuringiensis		**ALP**		**P**
Microsporidia		**A**		

Table 1.

Naturally occurring pathogens on cabbage root fly (*Delia radicum*) and turnip fly (*Delia floralis*) in Denmark and Norway.

A = Adults
P = Pupae
E = Eggs
L = Larvae

Con. = Conidia
Res. = Resting spores

Isolation of Bt from adult, larvae and pupal stages by plate dilution proved further the presence of the pathogen. The strains are currently subjected to characterization. It needs, however, to be proven, if the isolated Bt strains are pathogenic to the fly species.

From the studies it can thus be concluded that from both species of *Delia* a number of naturally occurring pathogens can be isolated. Additional biological studies are, however highly needed in order to clarify, which species will have highest potential for biological control.

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Biological control of *Popillia japonica* on Terceira Island (Azores, Portugal): Potential of *Bacillus popilliae*

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ABSTRACT

Bacillus popilliae Dutky, the causal agent of milky disease in *Popillia japonica* Newman, was evaluated for efficacy in controlling the Japanese Beetle on Terceira Island. The entomophatogenic activity of milky spore powder from commercial and laboratory sources was studied under both laboratory and field conditions.
B. popilliae was pathogenic to Japanese beetle larvae under laboratory conditions, but in the field, there was no reduction of larvae in treated plots and patently infected larvae were extremely rare.
The discrepancy between the results obtained in laboratory assays and under the field conditions observed on Terceira Island may be due to relatively low soil temperatures and lack of stress due to lower larval density and uneven distribution in the soil.

INTRODUCTION

The Japanese beetle (JB), *Popillia japonica* Newman, was introduced on to Terceira Island in the early 1970's (Martins and Simões, 1986). In the absence of a diverse complex of natural enemies the beetle has steadily increased and spread across the island. Prior to 1991 the main method for reducing the number of adult beetles was aerial application of insecticide (carbaryl). Due to the low residual effect of this insecticide for control of adult JB, its questionable efficacy in preventing a build up in beetle populations, potential detrimental environmental effects and the high cost of recurrent application, other sustainable methods for the long term control of the beetle have been initiated on Terceira Island. Biological control through the use of insect parasites and pathogens of *P. japonica* offers significant potential for sustainable control on an island habitat such as that found on Terceira.
Based on the efficacy and persistence of milky spore, *Bacillus popilliae*, as a microbial control agent of the JB in the United States (Fleming, 1968, Klein, et al. 1976, Klein, 1981, 1992), it was considered a prime candidate for use in the biological control of the beetle in Terceira. From 1985 to 1989, however, several field trials did not reveal promising results against the JB on the island.
Our studies were conducted to determine the efficacy of milky spore powder from commercial and laboratory sources applied at elevated rates, under laboratory and field conditions, for controlling the JB.

MATERIAL AND METHODS

Laboratory assays. Five strains of *B. popilliae*: Ohio (1990), Ohio (1975a), Ohio (1975b), Maryland (1952) and Connecticut (1986), isolated from JB larvae during epizootics, were used to determine pathogenicity against JB larvae. The spores, preserved on slides as dried blood smears, were suspended in sterile water and tested on 3rd instar larvae by intrahemocoelic injection. Thirty larvae/strain were

injected with $5x10^5$ spores/larva following the procedures of Dutky (Dutky, 1941) and 3 replicates were made. Thirty larvae injected with sterile water and thirty uninjected larvae were used as controls. The larvae were incubated for 14 days at 25±2°C in covered 0.95 l plastic containers (10 larvae/container) with 500 g of sterile soil seeded with 1 g of white clover for a total of three replicates for each treatment and control.

Spore powder of the Ohio (1990) strain were produced *in vivo*, as described by Dutky (1942),through intrahemocoelic injection of 3rd instar larvae. To test the *per os* pathogenicity of the Ohio (1990) strain, the spore powder was added to sterile and to non sterile soil at five dosages ranging from $5x10^8$ to $30x10^8$ spores/kg soil. The soil was then seeded with 2.5 g of white clover/kg of soil and tested against 2nd and 3rd instar larvae. Plastic cups (150 ml) were filled with 50g of the inoculated soil and one larvae was placed in each. For each concentration 3 replicates of 30 larvae each were made. Control larvae were held in cups with non inoculated soil. The cups were incubated for 5 weeks at 25±2° C. The larvae were examined at weekly intervals and dead or diseased larvae were counted and removed. The percent mortality obtained for the $15x10^8$ and $20x10^8$ dosages were corrected using Abbott's formula (Abbott 1925), and compared using Duncan's Multiple Range Test using the Systat computer program (Systat: The system for statistics, Evaston, IL Systat, Inc., 1988) following arcsin transformation.

Field assays. During 1990 and 1991 over 40 sites on Terceira Island were treated with DOOM® (Fairfax Bio. Lab, Clinton Corners, NY) containing 10^8 spores of *B. popilliae*/g of powder. At each location the spore powder was applied by two methods, subsurface application of 50 kg/ha and 200 kg/ha to two 5x5 m plots, and surface application of 50 kg/ha to a 10x20 m plot. An additional 5x5 m plot was established at each site as a control. The treatments were assessed in September 1991 and again in October 1992, by taking five soil samples of 30x30x10 cm/plot, counting the JB larvae found and checking for milky disease symptoms.

In September 1991, six 0.25 m^2 plots were delimited by an iron rim to a depth of 0.25 m. The soil inside the rim was removed and replaced by 20 kg of inoculated soil with $20x10^8$ spores of the Ohio (1990) strain/kg of soil and seeded with a mixture of white clover and grass. Another four plots were prepared with non inoculated soil as controls. Two hundred JB larvae (50 2nd and 150 3rd instar) were placed in each plot. After five weeks all the larvae were removed, counted and examined for milky disease symptoms. After the initial assessment another 200 3rd instars were placed in each plot to be checked the following spring.

During 1992, soil temperatures were recorded, in 5 minutes intervals with a Omnidata Easy Logger Portable Weather Station at two sites: Agualva (120 m elev.) and Cinco Picos (315 m elev.). Temperature probes were placed in the soil at 2, 10 and 20 cm below the surface. The weekly average temperatures were calculated for graphical presentation. The mean weekly soil temperatures that were recorded are shown in Fig.1. Mean temperatures above 21° C were recorded for 9 weeks from June to August at the lower elevation. At the higher site that period was only 5 weeks.

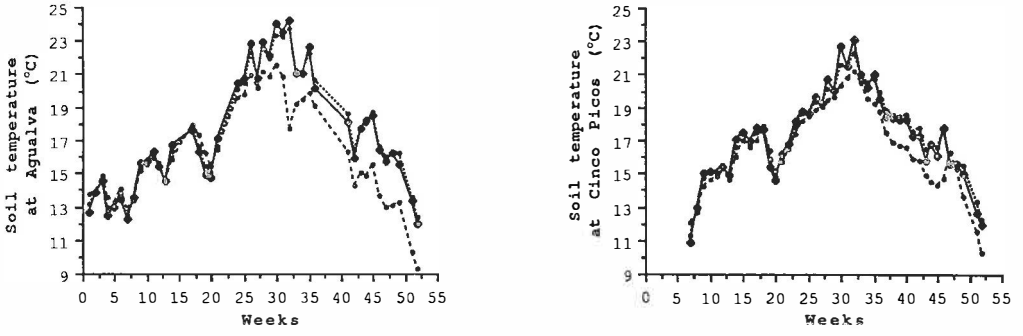

Fig.1. Mean weekly soil temperatures recorded at Agualva and Cinco Picos during 1992 (—•—2 cm,·····•·····10 cm,- - -•- - 20 cm).

RESULTS AND DISCUSSION

Laboratory assays. The results of bioassays of injected larvae are summarized in table 1. Of the five strains tested, only the Ohio (1990), the Ohio (1975b), and the Connecticut (1986) were pathogenic to JB larvae. The Ohio (1990) strain proved to be the most pathogenic, producing patent infection in 74.5% of the larvae. This was slightly inferior to the results reported by Beard (1945) and Dutky (1963).
The results for the *per os* assays are summarized in table 2. The Ohio (1990) strain was pathogenic to both 2nd and 3rd *P. japonica* larvae, the dosage of $20x10^8$ spores/kg soil produced 60% and 34.6% corrected mortality in 2nd and 3rd instar respectively. These results are slightly inferior to the 60-75% infection cited by Dutky (1941).
The difference in the dose response of 2nd and 3rd instars may be due to the maturation and nutritional states of the larvae (Fleming, 1968). There were no significant differences in mortality in assays conducted with sterile and non sterile soil.

Field assays. The commercial spore powder, although applied at very high dosages, did not result in larval reduction in field plots. Of the thousands of larvae observed in the treated plots, only one infected grub was found. The results obtained for the highest application of DOOM and the control from 18 selected sites were not significantly different (T-test).
Despite the level of infection and mortality observed in laboratory assays of the Ohio (1990) strain, there was no significant reduction

TABLE 1. Pathogenic activity of several strains of *Bacillus popilliae* injected in 3rd instar *Popillia japonica*.

Strain (collection date)	Dose (x10^5spores/larva)	Patently infected larvae mean (%) ± s.e.	Total mortality[1] mean (%) ± s.e.
Ohio (1990)	5	74.5 ± 6.2	84.5 ± 5.9
Ohio (1975a)	5	33.3 ± 8.8	47.8 ± 7.8
Connecticut (1986)	5	10.0 ± 1.9	40.0 ± 1.9
Maryland (1952)	5	0.0 ± 0.0	18.9 ± 6.7
Ohio (1975b)	5	0.0 ± 0.0	33.3 ± 6.9
Control[2]	0	0.0 ± 0.0	25.5 ± 4.0
Control[3]	0	0.0 ± 0.0	20.0 ± 3.9

[1]After two weeks held at 25±2° C
[2]Larvae injected with sterile water
[3]Non injected larvae

TABLE 2. The effect of spore concentrations, larval age and soil sterility on the *per os* pathogenity of the *B. popilliae* strain Ohio (1990) against *P. japonica* larvae under laboratory conditions[1/].

dosage[2/]	2nd instar (% mortality)		3rd instar (% mortality)	
	sterile soil mean ± s.e.	non sterile soil mean ± s.e.	sterile soil mean ± s.e.	non sterile soil mean ± s.e.
0	17.8 ± 9.5	18.9 ± 6.2	10.0 ± 5.1	25.6 ± 8.7
5	14.4 ± 8.0	-	22.2 ± 4.0	-
10	26.7 ± 15.0	40.0 ± 5.1	28.9 ± 4.0	-
15	25.6 ± 5.6	61.1 ± 1.1	47.8 ± 20.2	42.2 ± 6.7
20	54.4 ± 17.9	68.9 ± 8.0	30.0 ± 10.0	54.5 ± 7.8
30	78.9 ± 6.2	-	38.9 ± 9.9	63.3 ± 10.2

1/ Incubated at 25±2° C, during 5 weeks, 3 replicates
2/ $x10^8$ spores/kg of soil

of larvae in the field plots treated with the extremely high dosage of this strain. In October 1991, however 23.2% of the larvae found in the treated plots were milky. In April 1992 there were no milky larvae found in the treated plots. The results of our trials indicate that *B. popilliae* has very low potential for control of JB on Terceira Island. Field tests conducted over several years in the US with much lower application of spore powder have resulted in considerable reduction of the JB populations in most of the treated areas (Klein, 1992).
The failure of *B. popilliae* in reducing *P. japonica* in Terceira Island could be due to one or more of the following factors: 1) the relatively low soil temperatures when larvae would be actively feeding may prevent the establishment and build up of milky disease (Dutky, 1963, Fleming, 1968, Klein, 1992), 2) the vertical distribution of actively feeding larvae in a wider band of the soil profile on Terceira than in the US, combined with the tendency for *B. popilliae* spores to concentrate in the upper soil horizon, and 3) a lack of stress due to lower larval densities (Fleming, 1968).

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A NEW PATHOTYPE OF *BACILLUS THURINGIENSIS* WITH PATHOGENIC ACTION AGAINST SAWFLIES (HYMENOPTERA, SYMPHYTA)

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Summary

A strain of *Bacillus thuringiensis* (K24) with some unique properties has been isolated from diseased sawfly larvae, *Cephalcia falleni* (Hymenoptera, Symphyta: Pamphiliidae), devastating Polish spruce forests. The isolate is pathogenic to pamphiliid species, as well as sawflies of two other families. It is toxic for *Aedes aegypti* and other Culicidae (Diptera, Nematocera). It is neither pathogenic nor toxic for *Drosophila melanogaster* or *Musca domestica* (Diptera, Cyclorhapha), four species of Lepidoptera, or six species of Coleoptera (including three species of Chrysomelidae). K24 produces 2 to 3 irregular parasporal bodies (δ-endotoxin) but no soluble ß-exotoxin.

Introduction

In 1983 the first author (G.B.) isolated a strain of *Bacillus thuringiensis* with some unique properties from moribund diapausing larvae of the sawfly *Cephalcia falleni* DALM. (Hymenoptera, Symphyta: Pamphiliidae) collected in the Polish Gorce National Park and taken to Switzerland. On inspection with the phase-contrast microscope the presence of freshly sporulated bacteria with parasporal inclusions was detected in some of the diseased larvae. One of the larvae contained an almost pure culture of the microorganism; only a few colonies of other bacteria developed on an agar plate that was inoculated with a diluted suspension of the haemolymph. The isolate was named K24.

In the years 1978 to 1980 *Cephalcia falleni* had defoliated large 80-year-old stands of Norway spruce (*Picea abies*) in the Gorce National Park, about 1100 to 1400 m above sea level. After 1980 the population density diminished gradually, but became locally high again in the years 1984 to 1986. Polish investigators had observed earlier that larvae, prepupae, pupae, and adults of *Cephalcia abietis*, *C. falleni*, and *Acantholyda nemoralis* may exhibit a pathological syndrome, which they called "brown disease", characterized by increasingly brown coloration and decreasing motility, resulting in death within days in the active stages and within weeks in diapausing prepupae and in pupae. (KUCERA and MADZIARA-BORUSIEWICZ, 1982) . The contagious nature of the disease was demonstrated by injecting healthy larvae of *C. falleni* with a small amount of haemolymph from diseased diapausing larvae of the same species, which caused "brown disease" in the injected insects (MADZIARA-BORUSIEWICZ, unpubl.). However, no pathogen could be detected in the diseased larvae.

To our knowledge K24 is the first *B.t.* isolate from a sawfly since SMIRNOFF and HEIMPEL (1961) described a strain of *B.t.* from a larva of the larch sawfly, *Pristiphora erichsoni*. But the *Pristiphora* isolate produced the usual bipyramidal δ-endotoxin crystals toxic for Lepidoptera but of very limited virulence for the larch sawfly. In fact the isolate was even less pathogenic than some crystal-less strains of *B. cereus* (HEIMPEL, 1955). Therefore, it was not further investigated. Moreover, KRIEG (1986) considered the toxicity for sawflies to be caused by the soluble ß-exotoxin of the isolate. Since K24 from *C. falleni* is not toxic for Lepidoptera and free of ß-exotoxin, but has the unique property of being pathogenic to larvae of several sawfly species and the rare property of being toxic for mosquitoes it will be shortly described here.

Material and Methods

Microbiological Methods

Standard microbiological methods were used for the isolation of colonies from agar plates, the production of a liquid culture for the production of spores and parasporal bodies as well as supernatant for tests of ß-exotoxin, and microscopical examination. For determining the flagellar serotype and the plasmid spectrum of K24 we are indebted to Dr. DENIS BASSAND of Sandoz Company, Basel.

Bioassays

1. Sawfly larvae

Since the bacterium was isolated from sawfly larvae, we tested a series of sawflies belonging to different families by feeding them with their specific food plants treated with the K24 spore/parasporal body complex.

2. Lepidoptera larvae

Since the *Pristiphora* isolate of SMIRNOFF and HEIMPEL produced the usual bipyramidal δ-endotoxin crystals toxic for Lepidoptera, we tested the larvae of 7 lepidopteran species: of the Pieridae *Pieris brassicae*, of the Tortricidae, *Cydia pomonella* and *Adoxophyes orana*, of the Pyralidae *Ephestia kuehniella*, and of the Noctuidae *Mamestra brassicae*, *Scotia segetum* and *Spodoptera littoralis*.

3. Coleoptera

Adults and larvae of the following beetles were tested: *Melolontha melolontha*, *Galerucella luteola*, *Gastrophysa viridula*, *Melasoma aenea*, *Sitophilus granarius* and *Tribolium confusum*.

4. Diptera Nematocera and Cyclorrhapha

Tests for ß-exotoxin in the autoclaved supernatant of a submerse culture were made with larvae of the cyclorrhaphous *Drosophila melanogaster* and *Musca domestica*. - Mosquito toxicity was tested with second and third instar larvae of the following

Nematocera species: *Aedes aegypti*, *Culex pipiens molesta* (of the Culicinae), *Anopheles stephensi* and *Anopheles albimanus* (of the Anophelinae), and *Chironomus tentans* (Chironomidae).

Results and Discussion

The isolate (K24) is not only pathogenic to other sawfly species [the pamphiliids *Cephalcia abietis* (L.) and *Acantholyda erythrocephala* L., the diprionids *Neodiprion sertifer* (GEOFFROY) and *Gilpinia hercyniae* (HARTIG), and the tenthredinids *Pristiphora abietina* (CHRIST.), *Nematus melanaspis* (HARTIG) and *Athalia rosae* (L.)] but also toxic for larvae of several nematocerous Diptera: highly toxic for the Culicinae *Aedes aegypti* (L.) and *Culex pipiens molesta* FORSK. [LC_{50} ca. 10^3 spores + parasporal bodies (S+P) per ml], less toxic for the Anophelinae *Anopheles albimanus* WIEDEMANN and *A. stephensi* LISTON (LC_{50} ca. 10^4 S+P/ml), and much less toxic for the chironomid *Chironomus tentans* DE GEER (LC_{50} ca. 10^7 S+P/ml). The vegetative cells are non-motile and do not produce ß-exotoxin, but on sporulation 2 to 3 irregular parasporal bodies are formed.

K24 is neither toxic nor pathogenic for larvae and adults of the cyclorrhaphous Diptera *Drosophila melanogaster* MEIGEN and *Musca domestica* L., nor for the Coleoptera *Gastrophysa viridula* (DEG.) and *Melasoma aenea* L. (both Chrysomelidae), *Sitophilus granarius* (L.) (Curculionidae), and *Tribolium confusum* DU VAL (Tenebrionidae), nor for all Lepidoptera tested: *Cydia pomonella* (L.), *Adoxophyes orana* F.V.R. (both Tortricidae), *Ephestia kuehniella* (ZELL.) (Pyralidae), *Pieris brassicae* L. (Pieridae) and *Scotia segetum* (SCHIFF.) (Noctuidae).

With its toxicity for culicid larvae (pathotype B) K24 resembles the *B. thuringiensis* ssp. *israelensis* with which it also shares the flagellar H serotype 14. Because of the non-motility of K24 the H serotype could not be determined easily. However, after 9 passages in Cragie tubes the motility increased slightly. The plasmid spectrum of K24, too, is similar to that of the ssp. *israelensis*, with which it shares 5 plasmids with the MWs of 4.0, 4.5, 68, 75, and 105 MDa., whereas an additional *israelensis* plasmid (MW 10.6 MDa.) is missing in K24. Instead, K24 seems to possess an extra plasmid of about 26 MDa. (Fig. 1).

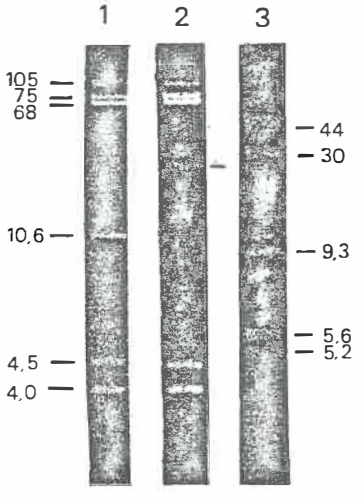

Fig. 1. Plasmid spectra of *B. thuringiensis* ssp. *israelensis* (1), K24 (2), and *B. thuringiensis* ssp. *kurstaki* (3).

These are not the only differences to the ssp. *israelensis*. Some of the differences concerning bioassays, the parasporal bodies and the δ-endotoxin are presented and discussed below in the paper of JOERESSEN and BENZ (1993). Differences concerning mammalian cell toxicity are presented in the poster of JOERESSEN et al. (1993) and in a paper by JOERESSEN et al. (1994). The latter two show that the ssp. *israelensis* has haemolytic and cytotoxic activity in mammalian blood and in a cell line *in vitro*, which K24 has not. These differences and the fact that K24 can infect and kill sawflies - which the ssp. *israelensis* cannot - lead us to postulate for K24 a further *B. thuringiensis* pathotype: **Pathogenicity for sawfly larvae and nematocerous Diptera larvae.**

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CHYTRIDIOPSIS TYPOGRAPHI (PROTOZOA, MICROSPORIDIA) AND OTHER PATHOGENS IN *IPS TYPOGRAPHUS* (COLEOPTERA, SCOLYTIDAE).

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Summary

Chytridiopsis typographi was found in *Ips typographus* collected at six of eight locations with great differences in infection rates depending on site and year. Furthermore, Gregarines were observed in beetles from six sites and Nematodes were relatively frequent in beetles from all locations.

1. Introduction

Ips typographus is a serious insect pest on spruce (*Picea abies*) in central and northern Europe. A heavy storm in 1990 caused a lot of wind thrown stems in Austria, which are best breeding material for bark beetles. At least since that event, bark beetle populations are increasing permanentely. This situation and the problems in bark beetle control were the reason to search for biological control possibilities. Till now very few is known about pathogens and parasites of *I. typographus* and other bark beetles (POSTNER, 1974; FÜHRER & PURRINI, 1981; BATHON, 1991). Therefore it is of interest to intensify the investigations on the potential antagonists of bark beetles and their role in regulating population density.

Chytridiopsis typographi is known as a protozoan pathogen developing in the cells of the midgut-epithelium of *I. typographus, Dendroctonus pseudotsugae* or *Hylastes cunicularius* (WEISER, 1954; 1963; 1966; 1970; PURRINI & WEISER, 1984). The spores of *Ch. typographi* are enclosed in cysts. Two types of cysts can be found, spherical and thick-walled with a remarkable refractive wall or slightly compressed and thin-walled. Each cyst contains 4 to 30 spores and is 10 to 20 µm in diameter respectively 30 to 40 µm long (FUCHS, 1915; WEISER, 1954; 1963; 1970).

2. Materials and methods

Adult beetles of *I. typographus* were collected from trap trees or pheromone baited traps at eight sites in Lower Austria (NÖ):
1 - Flatz/Hettmannsdorf; 2 - Gainfarn; 3 - Hainfeld/Ramsau;
4 - Prinzersdorf; 5 - Amstetten/Forstheide; 6 - Amstetten/Oed;
7 - Horn/Mödring; 8 - Rothwald.

First examinations of beetles from site no. 1 started in 1989 at the other sites in 1991, 1992 or 1993 (just now running). Specimens were collected from their first appearence in spring to the end of swarming in autumn. 4631 beetles were examined in total.

The whole gut, the gonads and parts of the fat body were dissected, from living beetles only and inspected in a drop of water under a coverslip by light microscope. The distribution of all pathogens or parasites in the organs or free in the hemolymph was recorded, with special regard to *Ch. typographi*. Afterwards the smears were fixed with methanol and stained with Giemsa or the organs were fixed in Dubosq-Brasil respectively in Glutaraldehyd-solution and embedded for histological sectioning.

3.Results

Chytridiopsis typographi was found in *I. typographus* from six of eight locations, depending on site and year, up to the current status in 1993. The percentage of infected beetles varied from 0.0% to 37.9% depending on location and year. The year with the highest infection rate (1990 at site no. 1) was followed by a rapid decrease of infection in the following years. The infection rates in the beetles from the locations no. 2 to 6 varied most of the time at a low level. No infection could be observed in the beetles from the sites no. 7 and 8 (Table 1).

Table 1: Infection of *I. typographus* by *Ch. typographi* (C), Gregarines (G) and Nematodes (N)(in %) depending on site (no. 1 to 8) and year (1993 represents the current status).

	1					2	3	4		
	1989	1990	1991	1992	1993	1993	1993	1991	1992	1993
C	26.4	37.9	6.4	2.5	4.0	1.3	5.0	0.0	16.6	0.3
G	5.1	24.3	18.2	9.7	19.6	7.9	18.9	0.0	0.0	0.0
N	13.8	66.4	81.8	39.7	60.9	46.1	70.9	65.0	57.1	73.4

	5		6		7	8		
	1992	1993	1992	1993	1993	1991	1992	1993
C	3.2	3.4	0.0	6.5	0.0	0.0	-	0.0
G	0.0	0.7	0.0	0.0	1.0	4.1	-	43.1
N	42.1	71.2	17.7	48.5	94.1	94.0	-	70.6

Thick-walled cysts were found in the former parts of the midgut (spherical type only, with a mean size of 8.8 µm in diameter) and in the hind parts (spherical cysts with a mean size of 12.5 µm in diameter and compressed cysts with a mean length of 15.1 µm and a mean width of 11.4 µm), thin-walled cysts also could be observed in the whole midgut. Whereever spore maturation started in the midgut, infection spread progressively over the whole midgut. Finally the midgut-cells dissolved totally. In an advanced stage of disease cysts also could be observed in the hindgut, transported

together with the intestinal juice and excreted together with the faeces.

Gregarines were found most of the time in the former parts of the midgut-lumen up to the region of the blind sacs (diverticuli). Sometimes they occurred in the whole midgut, maybe as a consequence of intestinal activity. Great differences in size and shape of Gregarines could be observed partly, till now exact determination is missing (only *Gregarina typographi*, or also a second species ?). Gregarines were observed in the beetles from six sites whereby in one site only in one beetle (site no. 5) and an outstanding high rate of beetles with Gregarines at site no. 8 (Table 1).

Nematodes were found in the beetles from all locations very frequently: In the body cavity were adults and larval progeny of *Contortylenchus diplogaster* and *Cryptaphelenchus macrogaster macrogaster*, whereas in the gut lumen was a population of invasive larvae and adults of nematodes frequently without pathological impact on host beetles. The general frequency of nematodes was rather high, peaks not in correlation with peaks of other pathogens. In some years some localities brought high incidence of infected bark beetles: no. 7 and 8 up to 94.1%, in others up to 81.8% (site no. 1) respectively 73.4% (site no. 4) (Table 1).

Spores of *Nosema typographi* were found in some few beetles, spores of *Malamoeba scolyti* in only one beetle (site no. 1).

Sometimes hyphae of fungi could be observed but examinating only living beetles, conidiospores of fungi could not be found. As a consequence of their absence, precise determination was impossible. Sometimes *Beauveria sp.*-infected bark beetles were observed in their galleries.

Hymenopterous parasites and mites could be observed, but were not very abundant.

Multiple infections with all the above mentioned pathogens and parasites were observed. No sex specific differences could be found. *Ch. typographi* was also found in some few pale and not hatched young beetles cut out of the bark. No infection could be observed in larvae. No relation of *Ch. typographi* appearence to season could be found.

Accumulation of *Ch. typographi* was obtained in *I. typographus* in a laboratory stock. It increased from 3.2% infection in the beetles of the fresh collected field population to 22.1% in the f1- and to 52.5% in the f2-laboratory generation.

Attempts to induce a *Ch. typographi* infection in *I. typographus* by feeding the cysts to the beetles worked not reliable till now.

4. Discussion

It is evident that the infection route for protozoan pathogens (Microsoridia, Gregarina) is different from the active way of nematodes, as it is evident from differing peaks of infections during the years. Impact of high density of a population in rearings is evident from growing infection rates with *Ch. typographi* in laboratory colonies. This confirms the density dependent appearence of *Ch. typographi* as found in the *I. typographus* field population from site no. 1.

Very few data are published about the pathogenicity of *Ch. typographi* for the hosts and there is no explanation yet why the

infection appears only in adults, not in larval stages. As mentioned, the infection could be identified in some freshly moulted adults, in gallery during maturation feeding but without a chance to get infected after hatch during migration.

The pathogenicity of all the pathogens and parasites to bark beetles, the involvement of larvae in transmission and the ways of transmission are open questions in this research.

5.Acknowledgements

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2. Interactions between insect defense mechanisms and pathogens.

INTERACTIONS BETWEEN INSECT DEFENSE MECHANISMS AND ENTOMOPATHOGENIC NEMATODES

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Summary

Insects defense mechanisms against pathogens consist of mechanical barriers, which protect invasion into the haemocoel, and systems, which eliminate invading particles in the haemocoel. This nonself-response system consists of humoral and cellular mechanisms. Haemocytes are involved in phagocytosis, nodule and capsule formation. A prerequisite for these reactions is the recognition of the invading particle as nonself, which is mediated by physicochemical properties and specific components on the surface of the invading object. Humoral factors like lectins and components of the prophenoloxidase activating system (proPO-AS) support this nonself recognition. Invading microorganisms can be eliminated by lysozyme and inducible antibacterial proteins (cecropins, attacins,sarcotoxins, diptericins). Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* evade nonself recognititon in many host insects. However they do not suppress the nonself-response system. In some dipterans and coleopterans encapsulation occurs but does not always prevent insect death, because the symbiotic bacteria can be released before complete encapsulation. The symbiotic bacteria *Xenorhabdus* spp. are recognized as nonself but tolerate the cellular defense mechanisms of *G. mellonella*. They are susceptible to inducible antibacterial proteins. Protein secretions of the nematode inhibit these enzymes ensuring survival of the bacteria. In *T. oleracea Xenorhabdus* spp. are probably eliminated by cellular reactions, which are possibly also suppressed by the nematode. Thus nematode and bacteria act together in overcoming the insect's defense mechanisms.

1. Introduction

Insects are relatively well protected against invasion of foreign organisms in their haemocoel. The cuticle covers the integument, the tracheal tubes and the fore- and hindgut. The midgut is protected by the peritrophic membrane. In addition, sieve plates or plugs cover the spiracles of many soil inhabiting insects, preventing the penetration of larger organisms. However, these structures do not completely prevent the invasion of organisms, so insects have evolved mechanisms to eliminate such invaders. In turn, pathogens, which must rely on the propagation in the host, have evolved mechanisms to overcome these nonself response systems in the insect's haemolymph. This is especially the case for entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*, which are characterized by a symbiotic association with gram-negativ bacteria of the genus *Xenorhabdus*. The high level of pathogenicity of these nematodes can in part be explained by the efficient reactions developed by the nematode and its bacteria to overcome the nonself response systems in the insect's haemocoel.

2. Defense reactions of insects against nonself particles in the haemocoel

The nonself-response system of insects consists of humoral and cellular factors. Both, humoral and cellular mechanisms act together in the elimination of invading objects. A prerequisite for the cellular elimination of nonself material in the haemolymph is the recognition as nonself. The **nonself recognition** is based on physicochemical properties as well as specific recognition receptors on the surface of foreign organisms. Lectins, proteids, which bind highly specific to carbohydrates, mediate the contact of foreign objects to haemocytes and support nonself recognition (Dunphy & Thurston, 1990). The prophenoloxidase activating system (proPO-AS), a complex enzyme cascade, which participates in wound healing and encapsulation, is also involved in nonself recognition (Dunphy & Thurston, 1990).

When haemocyte contact is achieved, smaller particles can be engulfed by haemocytes (**phagocytosis**) and by formation of a particle-haemocyte coagulum, which is finally walled of by plasmatocytes (**nodule formation**). Larger particles, like nematodes, can be entrapped by a multilayer capsule of haemocytes (**cellular encapsulation**). Both, nodules and cellular capsules are normally hardened and melanized by the activation of the proPO-AS, which is released from attached or free haemocytes (Dunphy & Thurston, 1990). Microorganisms can also be eliminated by bactericidal enzymes like the constitutive **lysozyme**, and nonconstitutive **antibacterial proteins** (cecropins, attacins, sarcotoxins, diptericins), which are synthesized *de novo* after vaccination or injury. Amino acid analysis of three major diptericins isolated from *Phormia terranovae* revealed their difference from the other three groups (Dimarcq *et al.*, 1986). An encapsulation of foreign bodies only with melanin (**humoral encapsulation**) could so far only be found in certain dipteran families characterized by a limited number of haemocytes (Götz, 1986).

3. Interactions of the nematode/bacteria complex with the defense mechanisms

3.1. Hemolymph nonself-response against nematodes

After nematode penetration, the insects nonself-response system only deals with the nematode. The bacteria are harboured in the intestine of the invading nematode and are released 2-5h post infection (Dunphy & Thurston, 1990). There is no visible defense reaction against the nematode in many insects. However, the absence of a response to the nematodes could not be attributed to a suppression of the nonself-response system in *G. mellonella*. Instead, nematodes evade haemocyte recognition. Dunphy & Webster (1987) showed, that lipoid components of the epicuticle of dauerjuveniles (DJs) are responsible for the lack of nonself recognition. The absence of the suppression of nonself-response systems is advantageous for the nematode-bacteria complex, since contaminating microorganisms can be excluded by wound healing or eliminated by antibacterial response. The symbiotic bacteria can thus be voided into a noncompetitive environment with favorable conditions for bacterial and, subsequently, nematode reproduction. The absence of defense reaction is obviously not restricted to the DJ, as axenic *S. feltiae* injected into *G. mellonella* larvae can develop to adults in the living insect without being eliminated (unpublished results).

Nonself responses achieved by humoral encapsulation of steinernematid DJs have been observed in dipterans of the family Culicidae (Poinar & Leutenegger, 1971). Besides, cellular encapsulation of *Steinernema* spp. is reported from the dipteran *Tipula*

spp. (Peters & Ehlers, submitted), from the chrysomelid beetle *Diabrotica virgifera virgifera* (Jackson & Brooks, 1989) and the weevil *Hylobius abietis* (Pye & Burman, 1978). Interestingly, encapsulation of *Heterorhabditis* spp. has so far never been reported. In larvae of *Tipula oleracea* and *T. paludosa* DJs of all tested *Steinernema* spp. were encapsulated while those of *Heterorhabditis* sp. were not (Sulistyanto *et al.*, 1994). Encapsulation of invading nematodes does not necessarily prevent insect death, especially when a large number of nematodes invade. In tipulid larvae infected with *S. feltiae* the symbiotic bacteria *X. bovienii* was detected in the haemolymph, although all nematodes had been encapsulated indicating that the nematodes have released their bacteria before complete encapsulation (Peters & Ehlers, submitted).

The encapsulation process is also influenced by the symbiotic bacteria. The proPO-AS, which is responsible for the hardening and melanisation of the capsule, is released into the haemolymph in *G. mellonella* during haemocyte lysis (see below). Lipopolysaccharides (LPS) from the surface of the bacteria, which are released in *G. mellonella* haemolymph, suppress the conversion of phenoloxidase from prophenoloxidase and thus can protect the nematode (Dunphy & Webster, 1991).

3.2. Haemolymph nonself-response against the symbiotic bacteria

The virulence of the symbiotic bacteria *Xenorhabdus* spp. against insects shows considerable variation between both bacteria and host species. *Xenorhabdus* spp. proved to be highly virulent against *G. mellonella* with the exception of *X. poinarii* which must be considered as nonpathogenic to this host (Yeh & Alm, 1992). The high level of bacterial virulence in *G. mellonella* could not be attributed to the absence of antibacterial response. Initially the bacteria are removed from the haemolymph by adhesion to haemocytes. They tolerate this adhesion and are even capable of multiplication during haemocyte contact. About 6 h post injection, the bacteria reenter the haemolymph. LPS, which dissociates from the bacteria cell wall, was determined to be the haemocytotoxin (Dunphy & Thurston, 1990). Insects other than *G. mellonella* are less susceptible to *Xenorhabdus* spp. which can be explained by differences in the defense reactions of these insects. *Xenorhabdus* spp. are not susceptible to lysozyme but are lysed by the antibacterial protein cecropin resulting in high LD_{50} (500,000 cells/pupa) against immune versus nonimmune pupae of *Hyalophora cecropia*. Protein secretions of the nematode, however, destroy the cecropin (Götz et al., 1981), another evidence for the close symbiotic relation of *Steinernema* and *Xenorhabdus*. The immunoinhibiting effect of *S. carpocapsae* secretions was also found in *G. mellonella*, *Bombyx mori* and *Tenebrio molitor*. Interestingly, the antibacterial proteins of *Phormia terranovae* (diptericins) were not inhibited (N. Simoes pers. comm.). Larvae of *T. oleracea* also showed a low susceptibility to *X. bovienii* (LD_{50} = 18,200 cells/larva). Since these larvae were not immunized, the bacteria were presumably removed by cellular mechanisms rather than by antibacterial proteins. After injection of 20 monoxenic nematodes, which harbour approximately 2,000 bacteria, 90% larval mortality is achieved, indicating that the cellular response against the bacteria is diminished by the nematode. The presence of the nematode may reduce bacterial removal either by active suppression of haemocyte phagocytic activity or by binding haemocytes in the cellular capsules and, subsequently, lowering haemocyte concentration.

4. Conclusions

Steinernematids and heterorhabditids together with their symbiotically associated bacteria have evolved ingenious mechanisms to evade the elimination by the insect's nonself-response mechanisms and at the same time take advantage of these mechanisms to achieve favourable conditions for reproduction. The interactions of nematode and bacteria demonstrates, that the nematode is not just acting as a living syringe introducing the bacteria, which then kill the host. Both elements of this symbiosis act synergetically in overcoming insect defense mechanisms and in killing the insect. Studies on the interaction of these nematodes with the insect's nonself-response system will be more fruitful with those insect species, which encountered these nematodes during their phylogenetic history. The model organism *G. mellonella*, which never contacts entomopathogenic nematodes under natural conditions might be an exception in its interaction with the nematode/bacteria complex and findings on this host cannot necessarily be transferred to other insects.

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QUANTIFYING INTERACTIONS AMONG HERBIVOROUS LARVAE, BACILLUS THURINGIENSIS δ-ENDOTOXIN, AND PLANT ALLELOCHEMICALS

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Summary
Qantifying mortalities of neonate larvae in plant and dietary bioassays can indicate on the type of interaction. Cotton condensed tannin antagonized the insecticidal activity of Bacillus thuringiensis (Bt) δ-endotoxin against Heliothis virescens. A detailed definition of types of interactions at tritrophic levels (insect-microbe-allelochemical) was made by recording the nutritional indices of relative growth rate (RGR) and relative consumption rate (RCR) in mature larvae. With these indices tannin antagonism to insect - microbe interactions was confirmed, whereas mandibular adduction force of the larvae in these interactions was not affected. This antagonism was obtained also with alginate encapsulated CryIA(c) δ-endotoxin and/or tannin. The encapsulation physically isolated the allelochemical and the endotoxin and avoided interaction with the diet. It also reduced larval chemosensing of the tannin. It is suggested that midgut effects on larval feeding and the microbial toxin may be included in the interactions.

Introduction

The role of tritrophic interactions in pest control became more realistic in view of the increased effectiveness and persistence of microbial toxins. Also, plant breeding for insect-hostplant resistance based on elevating levels of allelochemicals may increase the effect of the phytochemicals on microbes and insects. So far, the knowledge of interactions among a crop plant, its target pests and the Bacillus thuringiensis (Bt) applied for pest control has been limited to few studies (Reichelderfer, 1991). Quantifying diverse effects at tritrophic levels would be useful to understand interaction mechanisms and may become helpful guidelines for developing rational microbial pest management strategies. A start in this direction was made with describing interactions among Heliothis virescens, CryIA(c) δ-endotoxin of Bt and cotton condensed tannin (Navon et al., 1992, 1993).

Insect - microbe - plant interaction bioassays

1. Insect- allelochemical

a. leaf bioassays. Leaves of high and low tannin cultivars of cotton plants were exposed for three days to neonates of H. virescens. The commercial cultivar Acala SJ2 was used as control. Larval survival was reduced to about 50% that obtained with the commercial cultivar. Tannin

levels in the high tannin cultivars averaged 0.89 ± 0.13 (SE) mg/g. The commercial cotton was essentially tannin-free.

b. Dietary bioassays. Cotton condensed tannin was bioassayed against H. virescens neonates for seven days. The EC_{50} of the tannin was 0.25 mg/g. At 2.0 mg the reduction in weight gain acceeded 90%. Mortality due to tannin ranged 10-20%.

2. insect-microbe

The dietary bioassay procedures were similar to potency bioassays (Navon et al., 1990). The LC_{50} determined for CryIA(c) δ-endotoxin in H. virescens was 27.0 ng/mg. The mortality doses recorded in this bioassay were used in combinations with effective levels of tannin in tritrophic interaction bioassays.

3. insect-microbe-allelochemical

a. Bioassays with neonate larvae. effective diatery concentrations of tannin and δ-endotoxin were used in this tritrophic bioassay among H. virescens neonate larvae, CryIA(c) δ-endotoxin of B. thuringiensis subsp. kurstaki strain HD-73, and cotton condensed tannin. At tannin concentrations of 0.4 mg/g and 0.8 mg/g the LC_{50} was 30 ng/mg and 34 mg/mg respectively, close to the mortality obtained with endotoxin alone. However, Tannin level of3.2 mg/g almost doubled the LC_{50} (59.1 ng/g), whereas tannin alone in the diet at these concentrations did not cause significant mortality. This means that the allelochemical antagonized insect-microbe interactions probably dues to either reduced feeding and/or direct interaction between the allelochemical and the Bt endotoxin. The reduced weight gain obtained with tannin may reflect low consumption of diet which means also less endotoxin ingested by the larvae.

b. Feeding bioassays with mature larvae. Quantifying larval feeding in tritrophic interactions was based on two indices. Relative growth rate (RGR) = larval weight gain / initial larval weight per 24 h. The relative condumption rate (RCR) expressed weight of diet consumed / initial larval weight per 24 h. The calculations were based ondry weights. In separate bioassays, the microbial endotoxin and tannin were encapsulated with an alginate gel. Two μl droplets of endotoxin or tannin in aqueous sodium alginate mixture were gelled in a setting solution of calcium chloride.The microcapsules were mixed into the diet. The encapsulation provided physical isolation among endotoxin crystals, tannin and the diet. The alginate capsules ingested indiscriminately. Disintegration of the alginate capsules in the larval midgut were observed.
Low RGR was obtained with both unencapsulated and encapsulated endotoxin (Table 1). This RGR was increased in the presence of tannin, indicating that cotton condensed tannin antagonized the microbial intoxication. RCR in diets with tannin were lower than in the control and even somewhat lower in the endotoxin + tannin. Probably tannin caused the larva to ingest less endotoxin due to feeding deterrency caused by the

allelochemical. However, also direct interaction of tannin with endotoxin may explain the reduced microbial intoxication. RGR and RCR values in both unencapsulated and encapsulated forms were similar (Table 1) and also the encapsulation avoided interaction between tannin and endotoxin in the diet. Therefore, it is suggested that the antagonistic interaction occurred also in at postingestion, probably in the midgut.

Table 1. Relative growth rate (RGR), relative consumption rate(RCR) and mandibular adduction force of fifth instar larvae H. virescens fed for 24 h on a diet with CryIA(c) δ-endotoxin and/or cotton condensed tannin.

Bt CryIA(c) and tannin	RGR (mg/mg/24 h)	RCR	Mandibular adduction Wt., g.	Force,dyne
10.0 μg/g CryIA(c)	-0.20d*	0.45cd	17.1a	33,516a
2.4 mg/g tannin	0.57b	2.73b	18.0a	32,280a
10.0 μg/g CryIA(c) + 2.4 mg/g tannin	0.10c	0.14c	16.8a	32,928a
Control	0.75a	5.75a	17.5a	34,300a
encapsulated δ-endotoxin and tannin				
10.0 μg/g CryIA(c)	0.14c	0.45c		
2.4 mg/g tannin	0.13c	2.64b		
10.0 μg/g CryIA(c) + 2.4 mg/g tannin	0.42b	0.15c		
Control	0.82a	4.35a		

* ($p > 0.05$) in each column, ANOVA and the SNK test.

4. Mandibular adduction force of mature larvae in tritrophic interactions

An assay of mandibular function was developed by means of a mandibular adduction force (MAF) gauge. The calibration of the gauge was made by measuring weight load and comparing with weight calculated from a formula of mechanical behaviour of elastic cantilevel beam. Fifth instar larvae from the tritrophic interaction bioassay were tested for MAF. The microbial endotoxin and tannin at various combinations (Table 1) did not affect the MAF although larval growth and consumption were significantly reduced. These results suggest that gut paralysis after ingestion of Bt δ-endotoxin is not associated with mandible dysfunction.

Concluding remarks. Antagonistic effects of tannin in insect - δ-endotoxin interactions indicate that possibly breeding cotton for high-

tannin cultivars may not fit into microbial pest management with Bt. On the other hand, increasing allelochemical defenses in plants may delay the development of resistance to Bt. Therefore, a rational use of the plant chemical defense system in combination with Bt insecticides or Bt transgenic crops would be needed. This use will require an increased knowledge of herbivore-microbe-allelochemical interactions.

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INDUCTION AND REGULATION OF IMMUNE REACTIONS IN *GALLERIA MELLONELLA* (LEPIDOPTERA)

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Summary

Phagocytosis and encapsulation by haemocytes along with the appearance of antibacterial proteins in cell free haemolymph are well known effector mechanisms of the insect immune response. In studies of the wax moth *Galleria mellonella* these defence reactions were elicited by synthetic material and by haemolymph factors. Intrahaemocoelic injections of hydrophilic silica beads, positively charged ion exchanger beads or protein loaded latex beads caused strong increases of antibacterial activities in haemolymph of injected larvae. In contrast, hydrophobic, negatively charged or unloaded beads provoked only slight reactions. Humoral immune reactions could also be induced by haemolymph transfer. The inductive haemolymph factors proved to be heat-resistant and molecule fractions >30 <100 kDa were most effective. Immune responses of larvae injected with synthetic beads or haemolymph factors exhibited no differences to larvae immunized with bacteria. Intensities of responses and protein patterns after SDS-PAGE or native PAGE were equal. In subsequent experiments it was shown that - during phagocytosis of silica beads *in vitro* - certain haemocyte types (plasmatocytes) release factors which likewise activate the humoral response in *G. mellonella.*
Background informations, description of basic methods, summarized results and some future perspectives for the possible utilization of such studies in biological control are given.

1. Background

Insects include about 80% of all known animal species. It goes without saying that this evolutionary success could only be achieved by organisms with powerful immune systems. But what makes the insect immune system so effective? Insects posses no specific antibodies, in all probability no complement cascade, no specific immunological memory, and no long-term immunity (Götz and Boman, 1985). All essential characteristics of the vertebrate immune system seem to be missing.
Insect haemocyte defence including phagocytosis and encapsulation of non-self targets is well documented (Gupta, 1991), but our knowledge about the induction and regulation of haemocyte behaviour in arthropods is still limited (Lackie, 1988; Söderhäll, 1992). The antibacterial haemolymph proteins responsible for the temporal increase of antibacterial activity during humoral immune reactions were intensively studied in Lepidoptera (Boman et al., 1991) and Diptera (Hoffmann et al., 1993). Numerous amino acid sequences have been established and encoding genes are under investigation. Despite of this, regulatory mechanisms are mostly unclear (Chadwick and Aston, 1991). The presented investigations were conducted with the intention of attaining more informations concerning the recognition of non-self and the induction of immune reactions. Synthetic provocators and haemolymph factors were used to induce immune responses in last instar larvae of the wax moth.

2. Basic methods

2.1. Detection of antibacterial activity

For measurements of antibacterial activity in cell free haemolymph an inhibitory zone assay against *Escherichia coli* (Faye and Wyatt, 1980) and a lytic zone assay against *Micrococcus luteus* (Mohrig and Messner, 1968) are widely used in our lab. Handling of the test plates is uncomplicate, the required testvolume is small (3 μl) and standardization can be done with antibiotics. These assays enable the quantification and statistical evaluation of antibacterial humoral responses (Wiesner, 1992).
A combination of PAGE (polyacrylamid gel electrophoresis) with the principle of the zone assays allows the detection of protein bands with antibacterial activity. After separation of the haemolymph probes gels are overlayed with an agar suspension containing bacteria and incubated overnight. Protein bands with antibacterial activity produce clear zones in the agar layer because of inhibition of bacterial growth or lysis of bacterial cell walls (Hoffmann et al., 1981).

2.2. Isolation of phagocytic active haemocytes

The main phagocytic haemocytes in *G. mellonella* haemolymph are the plasmatocytes which account for about 52% of the haemocyte picture (Wiesner, 1991). Plasmatocytes can be isolated by density gradient centrifugation (Mead et al., 1986; Wiesner, 1986) or by using nylon wool columns (Wiesner and Götz, 1993). The resulting monolayers consist of >90% plasmatocytes and are best suited to study phagocytosis (Wiesner and Götz, 1993).

3. Induction of immunity by synthetic provocators

In comparison to biotic provocators like bacteria, inert synthetic material comprises several advantages: The surface characteristics are defined, modifiable to meet specific requirements and no soluble provocator substances can interfere with the test system.
In experiments with *G. mellonella* synthetic provocators (latex-, silica-, ion exchanger-beads) were injected and the subsequent onset of antibacterial activity in haemolymph was determined. It could be shown that hydrophilic or positively charged beads provoked stronger immune reactions than hydrophobic or negatively charged ones. Furthermore, the induction capacity of latex beads could be enhanced by surface coupled proteins. This effect was reached with fibronectin, peptides possessing the cell adhesion sequence RGD, haemolymph molecules, and also with mixtures of foreign proteins like bovine serum albumine. Further results and detailed discussions are given in Wiesner (1992) and Wiesner and Götz (1993).

4. Induction of immunity by haemolymph factors

Immune responses in insects are inducible without the involvement of any non-self target like bacteria or synthetic material but only by the transfer of haemolymph factors. In experiments with *G. mellonella* it was shown, that the injection of haemolymph preparations led to the induction of strong humoral responses. The inducing capacity of cell free haemolymph was higher if donors were preinjected with latex beads (Wiesner, 1991), lysis of haemocytes in donor haemolymph enhance the induction capacity, the inducing activity is heat resistant and molecule fractions $<100 >30$ kDa are most effective (Wiesner, 1993).

5. Comparison of differently induced humoral responses

The humoral responses induced by injection of synthetic beads or haemolymph factors exhibited no differences to bacterially induced immunity. The intensity of antibacterial activity as well as the haemolymph protein patterns in SDS-PAGE and the position of antibacterial proteins detected in the overlayed gels after native PAGE were equal. At least six new or enhanced bands (in the range of 15, 17, 19, 25, 52, 135 kDa) were detectable in haemolymph of immune animals after SDS-PAGE (Wiesner, 1993). The overlayed gels exhibited up to four bands with growth inhibition activity against *E.coli* and one band with lytic activity against *M. luteus*. This is in good accordance with the results of Hoffmann et al. (1981).

6. Signal connections between cellular and humoral response

During the experiments cited above it could be noticed that enhanced humoral reactions occurred only if there was strong cellular defence against the injected material. This fact together with the results from the haemolymph transfer experiments supported the idea that defending haemocytes release factors which induce the synthesis of antibacterial proteins. Therefore the following experiment was designed: Supernatants from monolayers of isolated plasmatocytes were injected into *G. mellonella* larvae and the induced humoral response was recorded. It could be shown that the inducing capacity of the monolayers was significantly enhanced if the plasmatocytes were active in phagocytosis (Wiesner and Götz, 1993). This result gives evidence for the existence of a haemocyte derived activating factor.

7. Possible utilization in biological control of insects

The studies outlined above offer the possibility to create experiments which couple basic research on insect immunity with the practical needs of biological control:
To 3.: Experiments with synthetic provocators make it possible to define which surface characteristics a pathogen should have to be effective in the avoidance or inhibition of the host defence. This would allow to search for new potentially useful pathogens by selecting those with suitable surfaces.
To 4.: The isolation of the haemolymph factors responsible for the induction of humoral immunity will be important for the development of more selective strategies in overcoming the insect defence system.
To 5.: The similarities between the immune reactions against bacteria and synthetic provocators prove the suitability of this artificial system for studying insect-pathogen interactions.
To 6.: The evidence for the existence of a signal connection between cellular and humoral responses is a very important aspect. Isolation of the haemocyte derived activating factors would enable the search for selective inhibitors of this compound which is probably of central importance in the induction process.

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Pathogenicity of new combinations of *Heterorhabditis* spp. and *Photorhabdus luminescens (Xenorhabdus luminescens)* against *Galleria mellonella* and *Tipula oleracea*.

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INTRODUCTION

Insect pathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are symbiotically associated with bacteria of the genus *Xenorhabdus* and *Photorhabdus*, respectively (Boemare *et al.*, Thomas & Poinar, 1979). The infective dauerlarvae of the nematode carry the bacterial symbiont in the intestine. The nematode penetrates an insect host, moves into the haemocoel, and releases the bacterium. The bacterium starts multiplying and kills the host, supported by excretion products of the nematode that repress the immune system of the insect. The symbiotic bacteria further produce antibiotics to inhibit growth of other micro-organisms in the insect cadaver and provide nutrients utilized by the nematodes (Akhurst, 1982; Gerritsen *et al.*, 1992). Each *Steinernema* species is associated with its own *Xenorhabdus* species (Akhurst & Boemare, 1988). All *Heterorhabditis* spp. have *P. luminescens* as the symbiont, but the species *P. luminescens* is composed of several DNA homology groups which might also be considered separate species (Smits & Ehlers, 1991). Ehlers *et al.* (1990) showed that it is possible to grow *Steinernema* spp. on bacteria other than their own symbiont. Han *et al.* (1990) exchanged bacteria between *Heterorhabditis* spp. with the aim of separating *P. luminescens* subspecies. In our experiments the purpose was to make new combinations of *Heterorhabditis* and *Photorhabdus* isolates and find possible differences in pathogenicity between these combinations. By combining the best nematode with the best bacterium the effectivity of the nematodes as biological control agent might be improved. This paper deals with the possibilities of exchanging bacteria between nematodes and the effect this has on the pathogenicity of these new combinations against a susceptible host, *Galleria mellonella*, and a more resistant host, *Tipula oleracea*.

MATERIALS AND METHODS

Nematode and bacteria isolates

Table 1 lists the sources of the nematodes and bacteria used. Bacteria were isolated from the haemocoel of waxmoth larvae (*Galleria mellonella*) infected by nematodes. The bacteria were cultured on nutrient agar (Lab Lemco, Oxoid) at 25°C.

Monoxenization of nematodes

The method for axenization of the nematodes was described by Gerritsen and Smits (1993). First-generation hermaphrodite nematodes with eggs were disintegrated in 0.4M NaOH, this way producing sterile eggs. J1 juveniles hatched from these eggs, were put on a 4-day-old bacterial lawn on lipid agar in a Petri dish. The Petri dishes were incubated at

25°C, in the dark. The infective juveniles produced on the Petri dishes were stored in sterilized tap water in tissue culture-flasks at 5°C (HE, Hjun and HF) or 10°C (*H. bacteriophora*). In the bioassay tests only nematodes that had been stored for 3 to 5 days were used.

Tests on pathogenicity and penetration of the combinations

Pathogenicity of the nematode/bacterium combinations was tested using a sand-column-bioassay as described by Gerritsen and Smits (1993). A cylindrical plastic container with a *Galleria mellonella* or *Tipula oleracea* larvae at the bottom was filled with moist sand. Infective juveniles were added to the sand surface in 1 ml tap water; the container was capped and incubated at 25°C. After 1 day the insect larvae were removed from the sand and incubated separately at 25°C for 6 days. Mortality was assessed. To assess the penetration rate of the nematodes, the dead insect larvae were dissected and the first-generation hermaphrodites were counted. Each treatment consisted of 30 containers and was done in three replicates. The results were statistically analyzed by analysis of variance. Pairwise comparisons were made by least-significant-difference-test (LSD).

Table 1
Origin of *Heterorhabditis* spp. and *P. luminescens* strains

Nematode species or strain	RFLP* group	Nematode code	Bacterial code	Original place of isolation
Heterorhabditis NLH-E87.3	1	HE	PE	Eindhoven The Netherlands
Heterorhabditis NLH-F85	1	HF	PF	Flevoland The Netherlands
Heterorhabditis NLH-jun	1	Hjun	Pjun	Papendal The Netherlands
H. bacteriophora	2	Hbac	Pbac	Brecon S-Australia
Heterorhabditis H-mol	2	Hmol	Pmol	Moldavia GIS

* RFLP groups of *Heterorhabditis*: 1. North-West-European (NWE) group.
2. *H. bacteriophora* group. From: Smits & Ehlers, 1991.

RESULTS AND DISCUSSION

Recombinants

The nematode/bacterium combinations can be separated into four groups (Tables 1, 2, 3, 4); I: a group of NWE-nematodes with NWE-bacteria (HE, HF and Hjun with PE, PF and Pjun) II: a group of NWE-nematodes with *H. bacteriophora* bacteria (HE, HF and Hjun with Pbac and Pmol), III: a group of *H. bacteriophora* nematodes with NWE-bacteria (Hbac and Hmol with PE, PF and Pjun), and IV: a group of *H. bacteriophora* nematodes with *H. bacteriophora* bacteria (Hbac and Hmol with Pbac and Pmol). The combinations within these groups mostly act alike and in the remainder of this paper I will refer to these groups.

Most combinations in group II can not be made (tried five times or more). HE, HF and Hjun are unable to develop on Pmol, and HF and Hjun are unable to develop on Pbac (Table 2,3). The combination HEPbac is the exception in group II, the only combination that can be made.

Pathogenicity against *G. mellonella*

Table 2 shows the pathogenicity of the combinations against *G. mellonella*. The combinations of *H. bacteriophora* nematodes with bacteria of the NWE group (group III) are not able to kill *G. mellonella* at a dose of 100 nematodes per larva. All other combina-

tions give 100% mortality with only 30 nematodes per larva (combinations with Pjun were not tested). Gerritsen and Smits (1993) show that this lack of pathogenicity is caused by the lack of bacteria in the gut of the nematode. Figure 1 shows that when you use HbacPE you need a hundred times more nematodes to get the same mortality than when using HbacPbac. From all *G. mellonella* larvae killed by HbacPE, PE could be isolated. This suggests that only 1% of the dauer juveniles of HbacPE actually carried the bacterium PE. The nematodes that do not carry any bacteria in their gut are not able to kill *G. mellonella*, this in contrast to *Steinernema* spp. that can kill insects without their bacterial symbiont (Akhurst, 1983; Dunphy *et al.*, 1985).

All pathogenic combinations (+ in table 2) have equal pathogenicity; 100% mortality with 30 nematodes, 30% mortality with 1 nematode (Gerritsen and Smits, 1993). This shows that *G. mellonella* is too susceptible to detect smaller differences in pathogenicity.

Table 2
Pathogenicity of *Heterorhabditis / Photorhabdus* combinations against *Galleria mellonella* in bioassays with 30 or 100 nematode per larva.

bacterium / nematode	PE	PF	Pbac	Pmol
HE	+	+	+	n
HF	+	+	n	n
Hbac	-	-	+	+
Hmol	-	-	+	+

+ = combination pathogenic, - = combination not pathogenic, n = combination not possible

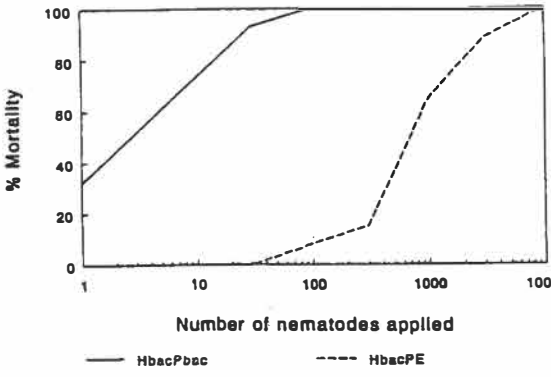

Figure 1
Mortality (%) of *Galeria mellonella* larvae exposed to different numbers of two nematode/bacterium combinations

Table 3
Mortality (%) of *Tipula oleracea* larvae exposed to different nematode/bacterium combinations in bioassays with 300 nematode per larva.

bacterium / nematode	PE	Pjun	Pbac	Pmol
HE	78	91	44	n
Hjun	75	68	n	n
Hbac	0	1	4	37
Hmol	0	7	1	54

n = combination not possible

Table 4
Penetration of J3 juveniles (% J3 penetrating) of different nematode/bacterium combinations in *T. oleracea* larvae in bioassay with 300 nematodes per larva

bacterium / nematode	PE	Pjun	Pbac	Pmol
HE	28	25	22	n
Hjun	25	13	n	n
Hbac	/	/	/	8
Hmol	/	2	/	9

n = combination not possible,
/ = no dead *T. oleracea* larvae

Pathogenicity against *T. oleracea*

Table 3 shows the pathogenicity of the combinations against *T. oleracea*. The combinations in group III have a very low pathogenicity. The combinations with PE are again not pathogenic at all, while the combinations with Pjun do have some pathogenicity. In these combinations probably more than 1% carries bacteria in their guts (not tested yet). The combinations with Pmol in group IV give a higher mortality than combinations with Pbac. Also the combination of HE with Pbac gave significantly less mortality than HE in combination with PE or Pjun. Pbac seems to be a less pathogenic bacterium, although in combination with a 'good' nematode (HE versus Hbac and Hmol) it still gives some mortality. When comparing the nematodes, regardless the bacterium, the NWE-nematodes give a higher mortality than the *H. bacteriophora* nematodes. Table 4 shows that the penetration rate of the NWE-nematodes is higher than the penetration rate of the *H. bacteriophora* nematodes (this is more clear in Gerritsen and Smits, 1993). So the more nematodes are able to find and penetrate the insect, the higher the mortality. Whether *H. bacteriophora* is less able to penetrate the insect, or moves more slowly because of its size (smaller than NWE-nematodes), is not clear.

Table 3 shows, as already said, that Pmol is more pathogenic than Pbac. Comparing PE and Pjun in the same way, we see other differences. In combination with HE, PE is less pathogenic than Pjun. But in combination with Pjun, PE is more pathogenic than Pjun. This shows that there is not just a 'better' bacterium and a 'better' nematode, but there is also interaction between bacterium and nematode.

CONCLUSIONS

- nematodes of the NWE-group are not able to grow and multilpy on *H. bacteriophora* bacteria
- some combinations do not carry bacteria
- *H. bacteriophora* nematodes without bacteria are not able to kill insect larvae
- the pathogenicity of *H. bacteriophora* strains against *Tipula* is low, probably because of the low penetration rate of these strains
- the pathogenicity of a combination is determined by the pathogenicity of the bacterium, the pathogenicity of the nematode and the interaction between them

Acknowledgements

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3. Meeting of the subgroup entomopathogenic nematodes.

THE EFFECT OF TEMPERATURE ON THE SUSCEPTIBILITY OF THE BLACK VINE WEEVIL, *OTIORHYNCHUS SULCATUS* TO DIFFERENT ISOLATES OF *STEINERNEMA* AND *HETERORHABDITIS*

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Summary

Temperature profiles were obtained for two isolates of *S. carpocapsae* and two isolates of *Heterorhabditis* against black vine weevil larvae. Efficacy was clearly delineated for each isolate: *S. carpocapsae* (14°C - 33°C), Nemasys-H. (14°C - 28°C) and Fightagrub (14°C - 33°C).

Introduction

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are lethal parasites of a wide range of insect hosts. They act as the vectors for specific bacteria in the genus *Xenorhabdus* which are harboured in the anterior intestine of the third stage infective juvenile (IJ). The IJs enter through the natural openings of the host and also through the cuticle (*Heterorhabditis*). Inside the haemocoel the bacteria are released, causing septicaemia: the development of the bacteria provide excellent conditions for nematode development, producing thousands of new IJs which is the basis of the commercial exploitation of these nematodes.

The black vine weevil, *Otiorhynchus sulcatus* is a major pest in nurseries, glasshouses and many outdoor crops world wide (Bedding and Miller, 1981) and due to the banning of organochlorine insecticides entomopathogenic nematodes have become an important alternative for control of this insect (Klein, 1990). Temperature is a major limiting factor for the effectiveness of entomopathogenic nematodes (Georgis and Gaugler, 1991) particularly against *O. sulcatus* (Dolmans, 1983). The present experiments were done to investigate the range of temperature activity of three commercial isolates against *O. sulcatus*.

Material and Methods

Late instar larvae of *O. sulcatus* were used for these experiments. IJs of the different isolates were reared in *Galleria mellonella*, extracted by the modified White trap (Poinar, 1975) and stored in a refrigerator at 7°C (*Steinernema*) or 15°C (*Heterorhabditis*) for not more than one week before use.

200 IJs were pipetted evenly onto sand which had been washed, autoclaved and oven-dried, in a 3.5 x 1.5 cm petri-dish: one *O. sulcatus* larva placed on top of the sand and the petri-

dish sealed; the water content of the sand was 15-18 %. The inoculated petri-dishes were placed on a temperature gradient plate (Murdoch et al., 1989) the overall dimension of which were 711 x 711 mm. The area of the plate was sub-divided into 169 small squares by a 13 x 13 polystyrene matrix so that each cell could contain a single petri-dish which was placed directly on the copper plate. To prevent loss of heat to the surrounding environment the whole of the polystyrene matrix was covered with a triple-glazed perflex lid.

The nematodes tested were:

a) *S. carpocapsae* - the ALL isolate, origin Georgia, USA and the UK isolate, origin South Wales, UK. Both isolates are commercialised by Biosys under the trade name BioSafe.

b) *Heterorhabditis* - the UK isolate NEMASYS-H commercialised by AGC, UK and the UK isolate FIGHTAGRUB commercialised by FARGRO, UK.

The *O. sulcatus* larvae were exposed to the nematode isolates for 6 days at temperatures from 8°C - 35°C: the vine weevil larvae were removed, washed and dissected. The number of developed nematodes were counted: replication was six-fold and data were analyzed using a SAS program. The effect on un-inoculated nematodes was investigated by exposing vine weevil larvae to the same temperature range and estimating mortality after 6 days.

Results

For the 4 nematodes investigated, establishment in vine weevil larvae occurred between 14°C and 33°C: the temperature profile of the *S. carpocapsae* isolates were very similar but those for the Heterorhabditis were dissimilar (Fig.1). More Heterorhabditis nematodes entered larvae than Steinernematids (Fig.1) and the mortality was greater (Fig.2): uninoculated vine weevil larvae were susceptible to increased temperature (Fig.2).

Discussion

Three of the isolates tested in these experiment, *S. carpocapsae* (ALL and UK) and Fightagrub established in vine weevil larvae over the temperature range 14°C - 33°C: Nemasys-H did not infect above 28°C. All four isolates did not establish below 14°C thus conforming the conclusion of Georgis and Gaugler (1991) that entomopathogenic nematodes are not very effective below 16°C. The temperature profiles for *S. carpocapsae* isolates are similar to these obtained by Otto et al., (unpublished) with *G. mellonella* as host. Since vine weevil larvae are rather susceptible to increased temperature on the gradient plate (Fig.2), *G. mellonella* may be a better host for testing nematodes above 25°C.

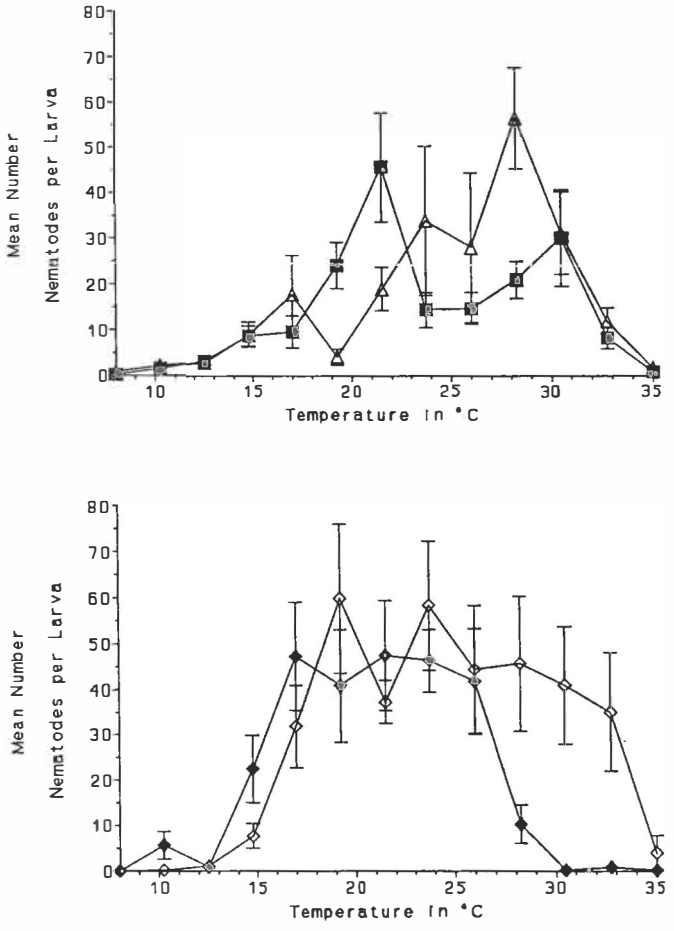

Figure 1. The mean number of nematodes established in *O. sulcatus* after 6 days exposure: ■ - *S. carpocapsae* ALL : Δ - *S. carpocapsae* UK : ◇ - Fightagrub : ◆ - Nemasys-H.

The two Heterorhabditis established better in vine weevil larvae than the Steinernematids and are more effective i.e. estimates of mortality are higher (Fig.2). These results confirm the findings of other researchers that Heterorhabditis are generally very effective for controlling vine weevil (Bedding and Miller, 1981) but as yet Heterorhabditis cannot be produced and formulated commercially in such large packages as Steinernematids. The two UK isolates of Heterorhabditis have very different temperature profiles which suggests that they may be different species.

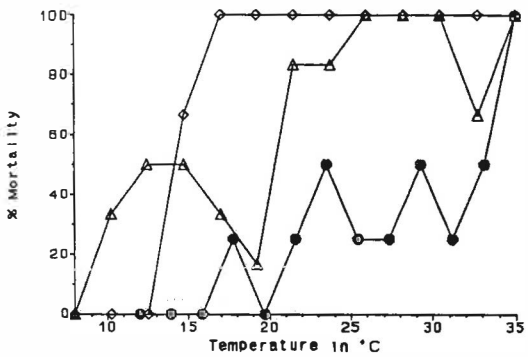

Figure 2. Mortality of *O. sulcatus* larvae after exposure to different temperatures for 6 days with and without nematodes:
◇ - Fightagrub : Δ - *S. carpocapsae* UK :
● - without nematodes.

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INFECTIVITY AND PATHOGENICITY OF THE INSECT PARASISTIC NEMATODES *HETERORHABDITIS* SPP. AND *STEINERNEMA* SPP. FOR *OTIORHYNCHUS SULCATUS* AT DIFFERENT TEMPERATURES

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Summary

A large number of isolates of insect parasitic nematodes were evaluated with respect to efficacy against black vine weevil, *Otiorhynchus sulcatus* in strawberries at low soil temperatures in climate rooms. No marked differences were found between heterorhabditids and steinernematids in efficacy at 20 °C. However, isolates responded very differently to low temperatures (9 or 12 °C). Three isolates were found to cause (almost) 50% control at 9 °C (HFsel, HUk211 and *S. kraussei* Mr) and these isolates, in addition to K122 and HB1'87, also performed well at 12 °C. Including a standard isolate for comparison of results between experiments is useless, because of the problem of interbatch differences.

1. Introduction

Insect parasitic nematodes of the genera *Heterorhabditis* Poinar and *Steinernema* Travassos are effective parasites of soil dwelling insect pests, however the nematodes do not operate well when soil temperatures are below 12-13 °C, which happens in North West Europe out of doors in spring and autumn (e.g. Dolmans, 1983; Westerman & Van Zeeland, 1989). In order to extend the application to periods and regions with lower soil temperatures, finding cold active isolates of the nematodes is necessary.

Control efforts focus on the black vine weevil, *Otiorhynchus sulcatus* F., because this is an increasingly serious pest of nursery stock, ornamentals and soft fruit such as strawberries, grapes and blackcurrants in most temperate regions in the world.

Heterorhabditids are generally more pathogenic towards *O. sulcatus* and other soil dwelling coleopterans than steinernematids (e.g. Bedding *et al.*, 1983; Georgis & Poinar, 1984). Steinernematids, on the other hand, tend to be more cold-active than heterorhabditids (e.g. Molyneux, 1986), although relatively cold active heterorhabditids have been found in the Netherlands (Simons & Van der Schaaf, 1986; Westerman & Van Zeeland, 1989).

The purpose of this study was to evaluate efficacy of a large number of *Heterorhabditis* and *Steinernema* isolates, including a number of commercially available isolates, for curative inundative control of *O. sulcatus* at low soil temperatures. A strain that was selected for infectivity at 9 °C for eight generations and its unselected counterpart were included. The influence of the bacterial symbiont at low temperatures was investigated by replacing the natural symbiont of a poor performing isolate by the symbionts of two well performing isolates, and including these recombinations in the efficacy experiments.

2. Materials and methods

Six experiments were carried out in the course of four years in climate rooms set at approximately 9 and 12 °C. In order to compare pathogenicity of steinernematids and heterorhabditids, one experiment was carried out at 20 °C (a non-limiting temperature) with six *Heterorhabditis* isolates (HF85, HNb87, HSH, HUk211, K122 and HP88) and seven *Steinernema* isolates (OBSIII, Uk76, SF1, Mr, Aus, U-M, and UK). For the first experiment at 9 and 12 °C a complete random design was used with 20 pots for both treatment and control. For experiment II a complete random block design was used with five blocks and again 20 pots both treatment and control. The final four experiments at 9 and 12 °C were carried out as complete random block designs with seven blocks, 21 pots per treatment and 49 pots for the control. The isolates used in each experiment are summarized in **Table 1**. For the 20 °C experiment a complete random block design was used, with 8 blocks, 32 replicates per treatment and 96 pots for the control.

The isolates HSH_{HF85} and $HSH_{HB1'87}$ were composed of the nematode HSH and the *Xenorhabdus* strain of either HF85 or HB1'87 (Gerritsen *et al.*, 1993). HFsel derived from HF85 but was selected for infectivity for *Galleria mellonella* (L.) in moist sand columns (4 mm high, 4 cm Ø) at 9 °C for eight selections rounds (Dr C.T. Griffin); HFIrl is the unselected origin.

In each experiment strawberry plants were used in potting soil in 0.7-0.8 l pots which were infested with 10 eggs of *O. sulcatus*. The plants were kept in the greenhouse at about 20 °C for approximately seven weeks. The plants were then transferred to climate rooms which were set at 9 and 12 °C, or 20 °C. A week after transfer groups of plants received nematodes of one of the isolates and one group of plants remained untreated. A dosage of 100 (living) nematodes per cm^2 was applied (7000-8000 nematodes per plant). At 9 and 12 °C the pots were sifted for living and dead insect larvae after six weeks. At 20 °C the experiment was terminated after four weeks. The actual temperature in the soil of the pots was measured using a CSU-thermistor temperature probe connected to a 1202 Grant squirrel datalogger.

Efficacy data were analysed using generalized linear regression after a logit-transformation on the number of living *O. sulcatus* larvae. Percentage effect was calculated (Abbotts formula). The percentage effect was related to the registered temperature by linear regression (models $y = a + b_1x_1 + \alpha$) for those isolates that were included in experiments more than once.

3. Results

Percentage effect at 20 °C was 100 % for all heterorhabditids and virtually 100 % for the steinernematids

The majority of the isolates gave poor efficacy results at 9 °C, but occasionally an isolate reached the 50 % control level: HFsel, HUk211 and *S. kraussei* Mr (**Table 1**). The nematodes of the selected HFsel were significantly better in controlling *O. sulcatus* than those of the unselected HF85 and HFIrl at 9 °C

At 12 °C generally a much higher level of control was achieved. Isolates responded differently to temperature; some performed poor at either temperature (e.g. *H. bacteriophora* B1 and *H. zealandica* NZH3), others did well at 12 but poor at 9 °C (e.g.

HB1'87, HKem, K122) and occasionally an isolate did relatively well at both temperatures (HFsel, HUk211, *S. kraussei* Mr). The recombinations HSH_{HF85} and $HSH_{HB1'87}$ gave higher control results than HSH with its natural symbiont, but these differences were not significant. Four isolates caused an almost complete control of *O. sulcatus* at, at least, one occasion; HF85, HUk211, K122 (exp. IV) and HB1'87 (exp. V).

Table 1. Control of black vine weevil, O. sulcatus, in strawberries (% effect) of various species and isolates of Heterorhabditis after 6 weeks at approximately 9 and 12 °C. (Percentages effect per experiment and temperature followed by the same letter are not significantly different from each other. Untreated control: 0%).

Exp. No.	isolate	efficacy [% effect] 9 °C	12 °C
I	HF85	19.2 a	71.8 b
	HFIrl	25.3 ab	65.9 b
	HFsel	53.5 b	80.0 b
II	HF85	28.1 ab	82.5 b
	HB1'87	38.6 b	76.3 b
	HB2'87	34.2 b	81.6 b
	HE87	18.4 ab	69.3 b
	HNb87	13.2 ab	61.4 b
III	HF85	7.2 a	58.0 cd
	HW79	3.1 a	79.5 de
	HKem	17.7 ab	89.7 e
	NZH3	-0.9 a	28.1 ab
	B1	-8.2 a	3.9 a
	HO1	13.6 ab	47.7 bc
	Mr	37.0 b	78.5 de
IV	HF85	36.3 ab	96.2 c
	HNb87	10.3 ab	63.6 b
	HSH	27.6 ab	57.4 b
	HUk211	47.4 b	94.0 c
	K122	22.2 ab	98.7 c
	HP88	15.7 ab	56.1 b
V	HF85	34.4 c	82.1 cd
	HB1'87	-4.6 a	98.5 d
	HSH	10.8 abc	59.8 bc
	HSH_{HF85}	11.7 abc	74.7 cd
	$HSH_{HB1'87}$	20.6 bc	73.9 cd
	M198	15.7 abc	71.7 cd
	OBSIII	15.7 abc	26.2 ab
VI	OBSIII	19.4 abc	41.0 b
	UK76	10.5 ab	39.8 b
	SF1	42.1 cd	87.5 de
	Mr	49.9 d	88.7 e
	Aus	43.9 cd	70.5 cd
	U-M	34.4 bcd	65.4 c
	UK	11.7 ab	59.7 bc

Efficacy at a certain temperature can be estimated from regression lines for efficacy versus the actual average temperature as registered during the experiments. This was done for the isolates

HF85, HB1'87, HNb87, HSH, *S. feltiae* OBSIII and *S. kraussei* Mr. (**Figure 1**). The results of HFIrl were included in the set of data for HF85 and the results of the recombinations HSH_{HF85} and $HSH_{HB1'87}$ were included in the set of data for HSH.

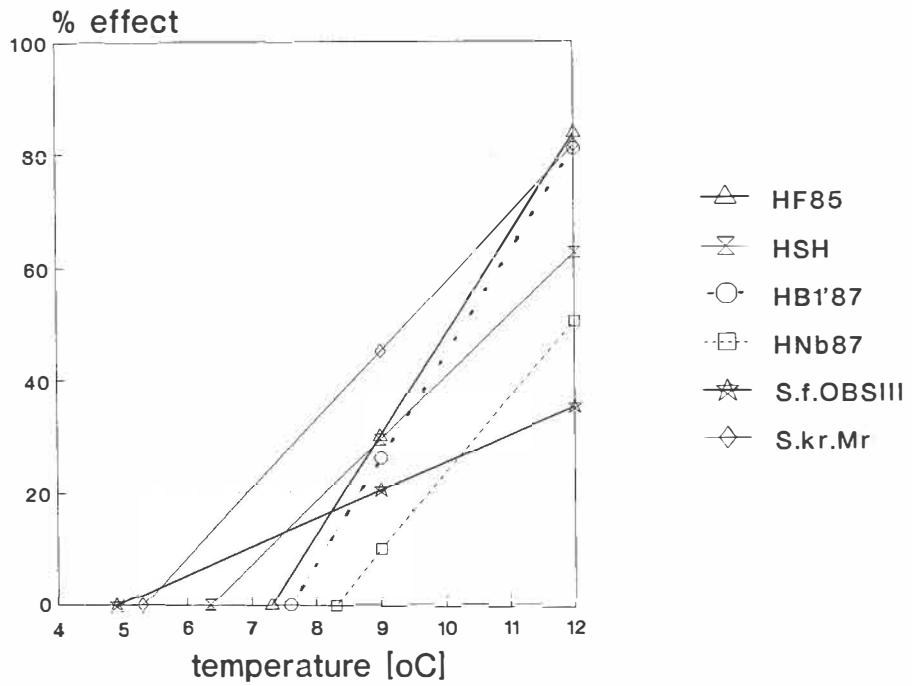

Figure 1. The estimated response in efficacy against black vine weevil larvae of six isolates of insect parasitic nematodes as influenced by temperature (based on linear regression analysis).

4. Discussion

Under optimal conditions (as in the 20 °C experiment) isolates from both genera were equally capable of infecting and killing larvae of *O. sulcatus*. Therefore differences at 9 and 12 °C can be ascribed to differences in cold activity of the isolates.

The data on HFsel suggest that selection for improved infectivity at low temperatures is possible and also that the nematodes respond rapidly to selection (eight rounds). Combining HSH with *Xenorhabdus* strains from the more cold active isolates HF85 or HB1'87 did not significantly improve efficacy of the former at low temperatures. Either *Xenorhabdus* plays a minor part in cold activity and the absence or presence of cold activity should be ascribed to the nematodes, or the HSH nematodes were so obstructed by temperature that the addition of a superior bacterium could not improve the general performance.

The data in **Table 1** and the slopes of the regression lines of the response of six isolates to temperature (**Figure 1**) showed that the various nematodes responded differently to temperature.

Isolates like HF85 and HB1'87 will stay highly effective until temperature drops below a certain critical temperature, while isolates like *S. feltiae* OBSIII will only be fully effective when temperatures are high, and efficacy declines slowly in response to a decrease in temperature.

The variation in efficacy caused by a particular isolate was enormous. A large proportion of this variation was accounted for by temperature but then still some 8 to 30 %, and for HSH even 37 %, of the variation was caused by other sources. Interbatch differences are most likely responsible. Considering this variation, little purpose would be served in using a standard isolate for comparison between experiments. Standardization of temperature in efficacy experiments should have priority.

Acknowledgement

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EFFECT OF PESTICIDES ON LONG-TERM SURVIVAL OF *Steinernema feltiae* IN THE FIELD

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Summary

The effect of five commonly used pesticides on long-term survival of the entomopathogenic nematode *Steinernema feltiae* was studied during three growing seasons, after a one-time inundative release in a spring turnip rape field.
The mortality of bait larvae due to entomopathogenic nematodes throughout the period of three summer seasons was highest in the soil samples taken from plots treated both with nematodes and chemical pesticides. During the first two growing seasons the plots which received only chemical pesticides contained more entomopathogenic nematodes than the control plots without plant protection. Nevertheless during the third summer the number of bait larvae killed by nematodes rose in control areas from the second year's lowest numbers above the number in areas treated with chemical pesticides. Possible reasons for these phenomena might be the shortage of natural enemies and other antagonists in areas treated with chemical pesticides, and the increase in the number of natural enemies and other antagonists when nematodes were applied to the plots, and without the supressive effect of chemical pesticides.

Introduction

The use of entomopathogenic nematodes in integrated pest management has received growing interest. The compatibility of the entomopathogenic nematodes and chemical pesticides has been studied in several laboratories during many years (e.g. Kovacs, 1982; Das & Divakar, 1987; Rovesti et al., 1988), whereas the effects of pesticides on long-term survival of nematode populations in the field has not been studied so thoroughly. The persistence of nematodes in the field, however, has been observed in many studies in different soils and environments. Vainio & Hokkanen (1993) found that augmented steinernematid populations were able to persist at least for two years in strawberry fields where no chemical pesticides were used after the release of the nematodes. Additionally, the nematodes survived best in these experiments in areas with heavy pest populations.
Smits & Wiegers (1991) could isolate one week after an inundative release on grassland only a few per cent of the number of *Heterorhabditis megidis*. However on other release sites they could still find *H. megidis* populations two years after application.

Materials and methods

Field: Two separate spring turnip rape fields, 30 meters apart were employed for the study, both consisting of two experimental

blocks. The fields were cultivated using good agricultural practice concerning the turnip rape, except that the turnip rape was sown every spring to the same blocks, compared with the surrounding areas, which were left fallow every other year between the rape crops. One block of both areas received all the chemical pesticide treatments as they are used in commercial farms, including herbicides, insecticides and fungicides, while the other blocks were not treated with chemical pesticides. Each block contained two 2*6 m^2 plots treated with nematodes, and two control plots of the same size with no nematode application. The plots were situated one meter apart to prevent mixing of the soil due to ploughing and other cultivation. All blocks received fertilizer in the spring. The soil type was fine sand, with pH about 6 at the beginning of the experiment. Mean soil temperature at the depth of 10 cm in May, June, July, August, and September are 8, 13, 16, 15, and 10 °C, respectively.

Pesticides: During the experiment the following pesticides were applied every growing season: insecticide + fungicide Oftanol (20g/1 kg seed, 12 kg seed/ha, isofenphos 400 g/kg + thiram 100 g/kg), insecticide Ambush (0.25-0.4 l/ha in 200-250 l/ha, permethrin 250 g/l), herbicides S-Treflan (2 l/ha in 400 l/ha, trifluralin 480 g/l), and Fusilade (2.5-3.0 l/ha in 200-400 l/ha, fluazifop butyl 250 g/l), and fungicide Rovral (3.0 l/ha in 400 l/ha, iprodion 250 g/l).

Nematodes: *S. feltiae* (SF-S22) was isolated from an apple orchard with a *Tenebrio molitor*, mealworm bait in 1988, and was propagated in *T. molitor* larvae at the Agricultural Research Centre of Finland. The infective juveniles were taken from fresh rearings one day before application. Dosage of 0.15 million nematodes/m^2 was applied on a cloudy and rainy day by watering on the soil surface. Soil samples were taken before the application, and then every second week during the following three growing seasons, in total ten times per season. The first samples were taken at the end of May, or on the first days of June after sowing, and the last samples at the beginning of October after harvest. From the plots 10 random samples were taken. Two consecutive soil cores (diam. 2.5 cm, depth 25 cm) were pooled. There were thus always five separate soil samples per plot. Three *T. molitor* bait larvae were placed in every soil sample in a small plastic pot. A total of 1670 soil samples were baited, with a total of 5010 mealworm larvae. 30 % of the samples were from control plots, and 70 % from the plots treated with nematodes. During a two week period after the beginning of the exposure, dead larvae were collected, and incubated for seven days in dark at room temperature before dissection. The combined data is presented in figures 1 and 2.

Results

The number of bait larvae killed by entomopathogenic nematodes throughout the period of three summer seasons was highest in the soil samples taken from plots treated both with chemical pesticides and nematodes.
During the first two growing seasons the plots which received only the chemical pesticides contained more entomopathogenic nematodes

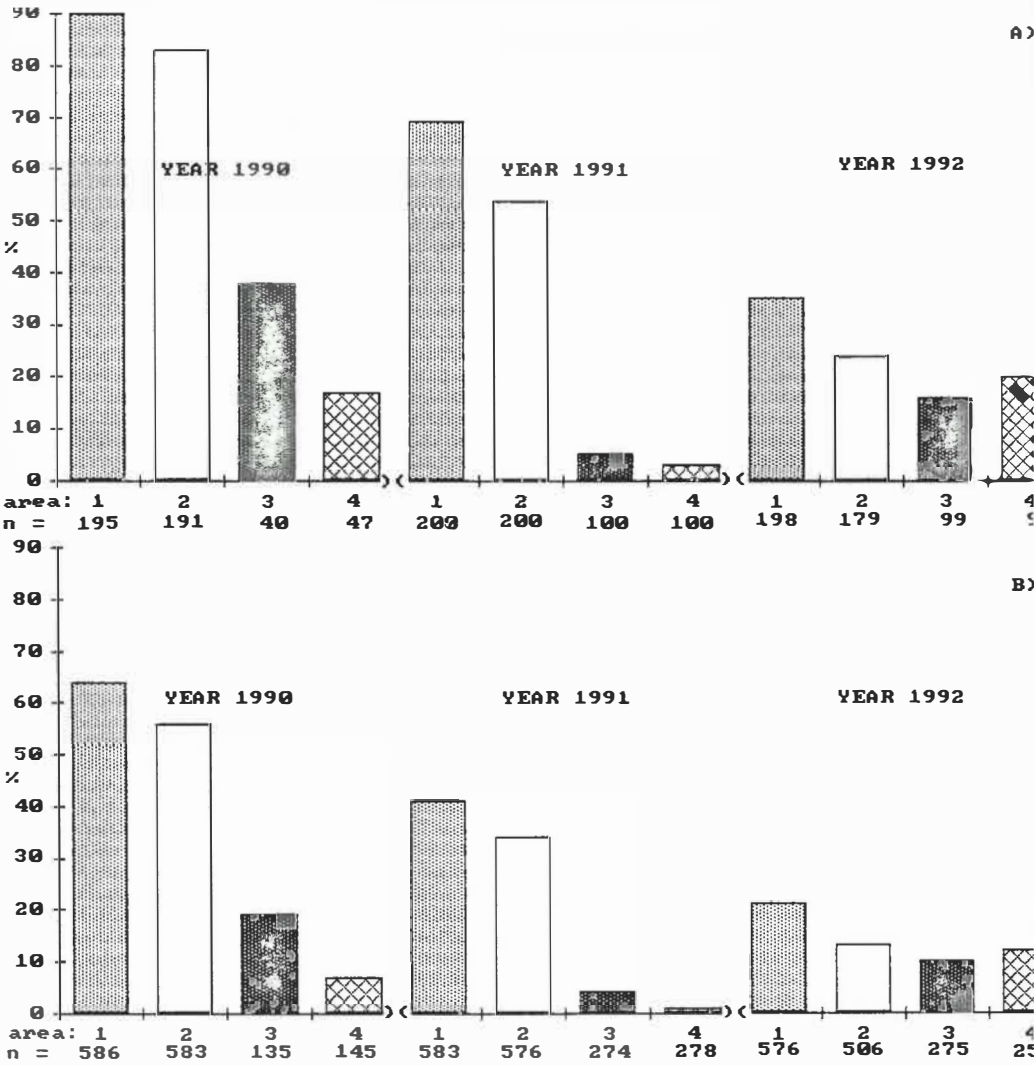

Figure 1A Occurrence of *S. feltiae* after an inundative release in the field. Columns indicate % of soil samples containing the nematodes. n = total number of the soil samples taken from the plots.

Figure 1B The proportion of *T. molitor* larvae killed by the nematodes in the soil samples.

Area 1 = plots treated with nematodes and chemical pesticides,
Area 2 = nematode treatment only
Area 3 = plots with chemical pesticides only
Area 4 = plots without any plant protection (untreated control).

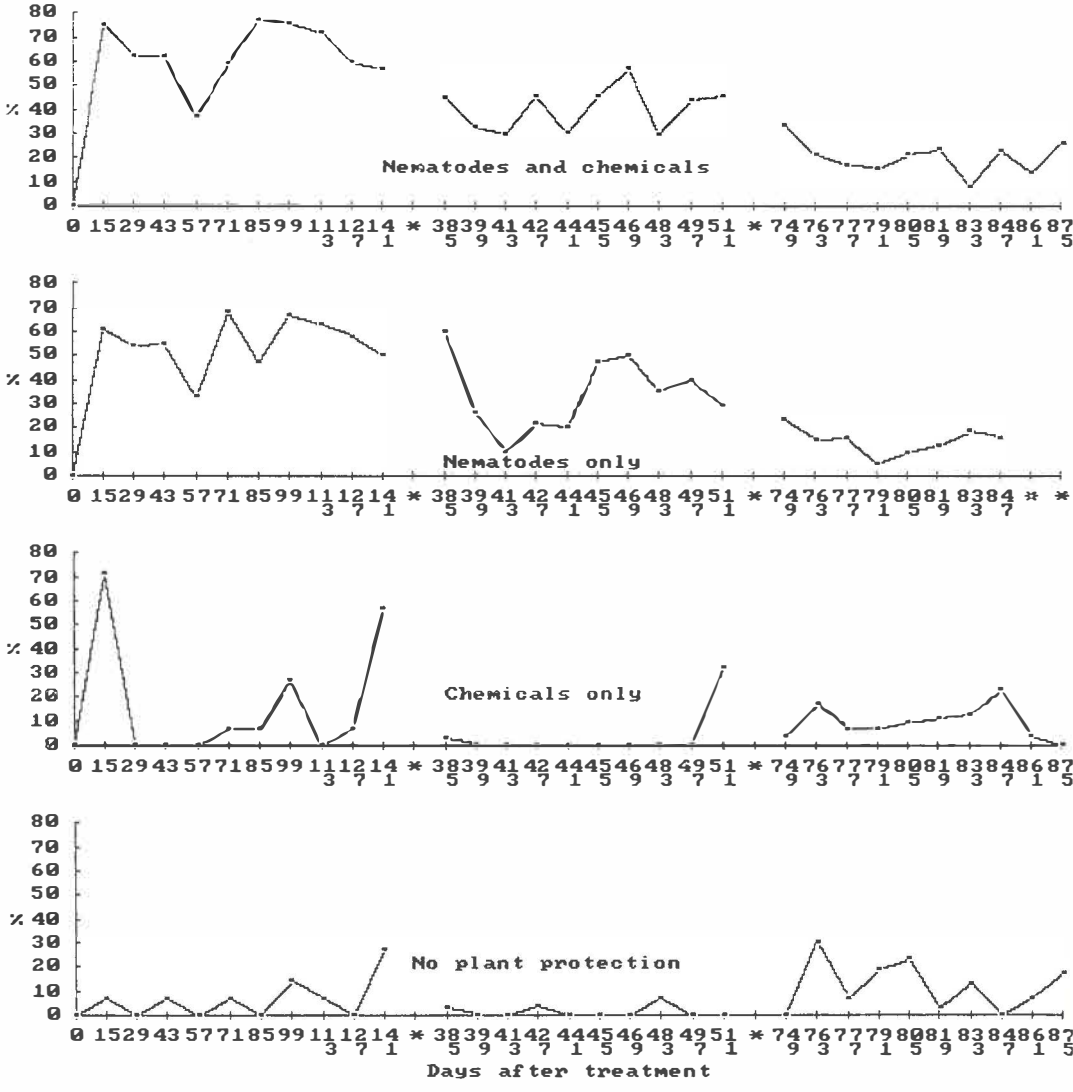

Figure 2 Relative amount of entomopathogenic nematodes in the field after an inundative release of *S. feltiae*. Curves indicate the proportion of *T. molitor* baits killed by the nematodes. Day zero is 16. May 1990, the day of nematode application. In soil samples taken a week before the nematode release no bait larvae were killed by nematodes. Timetable of the chemical plant protection is as follows (vertical lines in the figure): * Oftanol: days 0, 378, and 747; * S-Treflan: days -1, 378, and 747; * Ambush: days 36, 403, 412, and 775; * Fusilade: days 61, 412, and 775; * Rovral: days 61, 427, and 806.

than the control plots without plant protection. Furthermore the proportion of the bait larvae killed by the nematodes two weeks after the application was as high in the samples taken from the areas with only chemical plant protection (i.e. without nematode augmentation) as in the soil samples taken from areas treated with the nematodes.
During the third summer the number of bait larvae killed by the nematodes rose in the control areas from the second year's lowest numbers even above the numbers in the area treated with the chemicals.
On the whole the differences between the treatments seem to decrease in the third year. This may partly be due to soil mixing with cultivation. Only two of the blocks, the one with chemical plant protection and the one without chemical treatments were designed to remain completely apart.

Discussion

To minimize the adverse effects of chemical pesticides on entomopathogenic nematodes, or any other biological control agents, and furthermore to maximize the effectiveness of plant protection we need to know a lot more about the compatibility of different control methods with each other. In this study the effectiveness of the plant protection measures was not assessed. The question was more theoretical: does chemical plant protection in our system have adverse or advantageous effects on the augmented populations of the entomopathogenic nematode, *S. feltiae*? The spring turnip rape field was chosen because it guaranteed pest invasion every year. Furthermore, the most important target pest for chemical control in Finland recently has been *Meligethes aeneus* (Hokkanen et al., 1988). Whether the nematodes are able to cut down the number of rape blossom beetle larvae in the soil during the summer, and could be employed to enhance IPM with this crop, is a question to be answered in the future.
The results of this study exemplify that entomopathogenic nematodes may actually benefit from the use of agricultural chemicals. The most probable reason for the good survival of the nematode populations in areas treated with chemical pesticides appears to be increased space available for them, which means either less competitors, or less antagonists/natural enemies of the nematodes are present. This corresponds with the results of Ishibashi and Kondo (1987), whose investigations infer that the persistence of infective juveniles of *S. feltiae* seems to be affected more by biotic than abiotic factors. In other words, the biocontrol of soil insects by nematodes is more effective in soil with low number of competitors and/or natural enemies present.
The chemical plant protection surely affects such phenomena as Forschler & Gardner (1991) observed in one of their trials: predaceous mite populations significantly increased after nematode application. On the contrary, they did not notice significant increases in numbers of nematophagous fungi after nematode application, although they could recover these fungi both from treated and untreated plots.
Morris (1987) found in laboratory tests that the herbicide trifluralin did not affect the infectivity of *S. feltiae*. Kovacs (1982) studied the effects of several insecticides, fungicides,

herbicides, and growth regulators on the mobility of *S. feltiae*. Among these pesticides there were trifluralin and permethrin, which both had an adverse effect on the nematodes only at very high concentrations. Rovesti and his colleagues (1988) tested among many other pesticides thiram, fluazifop butyl, trifluralin, and isofenphos + phoxim in the laboratory. At their laboratory tests the thiram based fungicide, and the fluazifop butyl based herbicide did not have adverse effects on either the viability or the infectivity of *H. bacteriophora*. The trifluralin based herbicide affected adversely the viability of the nematodes, but it did not affect the infectivity. The isofenphos + phoxim based insecticide was deleterious to the nematodes. This compound limited the movement of nematodes, and inhibited their infectivity. Hara & Kaya (1982) indicated that organophosphates and carbamates incorporated into a nematode rearing medium adversely affected development and reproduction of *S. feltiae*.

Based on the results derived from the laboratory with individual compounds it would have been impossible to foresee their combined effects in the field.

Agrichemicals might influence nematodes by affecting the pH range of the soil, as well. Fuehrer and Fisher (1991) stated that the ranges between pH 5.0 and 7.0 are favourable for steinernematids. According to them the use of fertilizers should augment nematode populations.

In general, in the laboratory insecticides appear to be less harmful to entomopathogenic nematodes than certain fungicides and herbicides (Das & Divakar 1987, Kovacs 1982). Rovesti et al. (1988), however, found that some insecticides are also very deleterious to the nematodes. These effects may nevertheless turn out to be just the opposite in the field, where all the biotic factors are present.

To avoid misleading results of these investigations, pesticide testing with entomogenous nematodes should take place under conditions as close as possible to the actual environment of their planned use. Results from laboratory tests are always only indicators of what might happen in the field.

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SURVIVAL OF ENTOMOPHILIC NEMATODES IN SOIL

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Abstract: Survival and pathogenicity of two Azorean isolates, *Steinernema carpocapsae* (Az20) and *Steinernema* sp. (Az26), as compared to *S. glaseri* (NC1) were tested in soil under laboratory and field conditions for 19 weeks. In sterile soil the median survival time (ST_{50}) was 2.9, 14.6 and 18.0 weeks, for *S. carpocapsae*, *Steinernema* sp. and *S. glaseri*, respectively. At the 19th week the survival in sterile soil was 1, 32.9 and 29.8%, for *S. carpocapsae*, *Steinernema* sp. and *S. glaseri*, respectively. The presence of the entomopathogenic fungus *Metarhizium anisopliae* (Az1) in soil under laboratory conditions did not affect nematode survival. In laboratory and field assays the pathogenicity of nematodes was significantly reduced with time - median patogenicity time (PT_{50}s) of 8.7 and 7.0 weeks, for *S. glaseri* and *S. carpocapsae*, respectively. Correlation between infectives survival and infectivity was better for *S. carpocapsae* (PT_{50} = 12.6 weeks) than for *S. glaseri* (PT_{50} = 21.8 weeks).

INTRODUCTION

The improvement of entomophilic nematodes, steinernematids and heterorhabdits, needs accurated knowledge of the caracterization of the various strains constantly being isolated from the different environments.

One important caracteristic of these nematodes is their ability to survive, maintaining their infectivity under natural conditions in the absence of an adequate host. The survival of steinernematids and heterorhabdits in soil has been correlated with different environmental soil conditions namely moisture, texture, temperature, and antagonists (Simons and Poinar, 1973; Georgis and Poinar, 1983; Molyneux, 1985; Kung et al. 1990; Kaya, 1990).

This study presents the first results on the survival of two new strains of *Steinernema* recently isolated on the Azores, a *S. carpocapsae* (Az20) and a *Steinernema* sp. (Az26), as compared with *S. glaseri* (NC1), towards the establishment of a base line for this trait, considering its importance as a tool in the genetic improvement of entomophilic nematodes.

MATERIAL AND METHODS

Infective juveniles (IJ) of *S. carpocapsae* (Az20), *Steinernema* sp. (Az26), and *S. glaseri* (NC1) were produced according to Dutky et al. (1964) and stored at 9°C up to 1 month. *Metharizium anisopliae* (Az1) was produced on solid medium plates of 1/3 strenght Sabouraud-dextrose agar containing 0.3 yeast extract (w/v). Wax moth larvae were reared in wax and polen (1:1).

The soil used on the assays was classified as a sandy-loam (15,4% sand, 19,3% silt, 65,3% clay; pH 7,8; conductivity = 510 µs/cm).

Survival assays. Eight kg batches of sterilized soil, and sterilized soil innoculated with *M. anisopliae* ($8x10^5$ spores/g) at 15% moisture by weight, were distributed through plastic vials

(70g/vial). 6000 infective juveniles of each nematode were pipeted on the soil surface in 0.2 ml of sterile water. The vials were then caped and incubated at 16±1°C during the time of the assay. At weeks 0, 1, 2, 4, 8, 10, 12, 16 and 19 post nematodes application, 4 vials/nematode/soil treatment were checked for surviving IJ. Nematode extraction was achieved by centrifugation in 50% of sucrose at 1800 rpm, 2 min. The supernant was rinsed through 5 µm openings and the IJ were counted under a dissection microscope. Extraction efficiency was for *S. carpocapsae* 49.1 ± 6,3%, for *Steinernema* sp. 66.7 ± 9.3% and *S. glaseri* 66.7 ± 12.1%.

Infectivity assays. The evaluation of *S. glaseri* and *S. carpocapsae* infectivity was followed under laboratory conditions and field conditions. In the laboratory, 4 kg batches of sterilized soil, sterilized soil with *M. anisopliae* ($8x10^5$ spores/g), and nonsterilized soil, at 15% moisture by weight, were dispensed in plastic vials (70g/vial). 6000 IJ of each nematode were pipeted on the soil surface in 0.2 ml of sterile water. The vials were caped and incubated at 16±1°C for 0, 1, 2, 4, 8, 12 and 19 weeks. At each date, 10 last instar *G. mellonella* larvae were placed in each of 4 vial/nematode/soil combination. The vials were held at 20°C for 3 days, and the cadavers were than dissected.

For the field assay, 4 plots (1x1m) were established with the following treatments: *S. glaseri*, *S. carpocapsae*, *S. glaseri* plus *M. anisopliae* and *S. carpocapsae* plus *M. anisopliae*. Nematodes and fungus were applied with a watering can $10^6/m^2$ at 10^{12} spores/m^2, respectively. Nematode infectivity was assessed at 0, 1, 2, 4, 8, 12, 16 and 19 weeks post nematode application, by collecting 4 cores (Ø=5 x h=10 cm) per plot. The soil samples from each plot were then homogenized, and 70 g of soil were dispensed in a plastic vial. Ten insect larvae were placed in each vial, incubated, and checked as described for the laboratory assay.

Data analysis. Median percentages of surviving nematodes and insect parasitism were separated by Newman-Keuls multiple range test, following arcsin transformation. Median survival time (ST_{50}) and median pathogenicity time (PT_{50}) were determined by probit analisys (SYSTAT).

RESULTS AND DISCUSSION

Nematode survival was considered as the ability of the IJ to persist in sterilized sandy-loam soil under suitable temperature and moisture conditions. Significant differences on survival were found between the Azorean isolates and *S. glaseri* (Fig. 1) - ST_{50} being 18.0, 14.6 and 2.9 weeks for *S. glaseri* (NC1), *Steinernema* sp. (Az26) and *S. carpocapsae* (Az20), respectivelly (Fig. 2).

The impact of the biotic environmental factors on entomophilic nematodes is an importante condition for their use in microbial control (Kaya, 1990). The interference of the entomopathogenic fungus *M. anisopliae* with these nematodes was studied. The presence of the fungus in soil affects *Steinernema* sp. and *S. carpocapsae* (Fig. 3). However, the increase of the ST_{50} from 14.6 to 18.9 and from 2.9 to 4 weeks, respectivelly, is not significative (Fig 2).

The infectivity of the *S. carpocapsae* and *S. glaseri* remaining in soil was tested in laboratory and field conditions against

G. mellonella. In the laboratory the PT_{50} of *S. carpocapsae* is not significantly affected on any case, with PT_{50}s of 12.6, 14.4 and 11.8 weeks, in sterilized soil, nonsterilized soil and with *M. anisopliae*. On the other hand, the results obtained with *S. glaseri* either in sterilized or nonsterilized soil were not significantly different. However, the presence of the fungus seems to affect de infectivity of this nematode reducing the PT_{50} from 21.8 to 10.9 weeks (Fig. 4).

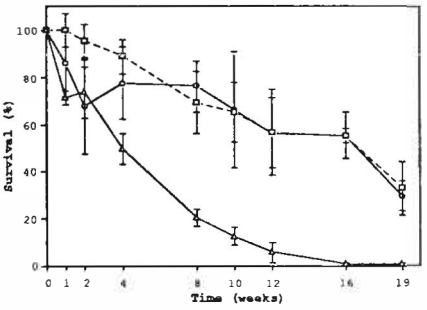

Fig. 1. Survival in sterilized soil of *S. glaseri* (NC1) (-o-), *Steinernema* sp. (Az26) (-□-) and *S. carpocapsae* (Az20) (-▲-). Brackets indicate standard deviations.

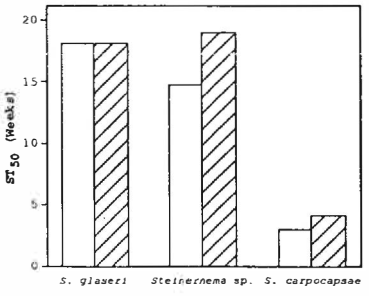

Fig. 2. ST_{50}s of *S. glaseri* (NC1), *Steinernema* sp. (Az26) and *S. carpocapsae* (Az20) in the absence (□) and in the presence of *M. anisopliae* (▨).

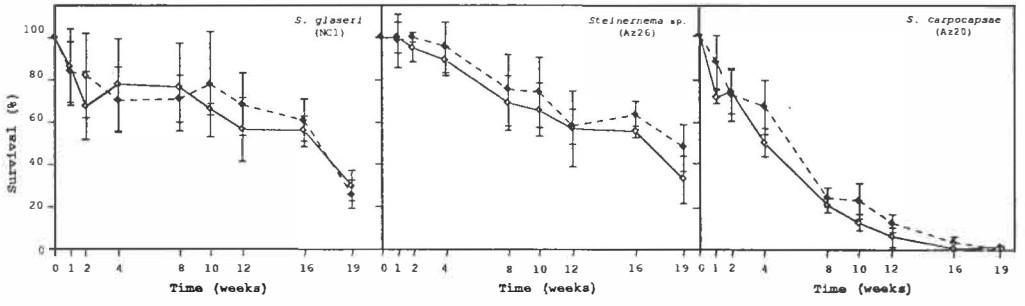

Fig. 3. Comparative survival of nematodes in the absence (-o-) and in the presence (-▲-) of *M. anisopliae*. Brackets indicate standard deviations.

Under field conditions the PT_{50}s of *S. glaseri* are 8.7 and 8.8 and the PT_{50}s of *S. carpocapsae* are 7.0 and 6.9 in natural occurring soil and in the presence of *M. anisopliae* at an epizootic level, respectively (Fig. 5).

Comparing the ST_{50}s of *S. carpocapsae* and *S. glaseri* (2.9 and 18.0 weeks) with their PT_{50} (12.6 and 21.8 weeks) obtained under laboratory conditions we can conclude that even though *S. glaseri* survives better in soil than *S. carpocapsae*, the latter remains infective longer. A strict correlation between survival and infectivity was found with *S. carpocapsae* (r^2 = 0.906), whereas with *S. glaseri* this correlation was only 0.743 (Fig. 6).

In this first aproach *Steinernema* sp. (Az26) seems to have a survival ability pattern similar to *S. glaseri* (NC1). *Steinernema carpocapsae* (Az20) although showing a lesser ability to survive in soil than the other nematodes, proved to be more pathogenic. This is probably due to a production of highly active virulence factors than

S. glaseri (NC1). Simultaneously, the infectivity of this isolate is less affected than *S. glaseri* (NC1) by the fungus *M. anisopliae*. This finding may be due to a diversity of factors which are currently under study.

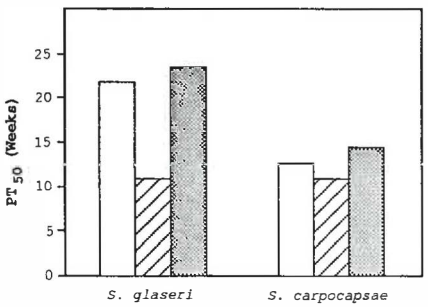

Fig. 4. PT_{50} of nematodes in laboratory conditions in sterilized soil (□), nonsterilized soil (▨) and with *M. anisopliae* (▨).

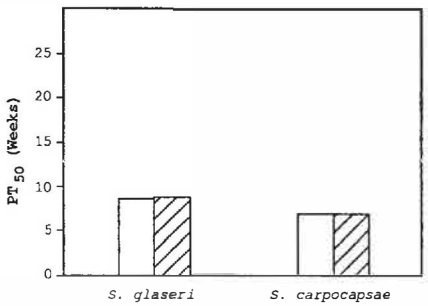

Fig. 5. PT_{50} of nematodes in field conditions in occuring soil (□) and with *M. anisopliae* (▨).

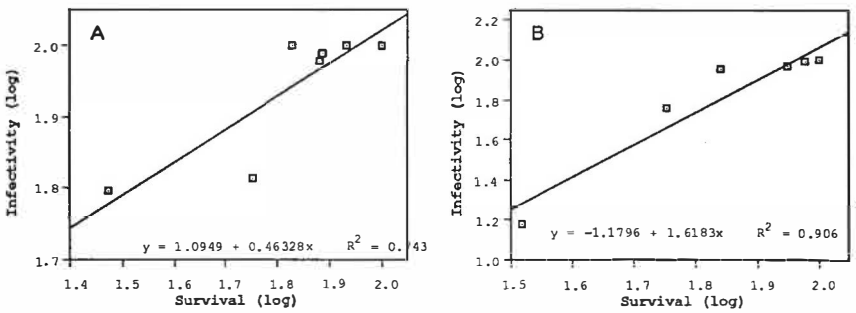

Fig. 6. Correlation between the survival in soil and the infectivity of *S. glaseri* (NC1) (A) and *S. carpocapsae* (Az 20) (B).

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THE VERTICAL MIGRATION OF *HETERORHABDITIS* SPP. AND *STEINERNEMA* SPP. AT 9 °C AND THE RELATIONSHIP TO EFFICACY AGAINST *OTIORHYNCHUS SULCATUS* AT 9 °C

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Summary

Sixteen heterorhabditid and three steinernematid species and isolates were tested with respect to dispersion and host searching ability towards larvae of the greater wax moth, *Galleria mellonella*, in 9 cm sand columns at 9 °C. A number of heterorhabditid isolates with superior migration at 9 °C were found; HUk211, K122, *H. megidis* HO1 and HL81. Activity at low temperatures was not related to a specific taxonomic group. The migration results at 9 °C were compared with the results from efficacy experiments at 9 and 12 °C. In addition, an efficacy experiment with six heterorhabditids and two steinernematids for control of black vine weevil, *Otiorhynchus sulcatus*, in strawberries at 9 and 12 °C, was combined with simultaneous testing of these isolates for migration at 9 °C. A strong relationship was observed. The value of migration as the basis of predictive tests for efficacy at low temperatures is discussed.

1. Introduction

A search was initiated for cold active isolates of *Heterorhabditis* in order to find an isolate that would be suited for control of *Otiorhynchus sulcatus* F. under the prevailing field conditions in NW Europe (Simons & Van der Schaaf, 1986; Westerman & Van Zeeland, 1989, 1994). This involved many time- and labour-consuming field experiments, because a reproducible laboratory assay with predictive value for efficacy in the field does not exist yet.

The most commonly used petri dish bioassay (Woodring & Kaya, 1988) lays emphasis on the pathogenicity of the nematodes for a particular insect when applied in close proximity. However it is likely that in many situations, where nematodes are applied against soil inhabiting pests, mobility and the ability to locate and reach the insects is equally or more important than pathogenicity testing.

There is reason to believe that migration may be related to efficacy (Westerman, in press; Westerman & Stapel, 1992). The purpose of this study was to investigate the influence of low temperature (9 °C) on migration and to see if migration at 9 °C is related to low temperature efficacy of *Heterorhabditis* and *Steinernema*. Migration in the presence and absence of a larva of the greater wax moth, *Galleria mellonella* (L.). was related to efficacy against *O. sulcatus* in strawberries in climate rooms at 9 and 12 °C by linear regression. For this reason the efficacy data obtained by Westerman & Van Zeeland (1994) were used. In addition, efficacy at 9 and 12 °C and migration at 9 °C were assessed

simultaneously in a combined set of experiments with six heterorhabditid and two isolates of the related genus of insect parasitic nematodes, *Steinernema* Travassos. Again, the relation between efficacy and migration was investigated.

2. Materials and methods

SEPARATE MIGRATION EXPERIMENTS
Migration was assessed in 9 cm vertical sand columns at 9 °C with or without a larva of *G. mellonella* at the bottom as described by Westerman (in press). The isolates used in these experiments are listed in Table 1. The fraction of nematodes in top layer (1.5 cm), middle section (6 cm) and bottom layer (1.5 cm) of the columns (n = 3, 4) were determined at 48 h and used to calculate migration and migration rate. Data were analysed per isolate using linear regression analysis and Tukey's test for comparison of means ($\alpha = 0.05$).

COMBINED MIGRATION AND EFFICACY EXPERIMENTS
Efficacy at 9 and 12 °C and migration at 9 °C were determined simultaneously in a combined set of experiments for six heterorhabditid isolates (HW79, HL81, HF85, *H. megidis*, K122, and M145) and two steinernematid isolates (*S. carpocapsae* UK and *S. feltiae* OBSIII). These experiments were carried out with one and the same batch of nematodes for each isolate, in contrast with the separate experiments which were done with different batches for efficacy and migration experiments.

a) Migration. Migration was assessed in the absence and presence of *G. mellonella* in 48 h at 9 °C as described above.

b) Efficacy at 9 and 12 °C. One efficacy experiment was carried out with 640 strawberry plants. They were potted and inoculated as described in Westerman & Van Zeeland (1989; 1994). A complete randomized block design (eight blocks) was used with 32 pots per treatment and 64 pots for the control, for each climate room. A dose of 100 (living) nematodes per cm^2 was used (approx. 8000 per pot). Half the pots were sifted for living and dead insects after three weeks and the other half was checked after 6 weeks. Data were analysed using generalized linear regression on the number of living black vine weevil (binomial distribution). Percentage effect was also calculated (Abbott's formula).

LINEAR REGRESSION ANALYSIS
Migration at 9 °C in the presence of a *G. mellonella* larva was related to efficacy at 9 °C after six weeks by linear regression (model $\varepsilon y = a + bx$). This was done both for the separately obtained migration and efficacy data, using different batches of isolates, and the data gathered in the combined experiment using identical nematode material.

3. Results

The results of the separate migration experiments are summarized in **Table 1**. The efficacy data of Westerman & Van Zeeland (1994) were plotted against these migration data in **Figure 1**, together with the estimated linear regression line. Regression was significant ($P \leq 0.05$) but only when the results on *S. feltiae* Mr were omitted from the regression; $y = 2.65 + 5.43\ x$ ($R^2_{adj} = 36\%$).

The data and the regression line of the combined experiment are plotted in **Figure 2**; y= -23.88 + 15.75 x (R^2_{adj} = 67%).

Table 1. Migration (average distance covered [cm]) at 9 °C in 48 h of a number of *Heterorhabditis* and *Steinernema* species and isolates in 9 cm sand columns without (-) and with (+) a *G. mellonella* larva. (*Migration per isolate followed by the different letters are significantly different from each other, P ≤ 0.05*).

	n	x±sem --	x±sem +
H. zealandica NZH3	3	1.1±0.0*a*	1.0±0.1*a*
H. bacteriophora B1	3	0.9±0.0*a*	0.9±0.1*a*
HP88	3	1.0±0.1*a*	1.3±0.1*a*
HI82	3	0.8±0.0*a*	0.9±0.0*a*
H. megidis HO1	4	3.3±0.3*a*	4.6±0.4*a*
Heterorhabditis sp. (Irish group)			
K122	4	3.3±0.3*a*	5.8±0.3*b*
M145	4	1.6±0.1*a*	2.2±0.3*a*
M198	3	1.2±0.1*a*	1.4±0.1*a*
Heterorhabditis sp. (NW European group)			
HW79	3	1.4±0.1*a*	2.7±0.2*b*
HL81	3	2.7±0.0*a*	4.6±0.2*b*
HF85	4	1.7±0.2*a*	3.3±0.3*b*
HNb87	4	2.6±0.1*a*	2.2±0.2*a*
HB1'87	4	3.1±0.1*a*	3.1±0.2*a*
HUk211	4	4.1±0.2*a*	5.4±0.2*b*
HSH	3	2.3±0.2*a*	3.0±0.4*a*
HKem	3	2.0±0.4*a*	2.3±0.2*a*
S. feltiae OBSIII	3	1.4±0.1*a*	1.5±0.0*a*
S. feltiae Mr	3	1.7±0.4*a*	1.6±0.2*a*
S. carpocapsae UK	3	1.1±0.3*a*	0.9±0.1*a*

4. Discussion

In the separate migration experiments some isolates were found with superior migration at 9 °C; K122, HUk211, *H. megidis* HO1, and HL81. However, three of these isolates (K122, *H. megidis* and HL81) did less well in the migration assay of the combined experiments, and HW79, HF85 and *S. feltiae* OBSIII did better (**Figure 2**). Differences between batches are a major source of variation in migration (Westerman, in press). Migration at 9 °C did not seem to be related to taxonomic grouping, as was observed for the 20 °C data (Westerman, in press). Ranking of isolates in order of descending migration was similar in the absence or presence of a wax moth larva. However, the presence of this host enhanced the differences between isolates (**Table 1**). Therefore these data were used in the linear regression analysis.

The description of efficacy at 9 °C by migration at 9 °C was poor (R^2_{adj}= 36%) when the data came from separate experiments

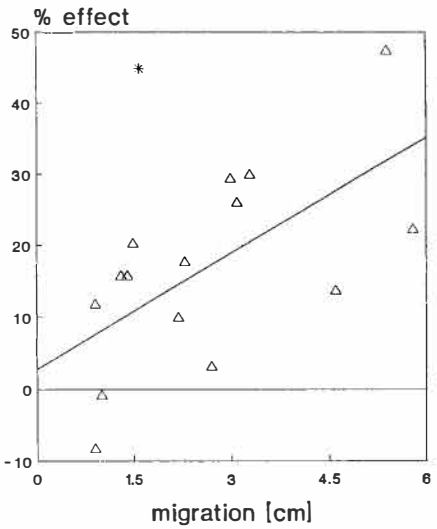

Figure 1. Efficacy of sixteen heterorhabditid and steinernematid isolates against black vine weevil in strawberry after six weeks at 9 °C (% effect) versus migration of these isolates (different batches) at 9 °C in the presence of a *G. mellonella* larva, and the estimated linear regression line based on these data (Δ), except those of *S. feltiae* Mr (*).

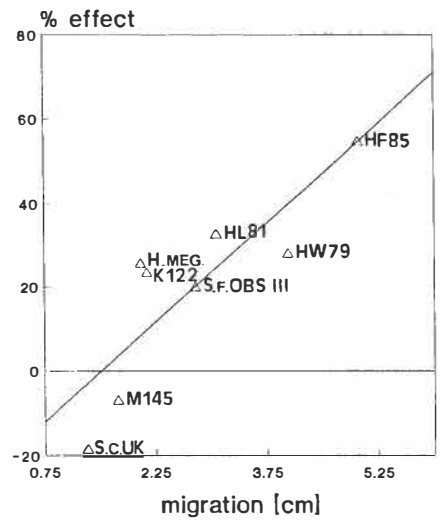

Figure 2. Efficacy of sixteen heterorhabditid and steinernematid isolates against black vine weevil in strawberry after six weeks at 9 °C (% effect) versus migration of these isolates (simultaneously, the same batches) at 9 °C in the presence of a *G. mellonella* larva, and the estimated linear regression line.

(Fig. 1). However, a much better relationship was found when data were gathered for efficacy and migration simultaneously and when

using the same batch of nematodes (**Fig 2**). Quality differences between batches of the same isolate are known to affect migration and efficacy against black vine weevil at 20 °C (Westerman, 1992, in press). Apparently the state of quality of a batch of nematodes affects efficacy and migration in the same way. So migration seems to be a sensitive tool for both quality and cold activity testing. Maybe the laborious field trials for finding cold active isolates can in the future be replaced by repeated migration assays on different batches of an isolate.

Acknowledgement

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ON THE ROLE OF THE BACTERIUM *XENORHABDUS LUMINESCENS*, THE MICROSYMBIONT OF THE NEMATODE CH-H-W79 (*HETERORHABDITIS SP.*)

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Summary:
The bacterium *Xenorhabdus luminescens (Enterobacteriaceae)*, microsymbiont of the nematode CH-H-W79, is characterised. A secondary form of *X.luminescens* could be detected and cultured in vitro. During the natural nematode cycle the secondary form only occurs after completion of the propagation of the nematodes within the dead insect larvae. Isolation of bacteria from the intestinal tract of the nematode only yielded primary forms of *X.luminescens*. The secondary form of *X.luminescens* could not be regularly obtained on artificial media. The secondary form of *X.luminescens* lost the ability to support symbiosis with the nematode. In cultivation experiments with axenic eggs of nematodes on artificial media the development of the nematodes was blocked completely.

1. Introduction

Insect parasitic nematodes of the families *Heterorhabditidae* and *Steinernematidae* are commercially used in Switzerland for biological control of all instar larvae of *Otiorrhynchus sulcatus* F. and *O. salicicola* Heyden. Since 8 years this method of biocontrol is applied in the field of home and garden as well as in horticulture and in special agricultural crops such as strawberries and nurseries of arboriculture and viticulture. A strain closely related to the Dutch nematode strain HW79 (*Heterorhabditis sp.* / NW-European group (Ehlers/ Smits 1991) was isolated from an experimental plot in Switzerland. This strain, designated as CH-H-W79, is now the basis of a nematode product for the Swiss market.
We report here results of a study, that was aimed to stabilize the primary form of the symbiotic bacterium *Xenorhabdus luminescens*. A secondary form of *Xenorhabdus luminescens* has been described by several authors (Boemare & Akhurst, 1988; Ehlers et al., 1990). If this form is present, in vitro production fails as shown in own experiments. A clear separation of the two forms of *X.luminescens* and the knowledge of the factors to stabilize the microsymbiont is required for reliable commercial production.

2. Material and Methods

Isolation of *Xenorhabdus luminescens*
Individual surface sterilised nematode larvae were placed into test tubes containing 9ml of peptonwater (15g pepton/litre) and incubated at 25°C on a rotary shaker (75 rpm). After three to four days the cultures were plated on Standard-1 agar (Merck) and checked for the typical properties of *X.luminescens* (pigmentation, luminescence, dye adsorption).
To enable isolation of *X.luminescens* over an extended observation period from the hemolymph of *Galleria mellonella*, the infected insect larvae were kept on sterile filter paper. After noticing the first signs of nematode infection (red pigmentation), the *G. mellonella* larvae were washed, surface sterilised with

Hibitane (0.05%) and transferred to sterile water traps. Probes of hemolymph were plated on Standard-1 agar and single colonies were checked for properties of primary and secondary forms.

Propagation of nematodes

The nematodes were cultured on the medium based on the Bedding-method (meat, fat and water homogenate, incorporated in rubber foam cubes, sterilised at 121°C/45min, Bedding 1984). Erlenmeyer flasks (80ml) were filled with 3.5 to 5g of this medium and preincubated with primary or secondary form of *X.luminescens*. Monoxenic cultures were prepared by adding surface sterilised nematode eggs to the preconditioned medium. The method of Lunau et al. 1993 was used to harvest the nematode eggs. The axenic state of the nematode eggs was confirmed by the absence of bacterial contaminants. The nematode development was determined after 12 to 14 days. In experiments to establish axenic nematode cultures, the medium was sterilised by gamma-irradiation. A minimum dosage of 5 kGray was required. In addition, attempts were made to substitute the Bedding-medium by purified cells of primary and secondary cultures of *X.luminescens*, respectively.

3. Results

Xenorhabdus luminescens and the natural nematode cycle

Isolation of bacteria from the hemolymph of *G. mellonella* larvae infected with nematodes yielded primary forms of *X.luminescens* until 10 to 15 days after infection. The secondary form appeared only after completion of the propagation of the nematodes within the dead insect. At that stage at least 90% of the nematodes had already left the insect cadaver. Isolations of *X.luminescens* from the hemolymph of the insect could be made up to 80 days. After that period the cadaver was completely disintegrated. The proportion of secondary and intermediate forms in the hemolymph showed a continuous increase.
On the other side, isolation of bacteria from the intestinal tract of the nematode only yielded primary forms of *X.luminescens*. This was also true for nematodes that had been stored at 4°C over a period of 1.5 years.

Characterisation of *X.luminescens* and its secondary form

Features	***X.luminescens***	**Secondary form**
Occurrence in vivo	-intestinal tract of nematode -hemolymph up to 10 days after infection (25°C)	-insect >20 days after infection
in vitro		-spontaneous appearance
Stability	variable low	high
Luminescence	high	low
Morphology	rods, length = 6.52 µm (±0.94)	rods, length = 3.5µm (±0.58)
Antibiotic activity	high	low, marginal
Pigmentation	red	yellow, olive brown
Inclusions	paracrystalline inclusions	--
Adsorption of Bromthymolblue	pronounced	weak
Colony form	doomed	flat
Consistency	mucoid	granular
Pathogenicity	pathogenic upon injection into *G. mellonella*	pathogenic upon injection into *G. mellonella*

Table 1: List of different characteristics of *X.luminescens* and the secondary form.

Occurrence of the secondary form in vitro

The secondary form of *X.luminescens* could not be regularly obtained from artificial media. It was noted that reduced oxygen supply during the exponential growth phase could lead to a higher proportion of secondary forms. Since in vitro cultivation gave rise to several intermediate forms, accurate identification of secondary forms was difficult.

Influence on the nematode propagation

The time of conditioning of the medium is essential for the successful multiplication of the nematodes. The optimum interval between inoculation of the bacteria and the addition of the nematodes is 5-6 days. The potential of reproduction of the nematodes decreases drastically, if the duration of conditioning is extended, reaching a zero level after 76 days.

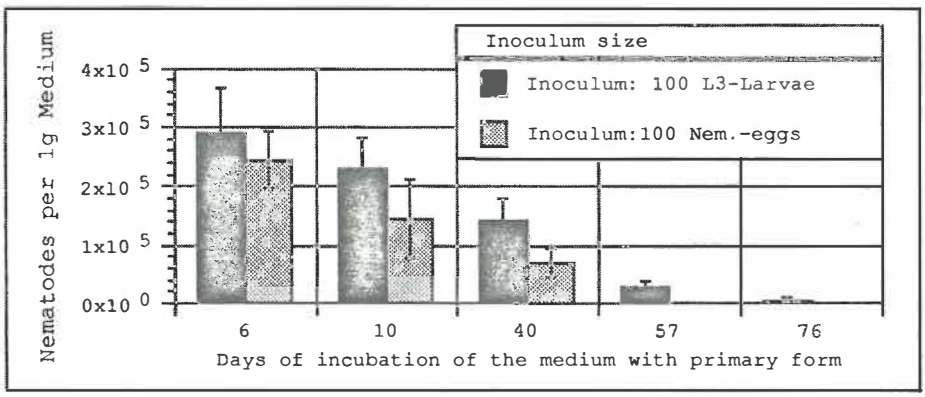

Figure 1: Influence of the incubation time of *X.luminescens*.

No development of axenic eggs could be detected, if the medium was inoculated with primary or secondary form of *X.luminescens* and sterilised again by irradiation after 6, 10, and 20 days of conditioning.
The addition of nematodes to the irradiated medium, preconditioned with the primary form showed consistently very low proliferation rates. It was observed, that the nematodes released their own bacteria leading to a reconditioning. Yields of nematodes grown on reconditioned medium were 40% to 60% lower. The same experiment with secondary forms showed no development, neither with inoculation of nematode eggs nor with nematode larvae.
Inoculation of sterile medium with axenic eggs showed no development of nematodes. An average yield was obtained, if infective nematode larvae were used as inoculum. The multiplication cycle of the nematodes was prolonged by 2-3 days, if the bacterium had to be released from the intestine and had to condition the medium.
In the corresponding experiments with substitution of the artificial medium by purified cells of primary or secondary forms of *X.luminescens* respectively neither the nematode eggs nor the larvae could survive or develop. Some nematodes hatched from the egg and died as L1-larvae.

4. Discussion

The two forms of *X.luminescens* described here, clearly demonstrate distinct properties. Only *X.luminescens* in its original state is able to support nematode development and to act as a symbiont. Furthermore only *X.luminescens* as primary form is suitable for in vitro production of nematodes.

The factors which lead to the appearance of the secondary form at the end of the nematode development in the insect host are unknown. We showed, that the secondary form lost the ability to support symbiosis with the nematode.
Symbiotic interaction between *X.luminescens* and the nematodes is essential for successful in vitro production. The absence of *X.luminescens* in the medium stopped the development of axenic nematode eggs, even if the medium was preconditioned with primary form of *X.luminescens.* On the other side the nematodes can not propagate only on cells of *X.luminescens*, primary nor secondary forms, respectively.
Under natural conditions the secondary form could be detected in the insect cadaver after the propagation of the nematodes. This form has no influence on the symbiotic interaction of bacteria and nematodes. Based on distinct abilities to use nutrient resources by primary or secondary form (Boemare et al 1990), the secondary form can be considered as an abortive stage of *X.luminescens.* This shift seems to be a reaction on the change of the nutrient conditions in the disintegrating insect cadaver. The role and the cycle of *X.luminescens* is shown schematically in figure 2.

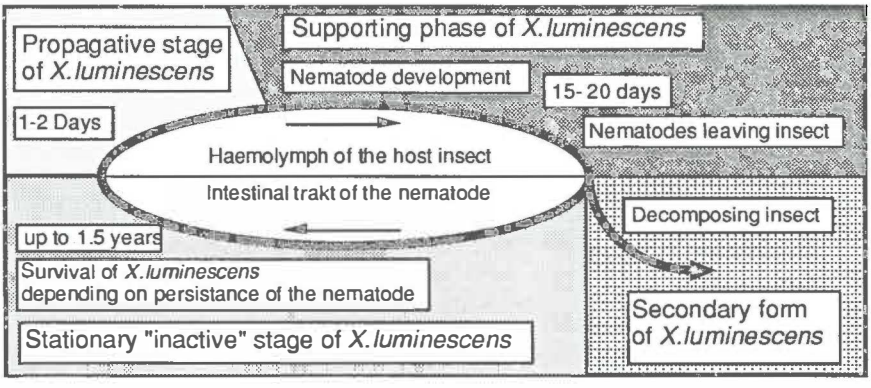

Figure 2: Natural life cycle of *X.luminescens* and the interactions with the symbiotic nematode and the insect host.

Within the nematodes only primary forms are found. The nematodes seem to have a special retention mechanism for the primary form. The primary form of *X.luminescens* is very likely selectively established in the intestine of the nematode. No clear reason for the appearance of the secondary form in the insect cadaver has been found. There is no possibility for the bacteria to return into a nematode cycle and into the hemolymph of another host insect.

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COST ACTION "ENTOMOPATHOGENIC NEMATODES": SCIETIFIC COOPERATION IN EUROPE

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COST ACTION 812 - how it started

ACTION 812 started in 1990 by initiative of Paula Westerman from The Netherlands. At the beginning seven countries were actively participating (CH, D, GB, IRL, NL, S, SOU). In the last three years objective of EC COST ACTION 812 ("Cold active lines of insect-parasitic nematodes") was the search and selection of low temperature active nematode and bacteria isolates in order to guarantee consistent outdoor control results. As ACTION 812 started without any financial support from the EC the cooperation was very much dependent on financial sources of scientific programs, which had been granted by the EC and national funds: An ECLAIR project (agre 0002) was granted by the EC to the Biology Department in Maynooth (Ireland), the Friesland College of Agriculture in Leeuwarden (The Netherlands), and the Agriculture Genetics Company in Cambridge (UK) aimed to develop novel strains of *Heterorhabditis* sp. and their symbiotic bacteria *X. luminescens* with improved activity at low temperatures, resistence to drought conditions and persistence in the absence of hosts. Another project aimed to improve *in vitro* culture techniques for the commercial production of entomopathogenic nematodes (EN) granted by industry and the German Ministery for Science and Technology to the Institute for Phytopathology, University Kiel (Germany). Both projects within the EC were of significance for the Commission to start the program. When it was finally incorporated national funds were available for collegues in Jokioinen (Finland) and Wädenswil (Switzerland). With the excess to these EC and national funds the cooperation within the ACTION 812 could start.

Results of the cooperative research

Comparison of bioassay systems to determine nematode efficacy at low temperatures was of major importance in order to select improved nematode strains. In 1991 five participating laboratories from NL, IRL, GB and CH agreed on a cooperative research to develop methods for screening nematode strains for low temperature activity. All laboratories used the same four nematode species/strains but variable test systems. On a workshop in Kiel Raisdorf, in May 1992 the results were presented and compared. The participating laboratories are currently working on a joint publication of the results.

Many new nematode strains were isolated throughout Europe and identification was urgently needed. In May 1992 a three day COST workshop, which was

mainly sponsored by the IOBC dealt with classical and molecular methods for identification of EN of both genera, *Steinernema* and *Heterorhabditis*. A key for identification of so far described species was developed and handed to the participants and 30 scientists learned during practical sessions and discussions how little we knew and still know about taxonomy of EN.

In October 23 to 27, 1993 a workshop on nematode genetics will be held at the Department of Biology, St. Patrick's College, Maynooth, Ireland which will benefit from presentations on genetics of *Caenorhabditis elegans* and further will train participants in *in vitro* culture methods, techniques for mutagenisis of EN and use of molecular methods in nematode research.

Where we are and where we want to go

The number of participating countries has now expanded to fourteen, involving 28 European laboratories. ACTION 812 was prolonged until December 1993 to guarantee the performance of the nematode genetics workshop and to give time for the organisation of a proposal for a new action. We are currently proposing for a new COST ACTION entitled "Entomopathogenic nematodes" with the main objective to combine interrelated European expertise in order to increase the use of EN in integrated pest management. The change of the title of the ACTION gives room for participation of any laboratory within Europe working with EN. Many research fields are shared by different laboratories. To improve coordination of research fields and objectives and achieve a maximum cooperation within Europe, different working groups shall be initiated (coordinators in brackets).

1: Isolation and identification (Bill Hominick, GB)
2: Production and application (J. Coosemans, B & R.-U. Ehlers, D)
3: Nematode ecology (C. Griffin, IRL & R. Gwynn, GB)
4: *Xenorhabdus* (N. Boemare, F)
5: Nematode biology and genetics (A. Burnell, IRE & A. Fodor, H)

The EC has learned also from other COST actions that the support of cooperation is a cheap and effective way to enhance scientific progress and has now decided to give financial support for workshops, publications and travel expenses of members of the management commitee (MC) and experts. Every participating country sends two representatives to meetings of the MC, which is organized twice a year (usually during COST workshops). On these meetings future programs and workshops are discussed and cooperative research is organized. The next and last meeting of the MC of ACTION 812 will be during the workshop at St. Patrick's College in Maynooth.

In the last years scientific activities in the field of EN have drastically increased. European scientists (including collegues from Eastern Europe) active in the field of entomopathogenic nematodes are invited to participate in the new ACTION. We are confident that the Commission of the EC will accept the proposal for the new ACTION, which will be starting, when at least five officers from the

national ministeries have signed the Memorandum of Understanding. This is expected for early 1994. It is our task to make them sign the document as quickly as possible in order to further improve our scientific cooperation. Another workshop is already planed for 1994 in Hungary. The success of the new ACTION will certainly depend on the input of the participating scientist. COST ACTION "Entomopathogenic nematodes" will provide use with a structure and generous financial support from the EC to further improve our cooperation. For further information contact the coordinators of the working groups or the chairman of ACTION 812 (Tel.: ..49-4307-7498; Fax.: 49-4307-7499).

CULTURE CONDITIONS DEFINE AUTOMICTIC OR AMPHIMICTIC REPRODUCTION OF ENTOMOPATHOGENIC RHABDITID NEMATODES OF THE GENUS *HETERORHABDITIS*

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Summary

Heterorhabditis sp. (strains HD01, HE and HSH) were cultured monoxenically in shaken liquid media inoculated with dauer juveniles (DJs). In all cultures some female phenotype adults produced offspring while others had only unfertilized eggs in their uteri. When *Heterorhabditis* spp. were grown on agar media they exhibited a "y" or "λ" type copulation behaviour. In contrast to steinernematid nematodes, heterorhabditid males were unable to attach to the vaginal region in liquid culture. Consequently, under liquid culture conditions only self fertilizing hermaphrodites reproduce and the amphimictic part of the F1 generation can be identified by the presence of unfertilized eggs without shell in their uteri. During daily observations of the nematode development (strain HSH) in liquid cultures containing approximately 20 F1 generation eggs in cell wells, 30% developed to amphimictic females, 38% to males and 32% to hermaphrodites. Single F1 generation first stage juveniles (J1s) were either directly inoculated into *X. luminescens* (XSH) cultures or starved for 24 h in Ringer's solution before inoculation. Starvation significantly increased the automictic part in the F1 generation (53.3% DJs, 40% hermaphrodites and 6.6% amphimictic adults). The ratio amphimictic : automicitic adults was 1.84 when J1 were directly transferred to culture medium and 0.07 when they were starved prior to transfer. In all experiments males developed a day earlier than amphimictic females and females 2-3 days earlier than hermaphrodites. Obligatory for automictic reproduction is a yet undescribed second juvenile pre-dauer stage (J2d) which can be morphologically distinguished from J2 developing to amphimictic adults by their corn cob like structure of the cuticle in the head region. A preceding development from a J2d to a DJ is not obligatory for automictic reproduction. In cell well cultures the F1 sex ratio males : amphimictic females was between 0.84 and 1,3 with no significant difference. The present results have a significant impact on liquid culture population dynamics of *Heterorhabditis* species.

For further information refer to a publication which was submitted to Journal of Fundamental and Applied Nematology.

QUANTIFICATION OF PHASE VARIANTS OF *XENORHABDUS LUMINESCENS* XSH1

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Summary

Methods to quantify the density of phase variants in cultures of *Xenorhabdus luminescens* (XSH1), the symbiotic bacterium of *Heterorhabditis* sp. (HSH1), were evaluated. Neither bioluminescent or antibiotic activity nor cell and colony morphology were useful characters to quantify the density of different phase variant cells in liquid culture. Differences in ethanol concentration in culture supernatants were found (primary 0,26 and secondary 0,6 g/l). Nine different lectins were tested for their specificity to adhere to the bacterial cell envelope. Wheat germ agglutinine (WGA) was the only lectin which labeled cells of *X. luminescens*. In secondary form more cells were labeled with FITC conjugated WGA than in primary form cultures.

1. Introduction

Nematodes of the genus *Steinernema* and *Heterorhabditis* are symbiotically associated with bacteria of the genus *Xenorhabdus*. *Xenorhabdus* spp. are polymorphic (Gerritsen & Smits, 1992) and produce phase variants. The primary form variant is found in the intestine of infective dauer juveniles whereas the secondary form variant appears after repeated subculturing, especially under *in vitro* conditions. The phase variants can be characterized by morphological and biochemical differences (Boemare & Akhurst, 1988; Hurlbert *et al.*, 1989). Both, primary and secondary *X. luminescens* (strain XSH1) are unstable and a culture always contains a mixture of primary, secondary and possibly intermediate form cells. The secondary form variant is unable to support nematode propagation (Krasomil-Osterfeld & Ehlers, 1992). Therefore phase shift to the secondary form must be avoided in order to obtain satisfactory nematode yields during *in vitro* propagation. A method to quantify phase variants in mixed populations of *X. luminescens* is necessary for investigations on the influence of culture conditions on the phase variation and stability of primary form.

2. Material and methods

The strain *Xenorhabdus luminescens* XSH1 was isolated from dauer juveniles of *Heterorhabditis* sp. (HSH1), endemic in Schleswig-Holstein/Germany. The antibiotic activity was assessed according to Akhurst (1982). Bioluminescens was detected by observation of cultures in the dark after 10 min adaptation of the eyes. Cell size and the presence of inclusion bodies was quantified microscopically at a 1000-fold magnification

with Normanski optics. Colony-morphology was observed on NBTA and McConkey Agar. The concentration of ethanol in the cell-free culture supernatant (YS, 48h, 25°C) was analysed with a gaschromatograph (Carlo Erla Vega 6300/01b, Perkin Elmer 10b Integrator and Interface) and the signals were compared with ethanol standards of known concentration. Nine different fluorescin (FITC)-conjugated lectins were tested for their ability to specifically adhere to the cell envelope of *X. luminescens* cells. Lectins were dissolved in phosphate buffered saline (PBS) at a concentration of 0,1 mg proteine per ml buffer. Primary and secondary phase variants were cultured on solid media (NBTA, 72h, 25°C) or liquid media (YS, 48h, 25°C, 180 rpm). The bacterial cells were washed three times with PBS and adjusted to a density of $0,5x10^9$ cells/ml buffer. 50μl of the lectin solution was incubated with 50μl of the cell suspension for 1h at 20°C. Subsequently 10μl of the lectin-cell solution was immobilized with 20% gelatine on a slide and the fluorescent-labeled cells were counted under the fluorescent microscope. Controls were untreated cells in PBS.

3. Results

Antibiotic activity is mainly found in the primary form cultures. However, the activity is highly variable and dependent on clone and growth phase. Bioluminescens is characteristic for the primary phase, but likewise variable in intensity and occurence. Morphological characters like cell size and the presence of inclusion bodies were quantified in primary and secondary cultures. A higher percentage of long cells and the absence of inclusion bodies was observed in secondary form cultures, whereas in primary form cultures middle-sized cells with inclusion bodies predominated. However, the majority of the cells lack these typical characters of primary or secondary form cells. Streaking samples from liquid cultures on NBTA or McConkey was used to distinguish between the variants. However, colony morphology is not representative for the distribution of phase variants in the original culture. Change of media and culture condition often resulted in changing population densities of phase variants in the subculture.

Differences in ethanol concentration were found in culture supernatants of primary and secondary form: the secondary form produced twice as much ethanol as the primary form. Of nine FITC-conjugated lectins only WGA labeled cells of *X. luminescens* (XSH1). Only few cells of primary phase cultures were labeled with WGA (12 ± 4 of 100 cells counted, mean of 8 test cultures), whereas secondary cells adhered to a significantly higher rate to this lectin (72 ± 12 per hundred cells).

4. Conclusion

Phase shift of *Xenorhabdus* spp. is of major importance for liquid culture production. Bacterial cultures composed mainly of secondary cells can severely inhibit nematode development and propagation. When the phase shift cannot be avoided during scale-up of *in vitro* cultures, decreasing yields are inevitable. Mechanisms of phase variation in *Xenorhabdus* are poorly understood. The genetic mechanisms of the phase shift could not yet be identified.

Xenorhabdus spp., symbionts of steinernematid nematodes, seem to be quite stable, once they have converted to secondary form. The same is said for *X. luminescens* (e.g. Akhurst & Boemare, 1990), however, for the strain XSH the opposite is true. Pure secondary cultures were not obtained even after subsequent selection over a period of two years. Cultures of *X. luminescens* (XSH), although exhibiting primary or secondary characters, always contain variable densities of primary and secondary cells, with the majority of the cells of the corresponding variant. Consequently a dynamic process of shift to both sides and, further more, the possibility to stabilize primary form by improvement of culture conditions is suggested. A prerequisite for the improvement of culture conditions is the quantification of primary and secondary form cells in mixed populations. The described procedure of labeling secondary form cells with FITC conjugated WGA is a first approach to develop a quantitative method. However, it is quite laborious and cannot easily be used for routine research.

Along with the phase shift the structure of the cell envelope is changing. Different absorption of bromothymolblue or neutral red give evidence for these changes (Akhurst, 1980). Recently Brehélin *et al.* (1993) recorded that primary cells of *X. luminescens* possess a two to three times thicker capsular material (glycocalyx) than secondary form cells. Furthermore they observed that primary cells have peritichous fimbriae which could not be evidenced on the surface of secondary form cells and cells grown in liquid culture. WGA is known to specifically adhere to terminal N-acetylglucosamine. The site of adherence of the WGA to secondary cells (if they are) is still unknown. However, it might be suggested, that the presence of fimbriae and a thick glycocalix in primary cells prevent passage of the lectin to the cell surface. Possibly the terminal saccharide of the O-side-chain of the LPS (lipopolysaccharide) on the surface of the cell envelope is N-acetyglucosamine and thus the target structure for the lectin WGA.

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PATHOGENICITY OF THE *STEINERNEMA FELTIAE-XENORHABDUS BOVIENII* COMPLEX TO *TIPULA OLERACEA*

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Summary

in order to study the pathogenicity of the separate symbiotic partners *S. feltiae* and *X. bovienii* in comparison to the virulence of the nematode-bacteria complex, cells of *X. bovienii* and axenic or monoxenic DJs of *S. feltiae* were injected into larvae of *T. oleracea* and *G. mellonella*. *X. bovienii* is highly pathogenic to *G. mellonella* but almost non-pathogenic to *T. oleracea*. Axenic DJs are able to kill larvae of both species. Monoxenic DJs are more virulent to leatherjackets than axenic DJs. This effect is due to the synergetic potential of the nematode-bacteria complex in interaction with the defense mechanisms of *T. oleracea*. Monoxenic nematodes were more frequently encapsulated by the hemolymph of *T. oleracea* than axenic nematodes.

1. Introduction

Larvae of the cranefly *Tipula paludosa* are important pest insect causing considerable damage in grassland, golf and football courses and tree nurseries. Ehlers & Gerwien (1993) and Sulistyanto et al. (1993) found that the nematode *Steinernema feltiae* (OBS III) was the most virulent strain against *T. oleracea* and *T. paludosa* among several nematode species/strains tested. Nevertheless control results are variable excluding the use of *S. feltiae* for biocontrol of tipulid larvae. Environmental and biotic factors influencing nematode performance during the pre-infectional and in the post-infectional phase of the pathogenesis are responsible for the inconsistency of the control results (Ehlers, 1992; Peters & Ehlers, submitted). In order to better understand the interactions of the nematode-bacterium complex with the insect's defense mechanisms the following experiments were done.

2. Material and methods

Leatherjackets were reared isolated from each other, in order to avoid non-self contact and variable defense potential due to inguries obtained by other tipulid larvae. Fourth instars of *T. oleracea* and last instars of *G. mellonella* were used.

X. bovienii was cultured in PGS broth (Götz et al., 1981). Cells were harvested by centrifugation, washed several times with fresh medium and 200 to 200,000 cells in 2µl PGS were injected into the insect larvae (control: 2µl cell-free medium). Cell concentration was assessed by counting (THOMA chamber). Viable cell counts were obtained by assessment of plating efficiency on PGS. The dosage of injected cells is given as the number of colony forming cells. Mortality of the larvae was recorded daily over a period of 2 weeks. LC_{50} was calculated by Probit analysis (Finney, 1971).

Axenic nematodes were cultured according to Lunau et al. (1993). Monoxenic *S. feltiae* were propagated in *G. mellonella*. DJs were washed several times in sterile tap water and 1 to 20 DJs/larva were injected in 2 to 10µl sterile tap water (control: sterile tap water). Larval mortality was evaluated each day after injection over a period of 2 weeks. Encapsulation of DJs was recorded in dead and alive *T. oleracea*.

3. Results

Mortality of the leatherjackets and the waxmoth larvae 7 days after injection of *X. bovienii* are given in Fig. 1. The same *X. bovienii* culture was used for both species. An injection of 68 viable cells into larvae of *G. mellonella* caused 100% mortality. In contrast, 68,000 viable cells of *X. bovienii* were necessary to obtain a mortality of 86% of the leatherjackets. Calculation of LC_{50} for *G. mellonella* was not possible. For *T. oleracea* the LD_{50} is 18,200 viable cells of *X. bovienii* per larva and thus must be considered as almost non-pathogenic to *T. oleracea*.

Mortality of the leatherjackets 7 days after injection of axenic or monoxenic DJs of *S. feltiae* is given in Fig. 2. A number of 20 injected axenic DJs per larva caused a mortality of 39% of the leatherjackets, whereas 20 monoxenic DJs resulted in 90% mortality. Injection of a single axenic or monoxenic nematode into *G. mellonella* always caused 100% mortality. However, axenic nematodes take longer to kill the host insect (up to 5 weeks).

T. oleracea can survive nematode infestation by encapsulating invading DJs. The number of encapsulated axenic or monoxenic nematodes found in dead and alive fourth instar *T. oleracea* is given in Fig. 3. The percentage of encapsulation is higher for monoxenic than for axenic nematodes.

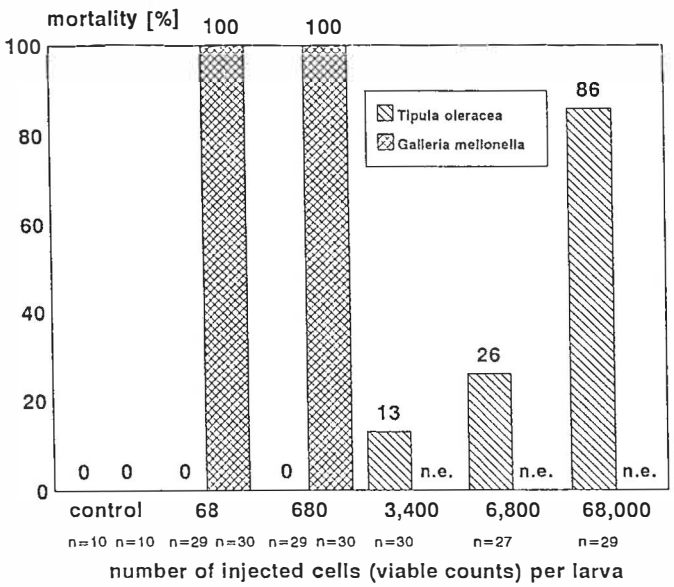

Fig. 1: Mortality of *Tipula oleracea* and *Galleria mellonella* 7 days after injection of 68 to 68,000 *Xenorhabdus bovienii* cells. Number of treated insects is given below the cell concentration (n.e. = not examined)

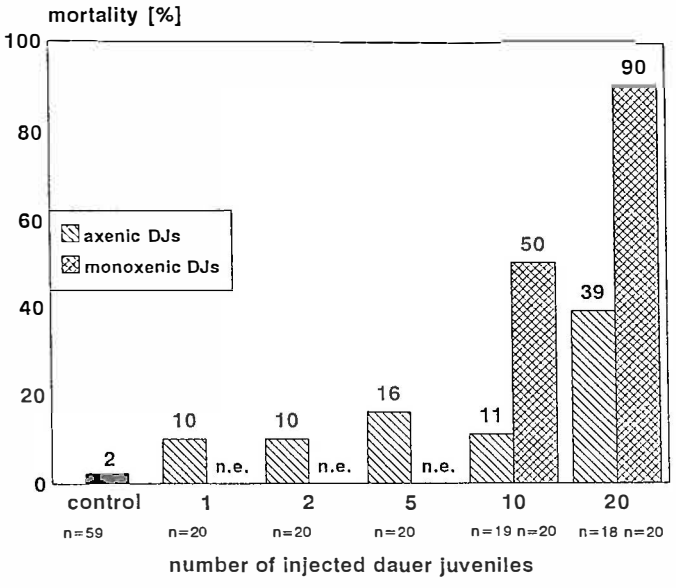

Fig. 2: Mortality of *Tipula oleracea* 7 days after injection of 1 to 20 axenic and monoxenic DJs. Number of treated insects is given below the number of nematodes injected (n.e. = not examined)

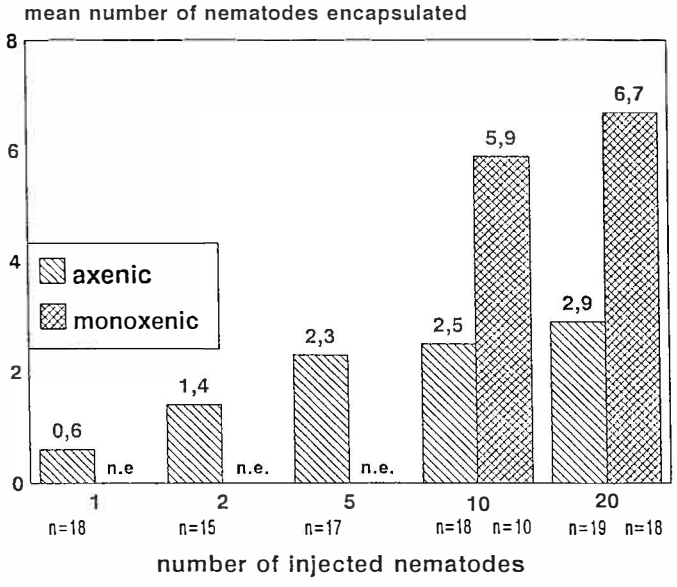

Fig. 3: Encapsulation of axenic and monoxenic nematodes in dead and alive *T. oleracea*. Number of treated insects is given below the number of nematodes injected (n.e. = not examined)

4. Conclusions

X. bovienii must be considered as almost non-pathogenic to *T. oleracea*. The defense system of leatherjackets is able to successfully eliminate cells of *X. bovienii*. Either humoral (antibacterial proteins) or cellular (phagocytosis) mechanisms are responsible for the elimination of the *X. bovienii* cells in the haemolymph. Preliminary results indicate that phagocytosis is the major defense mechanism against *X. bovienii*.

Axenic nematodes possess mechanisms to kill both, *T. oleracea* and *G. mellonella*. A single axenic nematode can kill larvae of *G. mellonella*, however, in comparison with monoxenic DJs the death of the insect is delayed. To achieve an equal degree of mortality among *T. oleracea* larvae like obtained with monoxenic DJs a higher concentration of axenic nematodes is necessary, although axenic nematodes are less frequently encapsulated than monoxenic DJs. Reasons for the reduced encapsulation of axenic nematodes need to be investigated.

The nematode-bacteria complex is more pathogenic than the single components of the symbiotic relation. Synergistic effects seem to enhance pathogenicity of both symbionts and/or support each other in their activity to overcome insect defense mechanisms.

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Sex ratio in *Heterorhabditis* spp.

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Infective dauer juveniles of entomopathogenic *Heterorhabditis* spp. always develop to hermaphrodites. The F1-generation, offspring of the hermaphrodites, consists of males and female phenotype adults. Contradictory results exist whether the female phenotype adults are automictic self-fertilizing hermaphrodites (Zioni *et al.*, 1992) or exclusively amphimictic females which can only reproduce by cross-fertilization (Dix *et al.*, 1992). Due to the lack of morphological characters hermaphrodites and females cannot easily be distinguished. However, for cross-breeding experiments it is essential to know if the female phenotype individuals are amphi- or automictic. In *Heterorhabditis* spp. (strains HSH1, HE, HD01) liquid cultures female phenotype adults were observed which carriy fertilized or exclusively unfertilized eggs. The latter were identified to be amphimictic females as males are unable to copulate under liquid culture conditions. The inability of the males to mate in liquid media makes it possible to distinguish between automictic hermaphrodites and amphmictic females by the presence of fertilized, respectively unfertilized eggs in the uteri. In liquid culture media eggs which were isolated from gravid hermaphrodites (strain HSH) developed to males, females and hermaphrodites (ratio was approx. 1:1:1, n = 483). Thus the F1 generation consists of both, self-and cross-fertilizing female phenotype adults. Single J1, offspring of hermaphrodites, were either starved for 24 h before transfer into bacteria suspensions or directly transferred to bacterial cultures. When starved, 93 % (n = 60) developed to hermaphrodites. Of J1 directly transferred to culture media only 32 % (n = 91) developed to hermaphrodites, indicating that the ratio automictic versus amphimictic adults is influenced by nutritional conditions.

4. Posters on entomopathogenic nematodes.

DISTRIBUTION OF ENTOMOPATHOGENIC NEMATODES IN THE SWISS ALPS

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INTRODUCTION

To control more efficiently outdoor insect pests at low temperatures, cold-active strains of entomopathogenic nematodes are required. Such strains are likely to be found in the Swiss Alps, since nematodes (and their bacteria) in the alpine environment must be adapted to long and cold winters. The faunistic data presented here refer to a field survey realised as part of COST 812 ("Selection and evaluation of cold-active lines of insect-parasitic nematodes for outdoor application").

SAMPLING

A total of 473 soil samples was taken between May and September 1991 at different altitudes in the Swiss Alps (woodland: 32%, grassland: 59%, others: 9%). Each sample consisted of 10 subsamples (to a depth of 10 - 15 cm), taken at regular intervals along a transect of ca 50 meters. Below the timber line, samples were taken along the edge of forests (one sample inside, one outside). Entomopathogenic nematodes were baited in the laboratory with larvae of *Galleria mellonella*. All strains were identified by morphometric characters and 35 selected strains also by restriction fragment length patterns (A. REID, GB-Ascot; and P. SMITS, NL Wageningen). The pH and the organic matter content of soil samples was determined electrometrically in distilled water (using a glass electrode) and by the WALKLEY BLACK method, respectively.

RESULTS

Steinernema spp. were present in 126 samples, while *Heterorhabditis* was recovered from only one site. The percentage of soil samples yielding entomopathogenic nematodes was thus 26.5%, and varied between 0.5% (lower Alps) and 53% (Engadine). The following species were identified (fig. 1): *Steinernema affinis* (BOVIEN, 1937), *S. feltiae*

(FILIPJEV, 1934), *S. intermedia* (POINAR, 1985), *S. kraussei* (STEINER, 1923) and *Steinernema* RFLP type E1 (a species close to *S. intermedia*).

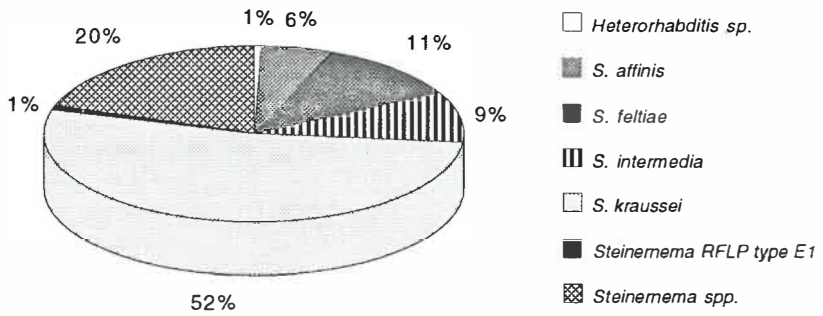

Fig. 1: Species representation of entomopathogenic nematodes in the Swiss Alps (n=127). *Steinernema spp.* denote unidentified strains.

Most strains were recovered in soil samples taken at altitudes between 1400 and 2200 m (fig. 2). The most elevated recovery site (with *S. kraussei*) was located at 2530 m near Zermatt. It seems that *S. kraussei* is the most important species in the alpine environment (altitude: 1833 m ± 310; mean ± std), whereas *S. feltiae* (1332 m ± 473) predominates in the lower Alps. The distribution of *S. affinis* (1611 m ± 474) and *S. intermedia* (1450 m ± 500) appears to be independent on altitude.

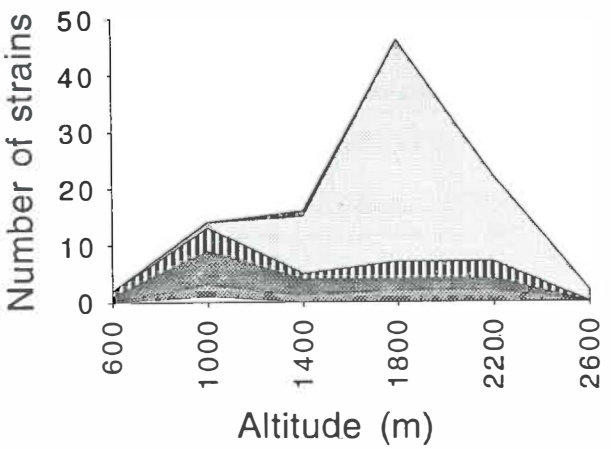

Fig. 2: Distribution of 102 identified nematode strains of the Swiss Alps with respect to altitude. For graphical patterns see fig. 1.

Visual examination of fig. 3 shows that *S. kraussei* is predominant in soils with relatively low pH values (5.4 ± 1.1; mean ± std), whereas *S. affinis* (6.8 ± 0.3) is rather confined to pH values near neutrality. *Steinernema intermedia* (6.2 m ± 0.7) and *S. feltiae* (6.4 ± 1.2) seem to avoid extreme pH conditions. The content of organic matter appears to have no decisive influence on the species distribution.

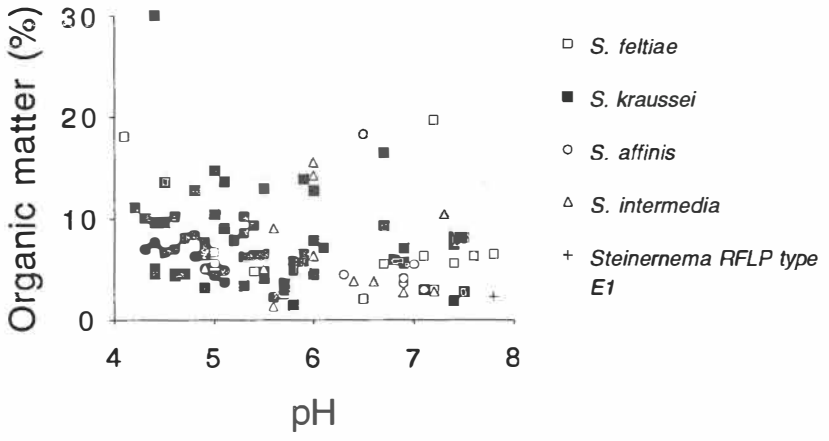

Fig. 3: Distribution of 102 identified nematode strains of the Swiss Alps with respect to pH and content of organic matter.

Woodland samples were relatively frequent (see above). Nevertheless, only two strains other than *S. kraussei* were recovered from forest soils (i.e. *S. feltiae* and *S. intermedia*). *Steinernema kraussei* was found in 33% of the woodland samples and in 63% of the grassland samples, which equals the overall frequency of samples taken in these habitats. It is concluded that *S. kraussei* shows no vegetational specificity.

DISCUSSION

Steinernema kraussei tolerates alpine climate, a wide range of soil conditions and is living in both grassland and forest ecosystems. Therefore, among the species recovered in the Swiss Alps, *S. kraussei* is the most promising candidate to control insect pests at low temperatures. *Steinernema affinis*, *S. feltiae* and *S. intermedia* were almost restricted to grassland ecosystems at relatively high pH values. Therefore, they should probably not be applied in acidic soil.

MORPHOLOGICAL DETERMINATION OF THE PRE-DAUER VERSUS PROPAGATIVE JUVENILES OF *HETERORHABDITIS* SPP..

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Summary
The infective dauer juveniles of entomopathogenic *Heterorhabditis* spp. develop to facultative automictic hermaphrodites. The filial generation of this hermaphrodites consists of males, obligatory amphimictic females and hermaphrodites, or DJs respectively. The occurence of automictic and amphimictic reproduction in one species make *Heterorhabditis* spp. ideal candidates for genetic improvement. Due to the lack of morphological differences between females and hermaphrodites, for cross-breeding experiments, they cannot easily be separated. Several morphological and ontogenetic characters were identified to be useful to distinguish between J2 and J3 juveniles developing to amphimicitc females or automictic hermaphrodites. The cuticle of the J2 of a hermaphrodite (so called J2d) has a corn cob like structure in the head region which is not found in the J2 stages developing to amphimictic males and females. The J2d is slender and the intestine appears darker. The DJ, respectively the hermaphrodite, remains longer in the J2 stage as those individuals developing to amphimictic females and consequently the development of a hermaphrodite takes about 2 to 3 days longer than the development to automictic females. A complete development of the DJ is not obligtory for the development to the hermaphrodite. The J2d can exit the DJ development by a molt to the propagative hermaphroditic J3. This stage is characterized by longitudinal lateral ridges, not found in amphimicitic J3.

Introduction

The infective dauer juveniles (DJs) of entomopathogenic *Heterorhabditis* spp. develop to facultative automictic hermaphrodites (Zioni *et al.*, 1992). These hermaphrodites have the female phenotype. The filial generation of these automictic individuals consists of males, obligatory amphimictic females and hermaphrodites or DJs (Fig 1). Developmental cues inducing the development to amphimictic adults are abundant food and to automictic hermaphrodites (or DJs) low food concentration (Strauch & Ehlers, 1993). The occurence of automictic and amphimictic reproduction in one species make *Heterorhabditis* spp. ideal candidates for genetic improvement. Cross-fertilization allows the transfer of benefical traits and self-fertilization produces homozygous inbreed-lines. Due to the lack of morphological differences self- and cross-fertilizing female phenotype adults of *Heterorhabditis* spp. cannot easily be distinguished which is a major obstacle for cross-breeding experiments. In this study several morphological and ontogenetic characters were identified to be useful characters to distinguish between J2 and J3 juveniles developing to amphimicitc females or automictic hermaphrodites.

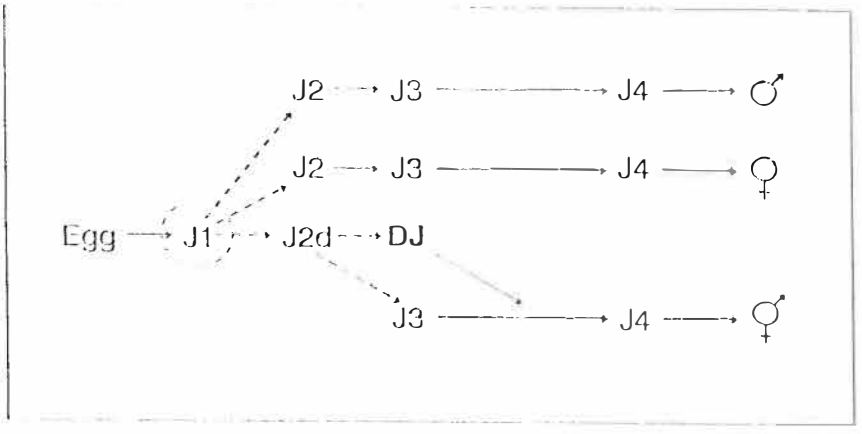

Fig. 1: Alternative developmental pathways of *Heterorhabditis* spp. from egg to male and female or automictic hermaphrodite and dauer juveniles (DJ). Solid lines indicate obligatory developmental steps. Dotted lines represent alternative pathways. Circle marks the first stage juvenile (J1) sensitive to environmental cues inducing development to either to amphimictic or automictic adults (J1-J4 = juvenile stages, J2d = pre dauer juvenile).

Method

Single first stage juveniles (n = 192), offspring of hermaphrodites grown in monoxenic liquid culture, were transferred to cell wells (6 mm diameter) filled with 40 µl *Photorhabdus luminescens* suspension. The bacteria were cultured 48 h in liquid lipid medium (Lunau *et al.*, 1992) and their density was adjusted to 10^9 cells/ml by dilution with cell free culture supernatant. The development of the individuals was recorded daily over a period of 9 days with an inverted microscope.

Results

Of the observed individuals 79 % developed to adults or DJs (Table 1) and the remaining juveniles died during development. Males occurred a day earlier than amphimictic females. Hermaphrodites took 2-3 days longer to develop than females. DJs were observed finishing the development within 3-7 days after transfer of the J1 from the parent liquid culture.

Table 1: Developmental time of the differend sexes of *Heterorhabditis* sp. (strain HSH) at 25°C in monoxcenic liquid culture (bacterial density: 10^9 cells/ml). Observations were started with 196 single J1 individuals in 40 µl medium (n = number of observed individuals). Dauer juveniles have the hermaphroditic sex.

Sex	Total developmental time from egg (mean in days)	Duration of the J2 stage (mean in days)
Male (n = 29)	4	1
Female (n = 34)	5	1
Hermaphrodite (n = 30)	8	3
Dauer juvenile (n = 58)	6	4

Of the 30 individuals which had developed to hermaphrodites only 3 had surpassed the DJ stage. All other J1s growing to hermaphrodites did not develop to DJs but surpassed a pre-dauer J2 stage, which is morphologically distinct from those J2 developing to amphimictic adults. The stage preceeding the DJ respectively the hermaphrodite is called J2d (Fig. 1). The cuticle of the J2d has a corn cob like structure in the head region and longitudinal ribs along the cuticle, which are not present in J2 stages developing to males and females. The structure of the J2d cuticle was described by Mracek & Bednarek (1992) as the sheat of the DJ. The J2d is slender and the intestine appears darker. Second stage juveniles developing to hermaphrodites remain longer in the J2 stage than individuals developing to amphimictic females (Table 1). Consequently the development of a hermaphrodite takes about 2 to 3 days longer.

After the development of the DJ cuticle the formation of the dauer juvenile is terminated with a radial shrinkage and by formation of a plug closing the mouth. A complete development of the DJ is not obligtory for the development to the hermaphrodite. The J2d can exit the development to a dauer juvenile by a molt to the propagative hermaphroditic J3 (Fig. 1). This stage is characterized by longitudinal lateral ridges like shown in Mracek & Bednarek (1992), which are not found in amphimictic J3.

Discussion

The described morphological differences of the J2 stages of amphimictic and automictic individuals are useful to separate females and hermaphrodites. The differentiation of juvenile females and hermaphrodites in a synchronous population is facilitated even more because of the prolonged duration of the J2d stage of the hermaphrodites. Due to the differences in the developmental time of the J2d and J2 stages, the J4 stages of the females and the J2d stages of the hermaphrodites occur simultanously in a synchronous population. J2d stages are easily distinguished from the J4 stages of females. Synchronous populations can be established by starting the nematode cultures from eggs (method see Lunau *et al.*, 1993).

The darker appearance of the J2d in relation to the J2 indicates an accumulation of food reserves essential for the survival of the non-feeding DJ in the soil. It can be suggested that the accumulation of reserves is responsible for the prolonged development of the J2d in comparison with the J2 stage.
The possiblity to terminate the development to the DJ in the J2d stage and directly develop to a hermaphrodite represents a well adapted strategy to changing culture conditions. The J1 enters the development to the J2d stage in order to secure survival in the dauer stage, however, if nutritional conditions do not get worse the J2d can terminate the development to the DJ in order to take up the reproductive life cycle again.

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MOVEMENT PATTERNS OF DAUER JUVENILES IN RESPONSE TO HOST CUES

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Summary
The behaviour of the entomopathogenic nematode *Steinernema feltiae* (OBSIII) was observed in an agarose plate bioassay. While chemotaxis has been documented by several authors, cues which induce host penetration are still unidentified. In the presented bioassay the dauer juveniles exhibited three types of locomotion: Rapid cruising, searching and curling behaviour. While the cruising locomotion occurred at any distance from the host cue and in the control plates, the searching and the curling behaviour was a response to attractants. The curling behaviour was mainly observed next to a host cue and possibly represents a response to a penetration stimulus.

1. Introduction

Dauer juveniles (DJs) of entomopathogenic nematodes respond to chemical and physical host cues. Chemotaxis has so far been documented by measurement of DJ migration (e.g. Schmidt & All, 1979; Gaugler *et al.*, 1980; Pye & Burman, 1981). However, cues inducing penetration are still unidentified (Peters & Ehlers, submitted). Different stimuli which induce host finding or penetration could result in different behaviour. The aim of the described bioassay was to distinguish between migration and searching behaviour on the one hand and the behaviour preceding penetration on the other hand.

2. Materials and Methods

In an agarose plate bioassay hydrous surface washings or cuticle pieces of *Galleria mellonella* (Lepidoptera) or *Tipula oleracea* (Diptera) were tested as host cues. Surface washings were made after Schmidt & All (1978) by shaking living insect larvae for 5 min in purified water (1 ml/1 g larval weight) in small Erlenmeyer flasks. Solid particles were removed by centrifugation (10,000 rpm, 10 min). Insect cuticle pieces (4 mm^2) were obtained by preparation from freeze killed larvae. Purified water or no test substance were used as control. On a Petri dish (5.5 cm diameter) with 1.5 ml agarose (1.5 %) either 3 μl of surface washings from *T. oleracea* or *G. mellonella* or a piece of

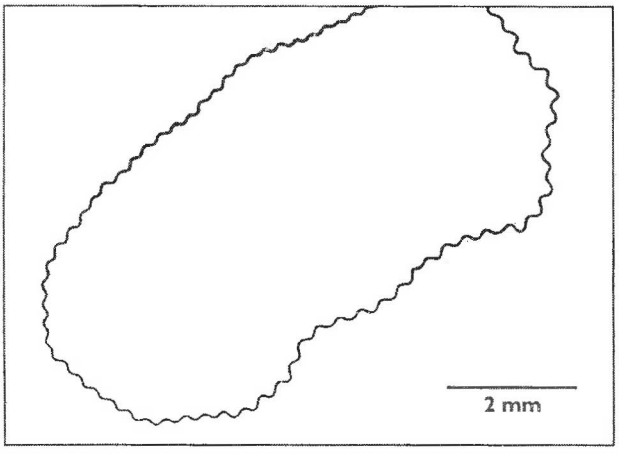

Figure 1: Cruising behaviour (type 1), scanned from the photographic print.

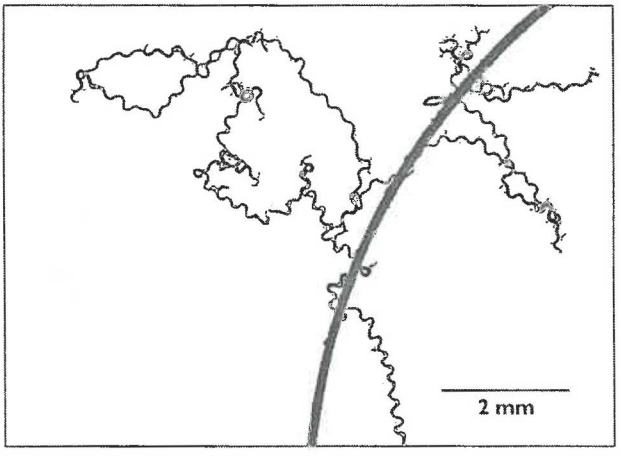

Figure 2: Searching behaviour (type 2), scanned from the photographic print.

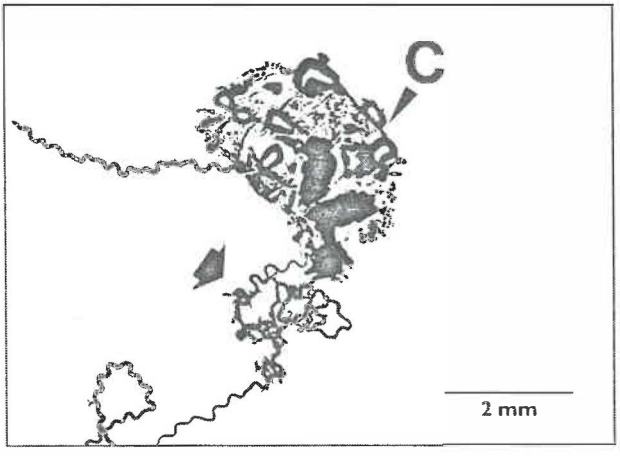

Figure 3: Curling behaviour (see arrow, type 3), scanned from the photographic print. C: Attractive source (piece of insect cuticle) removed before photographic documentation.

insect cuticle was placed in the centre. After one hour 2 DJs/plate of *Steinernema feltiae* (OBSIII) were placed on a circle at 1 cm distance from the centre. One hour later the DJs were removed from the plates. For recording of nematode tracks the Petri dishes were placed on Kodalith Ortho Film (type 3) and were exposed for 2 seconds (Ward, 1973). From the resulting negatives magnificated prints were made (see figures 1 to 3) and different movement patterns were documented. Each variant was repeated 6 times.

3. Results and Conclusions

On all agarose plates with test substances the nematodes showed three types of movement patterns, here called type 1 to 3.

Type 1 represents a rapid cruising behaviour with little changes of direction (see fig. 1). It occurred on all test and control plates at any distance from the centre. Therefore this pattern is interpreted as non-chemotactic migration behaviour.

Type 2 consists of slow forward and backward moving and head twisting to the left and the right (see figure 2). This behaviour seems to be an orientation to a gradient by proving differences in the concentration of attractants around the DJ. It occurred on the whole plate but it was less frequent in the periphery.

Type 3 resembles curling and uncurling several times within a small area (see fig. 3). It occurred mainly in the centre of the test plates and was hardly recorded on the control plates. This movement pattern may be a response in direct contact to host cues and thus may resemble penetration behaviour.

The control potential of a nematode strain/species is mainly defined by the number of penetrating DJs (Peters & Ehlers, submitted). Further investigations are necessary to identify cues inducing host penetration which possibly define host specifity. The presented bioassay might help to characterize host specific cues if the curling behaviour can be proven to be a response to a penetration stimulus.

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INFLUENCE OF TEMPERATURE ON THE CONTROL OF THE BLACK VINE WEEVIL WITH STRAINS OF SOME INSECT-PARASITIC NEMATODES

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Summary

In a climate room experiment two of the three strains of *Heterorhabditis* sp.(NWE) tested reduced the number of vine weevil larvae by 80 to 100% after six hours of 12°C soil temperature followed by 9°C until the end of the experiment. These two strains gave no control at continuous 9°C but achieved total control at continuous 12°C. A laboratory experiment with larvae in small tubes confirm the results of the experiments in the climate rooms. One strain infected in this experiment also at 9°C which suggests that the temperature limit for penetration is lower than the limit for successful migration to the larvae with this strain. A strain of *Steinernema* tested was not even effective at continuous 12°C.

1. Introduction

The black vine weevil (*Otiorhynchus sulcatus*) is a major pest in nursery stock. The larvae damage the roots of many plants and are difficult to control. Hatched larvae overwinter in soil and continue to feed on plant roots. In winter large larvae start to feed on the main roots and the root collar of the plants, causing economically important damage. Outdoor application of insect-parasitizing nematodes in autumn, as late as possible, would be most effective to reduce the larval population. Weevils have then stopped laying eggs, most larvae are big enough to be successfully infected by the nematodes and the plants have not yet suffered economic damage. The problem with late applications of nematodes outdoors is the low soil temperature, which reduces the efficacy of many strains. In earlier experiments in 1991 it was demonstrated that several strains of *Heterorhabditis* sp. are effective in pots outdoors. A few of these strains were also effective in the open ground. Climate room experiments showed that there is a relation between nematode activity at low temperatures and the efficacy against the larvae of the black vine weevil (Van Tol, 1993a; Van Tol, 1993b). A soil temperature of 9°C was not sufficient to obtain any control but a continuous temperature of 12°C was enough to achieve control with the better strain.

The study reported in this paper aimes to determine the critical period at 12°C required to achieve successful infection of the larvae.

2. Material and methods

Four nematode strains of different origins were tested (Table 1). The trials were carried out in climate rooms and in climate chambers. The substrate used in pots consisted of 55% peat pellets, 40% sphagnum-moss peat and 5% aeolian sand. The test plant was *Thuja occidentalis* 'Brabant'. The data were statistically processed using ANOVA.

Table 1 - Biological agents used in the experiments

nematode	origin	code	experiment*
Heterorhabditis sp.+	United Kingdom	UK-H-211	I,II
Heterorhabditis sp.+	Germany	D-H-SH	I,II
Heterorhabditis sp.+	Netherlands	Nl-H-F85	I
Steinernema carpocapsae	U.S.A.	US-S-25	II

** I = climate room experiment; II = laboratory experiment*
\+ nematodes belonging to the North-West European group (NWE)

I - Climate room experiment

Four treatments (Table 1), including "untreated", at five different temperature schedules were carried out in fourfold, using four test plants per replicate. Plants in one-litre containers were inoculated with 30 weevil eggs per plant and incubated at 20°C in a climate room for two months. On 30 October 1992 the plants were divided into three series and transferred to climate rooms at 9 and 12°C. At 2 November 1992 each pot was inoculated with 15 000 nematodes and placed on a grid to prevent the nematodes from migrating to other plants. Five temperature treatments were applied. In two treatments the plants, after inoculation with nematodes, were kept at either 9°C or 12°C until the end of the experiment. In the other treatments plants were kept at 12°C for 6, 18 or 96 hours after inoculation and then placed at 9°C until the end of the experiment. The nematode suspension applied was cooled to 9°C or 12°C before inoculation. On 14 December 1992 all plants were checked for the presence of living larvae.

II - Laboratory experiment

Three treatments (Table 1) at five different temperature schedules were carried out in tenfold. Tubes of 50 ml were filled with a mixture of peat and roots from Thuja-plants. In each tube one larva was placed. Moisture of the soil was kept constant in all treatments. The tubes were cooled down to 9 or 12°C. At the start of the experiment each tube was inoculated with a cooled down suspension of 500 nematodes. In two treatments the tubes were kept at 9°C or 12°C until the end of the experiment. In the other treatments tubes were kept at 12°C for 6, 12 (only strain D-H-SH and US-S-25) or 18 hours after inoculation and then placed at 9°C until the end of the experiment. Every 3 to 4 days the larvae were checked. Dead larvae were removed and checked for infection.

3. Results

The results of the two experiments are presented in table 2 and figure 1. The experiment in the climate rooms (I) shows that temperature affects the efficacy of the nematode strains. It also appears that the efficacy of the three strains of the NWE group differs at 12°C. At that temperature the strains UK-H-211 and Nl-H-F85 performed best. Strain D-H-SH gave no statistically significant control at 12°C. At 9°C there was no control with any of the three strains tested. One temperature shock of 12°C for 6 hours was already enough for almost complete control of the larvae with the strains UK-H-211 and Nl-H-F85.

The lab experiments (II) confirm the results of the climate room experiments. In the lab experiment, however, the nematodes of UK-H-211 were also able to infect and kill larvae at 9°C. The strains D-H-SH and US-S-25 were not effective at 9°C and also not after a temperature shock of 12°C. Only strain D-H-SH gave some infection at 12°C continuous.

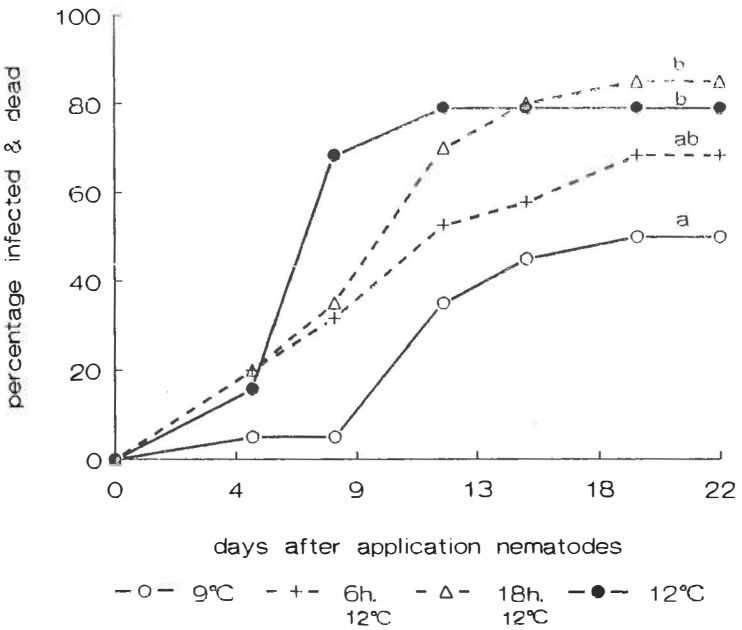
Table 2 - Percentage reduction of *O. sulcatus* larvae compared with "untreated" (untr.), achieved by three strains of *Heterorhabditis* sp. under different temperature conditions in climate rooms (exp.I), 42 days after inoculation with nematodes.

treatment@	O.s.#	Percentage reduction			
		untr.	UK-H-211	D-H-SH	Nl-H-F85
9°C	1.9	0a*	16a	0a	0a
6h.12°C	2.7	0a	100b	44a	82b
18h.12°C	2.4	0a	79c	38ab	63bc
96h.12°C	1.3	0a	100c	39ab	85bc
12°C	0.5	0a	100b	40ab	100b

* *Figures in the same row followed by the same letter are not statistically significantly different, with a 95% confidence limit.*

\# *average number of O. sulcatus larvae in the untreated plants.*

@ *soil temperature during the experiment. After 6 to 96 hours at 12°C the temperature was maintained at 9°C until the end of the experiment.*



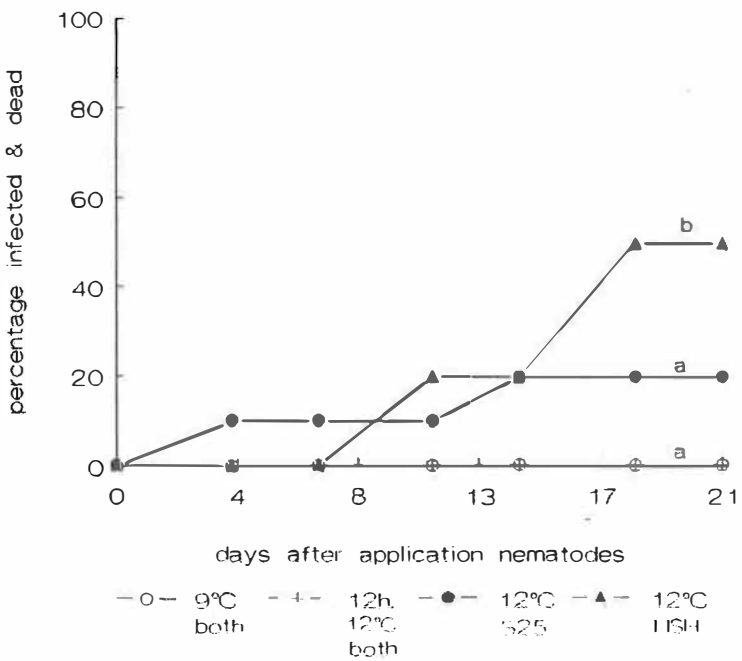


Figure 1 - Percentage infected dead larvae in time (exp.II) after application of UK H-211 (top figure) and D-H-SH and UK 5 25 (bottom figure).

4. Discussion

The results from the climate room experiment confirm that temperature influences the efficacy of the tested strains of the NWE group. At 9°C no strains were effective and at 12°C two strains (UK-H-211 and Nl-H-F85) gave 100% control. As found earlier in 1991 (Van Tol, 1993a), the D-H-SH strain was not effective, even at 12°C. It is remarkable that only one temperature shock of 12°C for six hours after application of the nematode strains UK-H-211 and Nl-H-F85 was enough to achieve 80 to 100% control. It seems probable that the temperature shock of 12°C for a few hours enables the nematodes to find the larvae in the soil. Penetration and successive infection and death of the larvae can then occur at 9°C soil temperature. In the laboratory experiment the nematodes of UK-H-211 were also able to infect and kill larvae at 9°C. In the lab experiment larvae were placed in a small soil volume (50 ml) so that the nematodes did not need to search for their potential hosts, but only had to penetrate the larvae. It seems therefore probable that the temperature limit for penetration is lower than the limit for successful migration to the larvae. Many researchers also found that different strains of *Heterorhabditis* sp. and *Steinernema* sp. differ in their efficacy to migrate and locate hosts and in their chances of penetrating the host (Barbercheck & Kaya, 1991; Gerritsen & Smits, 1993; Westerman & Godthelp, 1990; Westerman, 1991). Gaugler et al.(1990) stated that the probability of successful penetration and establishment of a single insect-parasitic nematode is low, even in very susceptible hosts. This means that there is a minimum number of nematodes needed to get a successful infection. Low soil temperatures probably limit the number of nematodes that can find the larvae in soil. In large volumes of soil (open ground) there is less chance of finding the hosts, and consequently also less chance of a successful infection.

The experiments discussed here indicate that every subprocess of the infection of the larvae by nematodes has its optimal temperature. Locating and finding the larvae in the soil is influenced by temperature, soil volume and probably by antagonism. Successful penetration and infection of the larvae probably needs a minimum number of nematodes per larva. Knowing more about the subprocesses of infection would help optimize the strategy and timing of nematode applications to control the larvae of the black vine weevil and makes it possible to select more specific for better nematode strains. The results of the different experiments done suggest that the searching ability of the nematodes is one of the most limiting processes for infection at low temperatures. Future research will focus on these different subprocesses.

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GROWTH OF SEVERAL *XENORHABDUS* AND *PHOTORHABDUS* SPP. ISOLATES AT LOW TEMPERATURES

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Summary

Entomopathogenic nematodes were isolated during a search of UK soils for cold-active nematodes. Bacteria were obtained from two of these isolates, *Steinernema feltiae* (L/128) and *S. affinis* (L/179). Bacteria were also obtained from three nematode isolates available commercially: *Steinernema feltiae* (Nemasys®) and *Heterorhabditis megidis* (Nemasys H®), both from Agricultural Genetics Company Ltd. Cambridge, UK and *S. carpocapsae* (MF252) from Biosys, Palo Alto, USA. Biochemical tests and fatty-acid profiling identified four of the isolates as *Xenorhabdus* spp. and the fifth one, from *H. megidis* as *Photorhabdus* sp.

The relative growth rates of these five bacterial isolates were compared in liquid culture, at low temperatures. The isolates were grown in 50 ml nutrient broth (Oxoid) in 250 ml flasks (baffled) and incubated in shaking incubators (150 rpm) at 10°, 6° and 2°C. Growth was assessed using a spectrophotometer to detect changes in optical density (600 nm) of the growth media.

Xenorhabdus spp. isolates L/128 and L/179 grew significantly faster than the other three isolates, at all the temperatures tested and were the only isolates to grow at 2°C.

Introduction

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have a symbiotic association with bacteria of the family Enterobacteriaceae. These nematodes have been exploited commercially, resulting in a range of products being made available for the biocontrol of insect pests of protected crops in Europe.

A major limitation of entomopathogenic nematodes for biocontrol is that existing products are ineffective on pests of unprotected crops at the low temperatures prevailing from October to April in temperate climates of the northern hemisphere. In unprotected field crops nematodes selected for pest control purposes will only be effective at killing if 1) they can parasitise soil dwelling insect pests at low temperatures and 2) their bacterial symbiont can successfully colonise the host in the same conditions.

Our research compared the growth characteristics of five isolates of symbiotic bacteria at three temperatures that are typical of UK soils from October to April.

Materials and Methods

The source of the five nematodes and their bacterial symbiont (*Xenorhabdus* or *Photorhabdus* spp.) are shown in Table 1. Symbiotic bacteria were obtained from the haemolymph of nematode-parasitised wax moth (*Galleria mellonella*) larvae and identified by biochemical tests and fatty-acid profiling.

Table 1. Source and origin of the five nematodes and their symbiont

Bacterium	Nematode (isolate)	Source
X. bovienii	*S. affinis* (L/179)	HRI, cold-active soil survey
X. bovienii	*S. feltiae* (L/128)	HRI, cold-active soil survey
X. bovienii	*S. feltiae* (Nemasys®)	Agricultural Genetics Company (AGC) Ltd., Cambridge, UK.
X. nematophilus	*S. carpocapsae* (252)	Biosys, Palo Alto, USA.
P. luminescens	*H. megidis* (Nemasys H®)	AGC Ltd.

To assess bacterial growth colonies of *Xenorhabdus* and *Photorhabdus* spp. were transferred to 10 ml of nutrient broth and incubated at 10°, 6° or 2°C, on a shaking incubator. When spectrophotometer readings of the optical density (OD) at 600 nm reached 0.5, aliquots of 0.5 ml of this 'starter' broth were transferred to 250 ml baffled flasks containing 50 ml nutrient broth and incubated as above. Spectrophotometer readings were taken at intervals, most frequently during the exponential growth stage. Samples from each flask were plated onto selective media to determine that cells were Phase I.

Results

The growth rates of the selected isolates are represented on Fig. 1. There were statistically significant differences between the growth rates of the five *Xenorhabdus* and *Photorhabdus* spp. assessed. The isolates are ranked on Table 2 (fastest to slowest growing). The ranking was the same at all three temperatures except at 2°C, when L/179 grew significantly faster than L/128. Phase II cells were not detected in any of the flasks.

Table 2. Ranking of growth rates of *Xenorhabdus* and *Photorhabdus* spp. isolates.

Temperature	Ranking of isolates*	
	slowest	fastest
10°C	L/128[a], L/179[b], Nemasys[c], Nemasys H[d], Biosys 252[d]	
6°C	L/128[a], L/179[b], Nemasys[c], Nemasys H[d], Biosys 252[d]	
2°C	L/179[a], L/128[b], Nemasys[c], Nemasys H[c], Biosys 252[c]	

* [abcd] isolates with different letters have significantly different growth rates ($P < 0.05$).

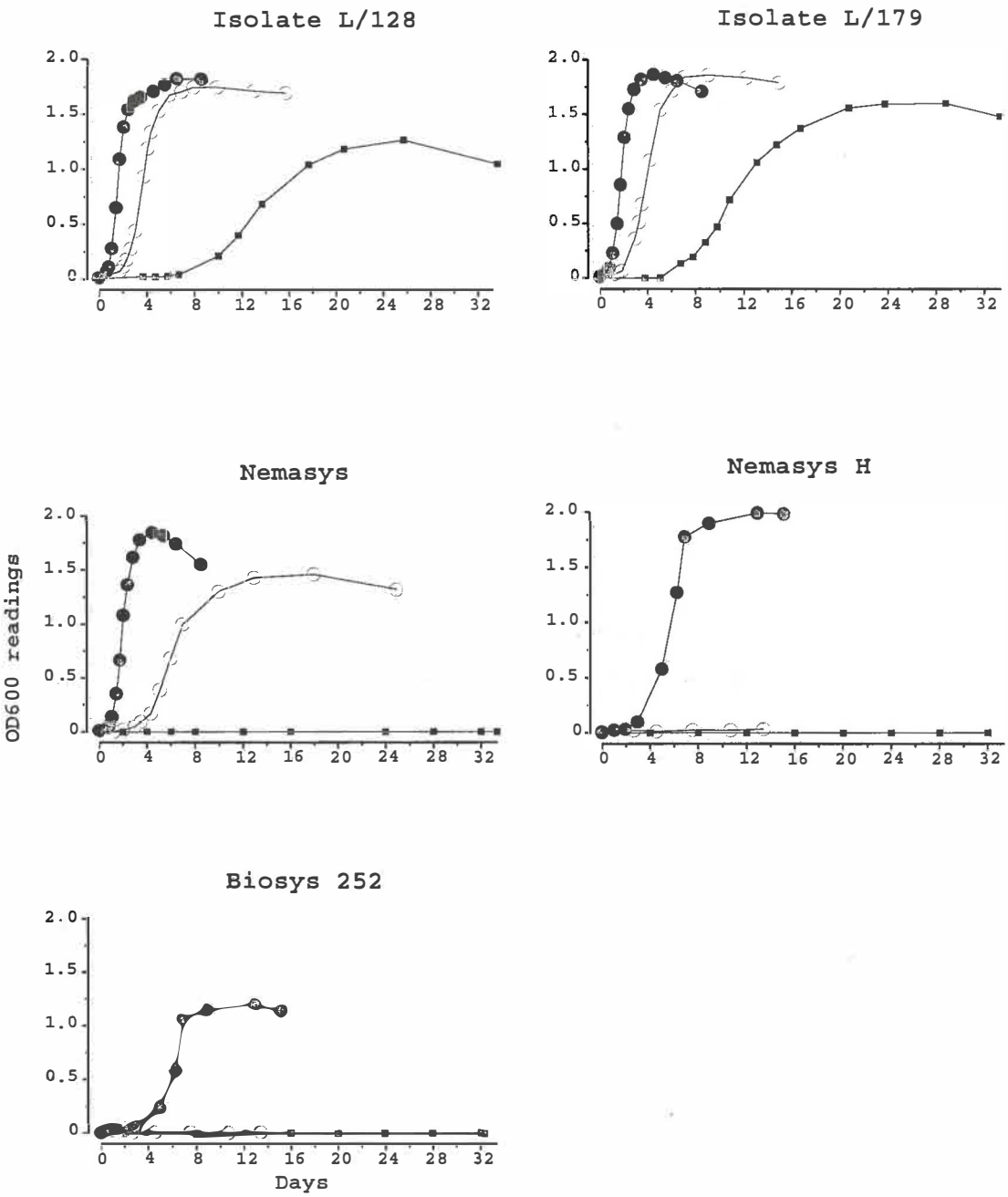

Fig. 1. Growth rates of Several *Xenorhabdus* and *Photorhabdus* spp. isolates at low temperatures —●— 10°C —○— 6°C —▪— 2°C

Discussion

It is significant that the symbiotic bacteria from the existing commercial strains showed little or no growth at the low temperatures used in this study. The lack of growth of these bacteria coincides with the ineffectiveness of their respective nematode hosts at parasitising insects at low temperatures: Nemasys® is not recommended for use below 10°C, Nemasys H® is not recommended for use below 14°C (product labels) and Biosys 252 is known not to be an effective parasite below 14°C (Dr. N G M Hague, pers. comm.). However, the two HRI *Xenorhabdus* spp. isolates were able to grow at low temperatures and the nematodes from which the bacteria are derived are able to parasitise vine weevil (*Otiorhynchus sulcatus*) larvae at low temperatures (unpublished data). This suggests that the effectiveness of entomopathogenic nematodes at parasitising insects at low temperatures may be dependent on the symbiotic bacteria being able to grow.

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THE INFLUENCE OF OSMOTIC STRESS ON INSECT PARASITIC NEMATODES

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Summary
The influence of gradual dehydration and subsequent gradual rehydration in steps of 24 hours in glycerol solutions of 5, 10, 15, 20 & 25 % (v/v) on infective juveniles of *Steinernema carpocapsae* (All), *S. feltiae* (UK76), Heterorhabditis sp. (HF85) and *H. bacteriophora* (HI82) was investigated. Changes in morphology and behaviour during dehydration, as well as survival after rehydration were assessed. Individuals of each species survived dehydration in concentrations up to 25 % glycerol. Dehydration resulted in shrinking. At the highest concentrations most nematodes were found in a motionless state, characterized by a straight position and typical surface structures.

1. Introduction

Osmotic stress is one type of environmental stress to which nematodes may respond by either slowing down or arresting metabolic rates. Nematodes with reduced metabolic rates are referred to as quiescent; cryptobiosis is a form of quiescence where there is no measurable metabolism. Both are reversible.

Research on insect parasitic nematodes sofar has concentrated on their ability to survive desiccation at reduced humidities (anhydrobiosis). The species studied were slow-dehydration strategists and entered a quiescent state rather than a cryptobiotic state (Womersley, 1990). Little attention has been payed to the effect of dehydration in hypertonic solutions on these nematodes. The present study provides information on the influence of osmotic stress on morphology, behaviour and viability of different species of insect parasitic nematodes.

2. Materials and methods

Steinernema feltiae (UK76), *S. carpocapsae* (All), *Heterorhabditis* sp. (HF85) and *H. bacteriophora* (HI82) were propagated and harvested according to standard procedures (e.g. Poinar, 1975). Before the start of the experiment, the infective juveniles were stored in 1 l bottles at 20 ^{o}C under constant aeration for one week. Samples of approximately $1{,}5 \times 10^{5}$ infective juveniles were concentrated on filter paper, added to 30 ml of 5 % (v/v) sterile glycerol in tissue culture flasks and left for 24 hours in a dark incubator at 20 ^{o}C. This was repeated for subsequent de- and rehydrations steps (10, 15, 20 & 25 %).

During the process of dehydration, samples of each species were examined for changes in morphology and behaviour and photographs

were taken (phase-contrast interference). Viability was assessed after the nematodes were rehydrated.

3. Results

In general, the four species responded very similar to dehydration.

Morphology

In 5 % glycerol, the body of the infective juveniles of the four species tested shrank. This was most clearly visible with ensheathed individuals, where the second stage cuticle marked the original volume. At 10-15 % glycerol many infective juveniles had lost so much water that their third stage cuticle started to fold. In 20 % glycerol or more most individuals entered a motionless state, characterized by a straight position and typical surface structures; regular arches and depressions especially in the area of the lateral fields.

Most individuals of UK76 were exsheathed and therefore shrinking was not so clearly visible unless concentrations were as high as 10 % or more. At 20 % this species was found in a half-moon shape and the typical surface structures were less pronounced. Infective juveniles of HF85 often lost their J_2 cuticle in 5 or 10 % glycerol and consequently many exsheathed individuals were found at higher concentrations.

Behaviour

Five % glycerol had little or no influence on the behaviour of infective juveniles compared to the behaviour in tap water. From 10 % glycerol on, the activity of the infective juveniles was reduced. The individuals of the four species moved slowly and tended to coil. At 15 % glycerol, the majority of the nematodes showed little or no activity. From 20 % glycerol on, all nematodes were immobile.

Activity of the All strain did not increase unless 15 % glycerol, in contrast to HI82, where many individuals were immobile from 5 % on.

Viability

Increasing glycerol concentrations caused a decrease in viability. *S. carpocapsae* All was superior in survival. From the other species approx. 60-70 % survived.

4. Discussion

The results showed that infective juveniles of various species of insect parasitic nematodes were able to survive dehydration in solutions that caused osmotic stress.

Some of the behavioural and morphological changes observed during the present study were comparable to those observed for other nematode species that were able to survive extended periods of desiccation (e.g. Reversat, 1981; Demeure & Freckman, 1981). That desiccation is accompanied by the formation of typical structures on the surface of the bodies especially in the area of the lateral fields was shown with *Rotylenchus robustus* (Rössner &

Porstendörfer, 1973). The authors suggested that folding of the lateral fields may be a mechanism of stabilizing the body and may be of great importance to the desiccation tolerance of many nematode species. Conclusively, it seems that insect parasitic nematodes are adapted to survive dehydration for extended periods.

Coiling of nematodes is thought to be a behavioural adaptation essential to dehydration survival (Womersley & Ching, 1989). Some individuals of *S. carpocapsae* for example, exhibited this adaptation when dried slowly at 97 % rh (Womersley, 1990). In the present study coiling of the infective juveniles was never observed when they had reached the motionless state. Maybe different mechanisms are involved in either anhydrobiosis and osmobiosis.

The presented results are encouraging, especially with respect to improving existing storage and shipment methods. A number of patents, based on the anhydrobiotic potential of steinernematids already exist (e.g. Yukawa & Pitt, 1985), but these methods are less successful with heterorhabditids.

If someone wants to determine the potential of insect parasitic nematodes to survive dehydration, it is helpful to assess the dehydration regimes occurring at their natural habitats (Womersley, 1990). Heterorhabditid isolates often originate from costal regions and due to the more salty soils in those regions, they may be better adapted to osmotic stress than to other types of dehydration stress.

Acknowledgment
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THE CURRENT VIEW ON THE TAXONOMY OF THE FAMILY STEINERNEMATIDAE

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SUMMARY

The history of the family Steinernematidae was beginning in 1923 when the nematode *Steinernema kraussei* was described by Steiner from the body cavity of spruce sawfly larvae, *Cephaleia abietis*. Since this year more than 30 species has been described, but many of them are considered as the younger synonyms. At present the following species are chronologically ranked in the genus *Steinernema: S. kraussei, S. glaseri, S. feltiae, S. affinis, S. carpocapsae, S. anomali, S. intermedia, S. rara, S. kushidai, S. scapterisci, S. ritteri, S. neocurtillis, S. riobravis* and new announced *S. longicaudum, S. serratum* and *S. cubana*.

Important diagnostic criteria in the taxonomy of Steinernematidae are following: infective juveniles (body length, number of ridges in lateral fields, distance from anterior to excretory pore /EP/, ratio EP to tail length); males (distance from anterior to EP, number and arrangement of genital papillae, presence of mucron, general shape of spicules, ratio EP to length of esophagus /ESO/, ratio length to width of spicule manubrium); females (protruding of vulva, ratio EP to ESO).

INTRODUCTION

Since Steiner's (1923) description of *Steinernema kraussei* more than 30 species were described in the genus *Steinernema* syn. *Neoaplectana*. Many of them are considered as a younger synonyms now. In 1929, Steiner erected the genus *Neoaplectana* and in 1934, Filipjev joined both these genera in the subfamily Steinernematinae which was later promoted to the family level. An examination of *S. kraussei* and several *Neoaplectana* species revealed no difference in regards to the number and arrangement of labial and cephalic papillae which was a main criterium for separating these genera and *Neoaplectana* was synonymized by Wouts et al. (1982) under *Steinernema*.

Until present, the taxonomy of the family Steinernematidae is not solved satisfactorily and it remains a puzzle for many insect nematologists. There are three approaches in a concentrated effort of taxonomists in last years. The first deal with a general morphology of infective juveniles as well adults, the second utilizes DNA analyses and the third is based on cross-breeding method.

The most used approach is based on general morphology. However, it fails sometimes even in a determination of species because of an incompatibility of selected criteria. In order to avoid further confusion and establish consistency, the most important characters and criteria valuable for the taxonomy are discussed here as an objective.

RESULTS AND DISCUSSION

Since 1923 more than 30 species were described in the genera *Steinernema and Neoaplectana*. Of these 16 species, described or in press, are considered as recognized species.

A list of the current Steinernema species

1. *Steinernema kraussei* (Steiner, 1923)
2. *Steinernema glaseri* (Steiner, 1929)
3. *Steinernema feltiae* (Filipjev, 1934)
4. *Steinernema affinis* (Bovien, 1937)
5. *Steinernema carpocapsae* (Weiser, 1955)
6. *Steinernema anomali* (Kozodoy, 1984)
7. *Steinernema intermedia* (Poinar, 1985)
8. *Steinernema rara* (Doucet, 1986)
9. *Steinernema kushidai* Mamiya, 1988
10. *Steinernema scapterisci* Nguyen & Smart, 1990
11. *Steinernema ritteri* Doucet & Doucet, 1990
12. *Steinernema neocurtillis* Nguyen & Smart, 1992
13. *Steinernema riobravis* Cabanillas, Poinar & Raulston, in press
14. *Steinernema longicaudum* Chang-peng Shen, in press
15. *Steinernema serratum* Jie Liu, in press
16. *Steinernema cubana* Mráček, Arteaga, Boemare, in press

Characters of the family value

These characters represent a diagnosis of the family Steinernematidae in the superfamily Alloionematoidea, order Rhabdita. They are relatively constant and they do not aid in the taxonomy on the genus or species level.

Adults: six fused lips, six labial and four cephalic papillae, two lateral amphids, indistinct phasmids, smooth cuticle with slight annulation (under SEM) lateral fields absent, stoma reduced, no tooth or stylet,pharynx with a cylindrical procorpus and spherical postcorpu ovaries opposed and reflexed, vulva opening in midbody portion, testi single, reflexed at tip,genital papillae present, usually 11 pairs and 1 single papilla.
Infective juveniles: cephalic papillae indistinct, mouth and anus clos pharynx and intestine collapsed, cuticle smooth with slight annulation (under SEM), lateral fields present.

Characters of the genus (group) value

These characteristics have the greatest significance in the taxonomy of Steinernematidae. In a dependency upon them all species can be separated in groups which may serve as a possible future genera.

Adults
- excretory pore position (male)

Posterior to nerve ring
(glaseri)
anomali
cubana

Anterior to nerve ring

a) at corpus swelling	b) at fore part of corpu
kraussei	(*carpocapsae*)
feltiae	*neocurtillis*
affinis	
intermedia	
rara	
kushidai	
scapterisci	

- Mucron

Absent	Present
glaseri	*kraussei*
anomali	*feltiae*
cubana	*carpocapsae*
intermedia	*rara*
neocurtilis	*kushidai*
	scapterisci

- Spicule manubrium length/width

ratio 1 : 1	ratio 1.5-2 : 1
kraussei	*glaseri*
affinis	*feltiae*
carpocapsae	*kushidai*
anomali	*neocurtilis*
intermedia	*cubana*
rara	
scapterisci	

Other characters: spicules coloration (coloured or colourless), spicule retinaculum (developed or absent), spicule tip (pointed or blunt), vulva protruding (slight, moderate, severe).

Infective juveniles

- body length

glaseri group (average length over 1000 µm)
glaseri
anomali
cubana

kraussei group (from 600 to 1000 µm)

kraussei	subgroup a/ 800-1000 µm	*kraussei*
feltiae		*feltiae*
neocurtillis		*neocurtillis*
affinis	subgroup b/ 600- 800 µm	*affinis*
intermedia		*intermedia*

carpocapsae group (less than 600 µm)
carpocapsae
scapterizci
rara
kushidai
ritteri

- number ridges in lateral fields

8 ridges	7 ridges	6 ridges
kraussei	*neocurtillis*	*affinis*
glaseri		*intermedia*
feltiae		
(carpocapsae)		
anomali		
kushidai		
rara		
scapterisci		
ritteri		
cubana		

Characters of the species value

These characteristics can be used for an accurate identification of species.

S. glaseri - hooked spicula tip
S. affinis - spine-like structure in infectives tail
S. intermedia - dorsal constriction in infectives tail
S. scapterisci - eliptically shaped structure, overflapping vulva
S. rara - double preanal papilla
S. neocurtillis - extraordinary anterior position of excretory pore

Important ratios
By Poinar (1986)
Ratios A - body length divided by width, B - body length divided by distance from anterior end to base of esophagus, C - body length divided by length of tail, D - distance from anterior end to base of esophagus divided by tail length, E - distance from anterior end to excretory pore divided by tail length

By Nguyen & Smart (1992)
EW - excretory pore from anterior end divided by body width at excretory pore, SW - spicule length divided by body width at cloaca GS - gubernaculum length divided by spicule length

E.g. Ratio D m a l e s - three groups

S. neocurtillis	0.19	*S. glaseri*	0.70
S. carpocapsae	0.41	*S. anomali*	0.93
S. scapterisci	0.38	*S. intermedia*	0.67
		S. cubana	0.70
S. ritteri	0.47		
S. kraussei	0.50		
S. feltiae	0.60		
S. affinis	0.61		
S. rara	0.50		
S. kushidai	0.51		

S. kraussei which was re-isolated and re-described from the original locality in Germany must be considered as the type of genus *Steinernema* as well this genus as the type of family Steinernematidae.

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DEVELOPMENT OF *STEINERNEMA FELTIAE* (STEINERNEMATIDAE: NEMATODA) IN *BRADYSIA PAUPERA* (SCIARIDAE: DIPTERA)

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Summary

S. feltiae entered larvae of *Bradysia paupera* within 3h, pre-adults formed after 18h, the first adults appeared after 27h and within 45h new infective juveniles were released. Only one generation of *S. feltiae* occured in sciarids, often small stunted females were produced and infective juveniles from stunted females were smaller than those from normal sized females.

Introduction

Sciarids are important pests of both mushrooms and ornamentals in glasshouses: in the latter they can increase to levels which are damaging to the crop. Although larval sciarids feed on organic matter, fungi and algae, they will also damage roots and tunnel into stems (Binns, 1973). The entomopathogenic nematode *Steinernema feltiae* is used commercially in many parts of the world to control sciarids on mushrooms, eg *Lycoriella mali* in the USA (Nickle and Cantello, 1991) and *L. auripila* in the UK (Richardson and Grewal, 1991): commercial strains of *S. feltiae* are also available in the Netherlands and Denmark for controlling sciarids in glasshouse flower crops (Gillespie, private communication).

In UK glasshouses one of the commonest sciarids is *Bradysia paupera* which can be controlled with *S. feltiae* (Gouge, unpublished).

The objectives of this study were:

1. To investigate the rate of development of *S. feltiae* in larvae of *B. paupera*.

2. To compare the development of *S. feltiae* in hosts of different size.

3. To investigate the pathogenicity of different sized infective juveniles exiting from different sized hosts.

Materials and Methods

The *S. feltiae* used in these experiments was isolated from soil at the University of Reading Farm, Berkshire, using a *Galleria mellonella* bait. The nematode was cultured on *G. mellonella*, extracted on a modified White trap and the infective juveniles (IJs) stored at 6°C for not more than one week before use in experiments.

Experiment 1

Fourth stage *B. paupera* larvae were exposed to 200 IJs in sand at 23°C in multi-well tissue culture plates. Ten sciarid larvae were dissected every 3 h for 48 h and the stage of development of *S. feltiae* noted.

Experiment 2

Larvae of three different insect hosts, *G. mellonella* (1st, 4th and 6th instars), *Phaedon cochleariae* (3rd instar) and *B. paupera* (3rd instar) were exposed to 500 IJs on filter paper at 23°C. After 72 h sciarid larvae were dissected in $^1/_4$ strength Ringers solution and their stage of development and adult nematode length measured.

Experiment 3

In the second experiment it was noted that some of the female *S. feltiae* produced in the smaller insects were reduced in size: these smaller *S. feltiae* produced shorter IJs.

Third stage larvae of *B. paupera* were exposed to 20 IJs from each of the following categories:

(a) Small IJs harvested from *B. paupera*.
(b) Normal sized IJs harvested from *B. paupera*.
(c) Normal sized IJs harvested from *G. mellonella*.

Exposure was in sand in 3.5 cm petri-dishes at 23°C for 72 h, replicated 10 times: the number of nematodes entering was counted.

Results and Discussion

Infective juveniles entered through the mouth and anus: entry through spiracles, which has been observed for other species of *Steinernema* and *Heterorhabditis* (Georgis & Hague, 1981; Mracek *et al.*, 1988) was not observed in sciarids: only one generation of *S. feltiae* occurred in *B. paupera*. The first IJs were found inside sciarid larvae after 3 h exposure, pre-adults had developed at 18 h, the first adults appeared after 27 h and IJs of the next generation were being produced in the sciarid larval cadavers after 45 h. The septicaemic tissues in sciarids disintegrate very rapidly so that nematode development must be accelerated to enable the production of IJs before the cadaver completely disappears. Very rapid disintegration of the host also occurred in 1st instar *G. mellonella* and 3rd instar *P. cochleariae*. Small stunted "pygmy" females are produced (Table) which in turn give rise to a proportion of stunted IJs. *S. feltiae* will persist in compost for several weeks after application (Gouge, unpublished) and the rapid development of the nematode in sciarids and the production of new IJs suggests that re-cycling of the nematode may occur in an environment where sciarids are continuously laying eggs and larvae are always present.

In the third experiment the IJs from the three categories all infected *B. paupera*, caused septicaemia and produced both normal and stunted females: the size of males remained relatively constant irrespective of the size of the host (Table).

Insect	Larval instar	Mean insect weight (g)	Mean length (mm)		Size range (mm)	
			Female	Male	Female	Male
Galleria mellonella	L1	0.0030	1.12	0.87	0.55-> 1.50	0.75-> 0.95
	L4	0.0698	2.98	0.91	2.79-> 3.25	0.80-> 0.94
	L6	0.1639	3.65	1.02	4.00-> 3.30	0.88-> 1.25
Phaedon cochleariae	L3	0.0083	2.39	0.96	1.28-> 3.52	0.64-> 1.56
Bradysia paupera	L3	0.0057	1.50	1.01	0.82-> 3.20	0.88-> 1.14

Table. The development of *Steinernema feltiae* in different sized larvae of three hosts: mean size in mm of males and females produced.

Acknowledgement

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The analysis of *in vivo* bioassay in entomopathogenic nematode research: a behavioural approach

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Introduction

The *in vivo* bioassay method is a fundamental tool in entomopathogenic nematode research and the need for robust techniques for the analysis of bioassay data cannot be over-emphasised. In this paper, several different approaches to analysis are discussed. The use of probits for the interpretation of host survival/mortality data is evaluated and an alternative, the exponential or "one-hit model" (Peto, 1953), is suggested. Simple linear models of nematode infectivity (Hominick & Reid, 1990) are reviewed and we propose a new model based on a modification of the binomial distribution. This is used to show that nematode behavioural strategies are an important component of nematode efficacy.

The analysis of insect mortality data

Probit analysis is conventionally used for the analysis of dose-response data from *in vivo* bioassays with entomopathogenic nematodes. However, probit analysis is a pharmacological approach and it is based on the assumption that all the toxin molecules included in a critical dose are necessary to elicit the response. Entomopathogens, on the other hand, act independently and are thus not analogous to drugs or toxic chemicals. A single nematode that successfully evades the host immune system is capable of killing its host because of the rapid proliferation of *Xenorhabdus* bacteria. Therefore, an exponential model (see Peto, 1953) that is based on the principle of particulate invasion (i.e. a discrete rather than continuous dose) is more appropriate. The discrete nature of the pathogen can lead to marked dosage error, particularly at low doses (Huber & Hughes, 1984), so that for virulent entomopathogens in particular, the exponential approach is both more robust and more defensible.

Counting nematodes in each parasitised host

Documenting insect mortality provides only data on the fate of the host relative to nematode dose. A more informative approach is to dissect insects at the end of an experiment and to record the number of nematodes in each. These data can be used to determine the actual numbers of successful infections necessary to kill a host; to assess the relative proportions of virulent and avirulent entomopathogens; and to estimate a measure of parasite aggregation. Such an approach is advocated by Hominick and Reid (1990), who describe a "universal bioassay" in which the number of nematodes infecting larvae of the greater wax moth, *Galleria mellonella* are assessed. They also cite the unpublished data of Fan and Mason (1989) who showed that highly significant regression coefficients can be obtained between the nematode application dose and the subsequent numbers of individuals establishing in this host (transformation of the data is unnecessary). The slope of the regression line can then be used as a comparative measure of virulence for each isolate tested. However, *G. mellonella* is one of the most susceptible insects to entomopathogenic nematodes (e.g. Bedding *et al.*, 1983) and these results have proved difficult to reproduce in other insect hosts. For example, Mannion and Jansson (1993), using five entomopathogenic nematodes, failed to record a significant correlation between dose and establishment in larvae of the sweet potato weevil, *Cylas formicarius*. Similarly, Epsky and Capinera (1991) tested two isolates of *Steinernema carpocapsae* for infectivity to the fall armyworm, *Spodoptera frugiperda*, but failed to observe a strong correlation between nematode dose and the number of infections per host.

The linearity hypothesis is equivalent to a simple binomial model for nematode infectivity, i.e. each nematode is assumed to have the same probability of infecting a host, a parameter that can be estimated from the slope of the regression line. Where linearity in infection data cannot be demonstrated, however, alternative models are required for statistical analysis. In our laboratories, a zero-modified binomial model (manuscript in preparation) has been developed to describe nematode invasive behaviour at different application doses. The number of nematode infections at a dose of one nematode to each host

(primary infection probability) is assessed and these data are then compared with the numbers of successful infections at higher nematode application doses. Thus, for each isolate two probability estimates are obtained: the primary infection probability and the probability of secondary infection (conditional upon the first infection having been achieved). Data for four isolates of *Steinernema feltiae* tested against the sciarid fly, *Lycoreilla solani*, show that the probability of a secondary infection is always greater than that of a primary infection (i.e. the nematodes do not behave independently as a linear model would suggest). The implication is that (a) a nematode-infected host is more susceptible to subsequent invasion than an unparasitized host; and (b) a population of infective-stage nematodes comprises both individuals that readily infect unparasitised hosts, and others that rely on prior infection before they invade. The functional explanations for these observations are probably the intuitive ones: first, that infected hosts lack protective immunity and are therefore more susceptible; and second, that nematodes select hosts that are already infected because this increases the likelihood of finding a potential mate.

Conclusions

Recently there has been a shift of emphasis in the entomopathogenic nematode literature towards a more behavioural approach. This should be welcomed because an improved understanding of nematode dispersion, host seeking behaviour and reproductive strategy is likely to contribute to the efficiency with which they can be exploited for pest management. In presenting this paper, we hope to have focused attention on the benefits of a behavioural approach to bioassay data interpretation. Bioassay experiments are a routine procedure in many laboratories, careful experimental design and analysis may mean that much more information than a simple ranking of isolate performance can be attained.

Acknowledgements

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EVALUATION OF ENTOMOPATHOGENIC NEMATODE STRAINS FOR CONTROL OF *DELIA RADICUM, TIPULA PALUDOSA* AND *T. OLERACEA*

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Summary

Entomopathogenic nematodes were tested in the laboratory for control of *D. radicum, T. paludosa* and *T. oleracea.* Mean larval mortality ranged from 22% to 87% for *D. radicum*, 14% to 71% for *T. paludosa* and 25% to 100% for *T. oleracea. S. feltiae* (OBSIII) caused highest mortality of third instar *D. radicum* (87%), second instar *T. paludosa* (71%) and *T. oleracea* (86%). Nematode caused mortality of first instar *D. radicum* ranged from 22% (*S. feltiae* SF-S22) to 39% (*S. carpocapsae* AII). Third instar *D. radicum* were significantly more susceptible than first instars only for *S. feltiae* (OBSIII and SF-S22). In *D. radicum* dauer juveniles never were encapsulated and a single nematode in the haemocoel always caused larval death. In *Tipula* spp. some larvae survived nematode infestation by encapsulation of penetrating DJs. Other than *Steinernema* spp., *Heterorhabditis* dauer juveniles were never encapsulated by *Tipula* larvae.

1. Introduction

The order Diptera contains a number of insect pests difficult to control. The cabbage root maggot, *Delia radicum* L. is a common pest in vegetables. Larvae of the crane fly *Tipula* spp. cause severe damage to grassland, pastures and meadows, as well as lawns and sportsfield in Europe and North America. Larvae of both species live in the soil (*Tipula* spp., L1 of *D. radicum*) or in roots of host plants (L2 to L4 of *D. radicum*) and thus are suitable for control with entomopathogenic nematodes. Objective of the presented study was the selection of a nematode strain with a high control potential.

2. Material and Methods

The susceptibility of first and third instar *D. radicum* and second instar *Tipula* spp. to different nematode species and strains was tested in the laboratory. The following species/strains were used: *Steinernema carpocapsae* (ALL), *S. feltiae* (OBSIII, SF-S22, NV17E, and Ruotsie), *S. affinis, S. glaseri, S. kraussei, S. anomali, S. intermedium* and *Heterorhabditis* sp. (HSH2). Nematodes were propagated in last instar *Galleria mellonella* at 25°C and stored at 15°C for about 2 weeks. All insect species were tested at the same time with the same nematode batch. Single test larvae (20 insects/strain) were placed in cell wells (1.8 cm, diameter) filled with silica sand (grain size: 200-400 µm; 15% moisture). Dauer juveniles (DJs) were added at dosages of 20 and 100/larvae for *D. radicum* and *Tipula* spp., respectively. After 15 h nematode exposure at 20 °C the

larvae were removed from the sand and washed with tap water. 2 days later nematode caused mortality was determined. Dead insects were dissected and only those with nematodes inside the haemocoel were counted as nematode killed larvae. Viable larvae were searched for encapsulated nematodes inside the haemocoel, which were visible through the transparent cuticle.

3. Results

Mean larval mortality ranged from 22% to 87% for *D. radicum*, 14% to 71% for *T. paludosa* and 25% to 100% for *T. oleracea* depending on the nematode species/-strain and the larval stage. The *S. feltiae* strains caused highest mortality of second larval stages of *T. paludosa* and *T. oleracea* (Fig. 1 and 2) and, with the exception of *S. feltiae* Ruotsie, they also were the most effective strains againt *D. radicum* L3 (Fig. 3). Mortality of first stage larvae of *D. radicum* did not differ significantly among the nematode strains tested, ranging from 22% (*S. feltiae* SF-S22) to 39% (*S. carpocapsae* ALL). In contrast, the susceptibility of L3 differed, depending on the strain tested. In comparison with L1 larvae the L3 were significantly more susceptible (Chi-square test, $p<0.05$) but only to *S. feltiae* (OBS III and SF-S22) (Fig. 3). Mean numbers of nematodes in the maggots were low (1-3) for all nematode strains tested and there was no correlation between mortality and number of invaded nematodes for the L1 ($r=0.33$, $df=5$, $p<0.05$) and L3 ($r=0.34$, $df=5$, $p<0.05$).

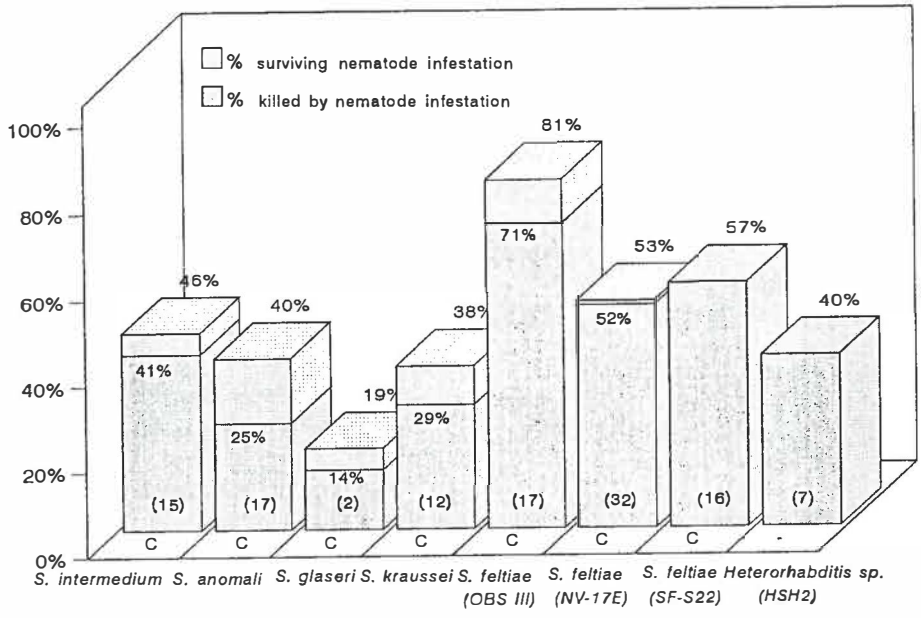

Fig. 1: Infestation and mortality of *T. paludosa* (L2) after application of 100 DJs/larva of different strains/species of *Steinernema* and *Heterorhabditis* (20 insects/nematode strain). Number in brackets are mean number of nematodes/larva. C = encapsulated nematodes observed

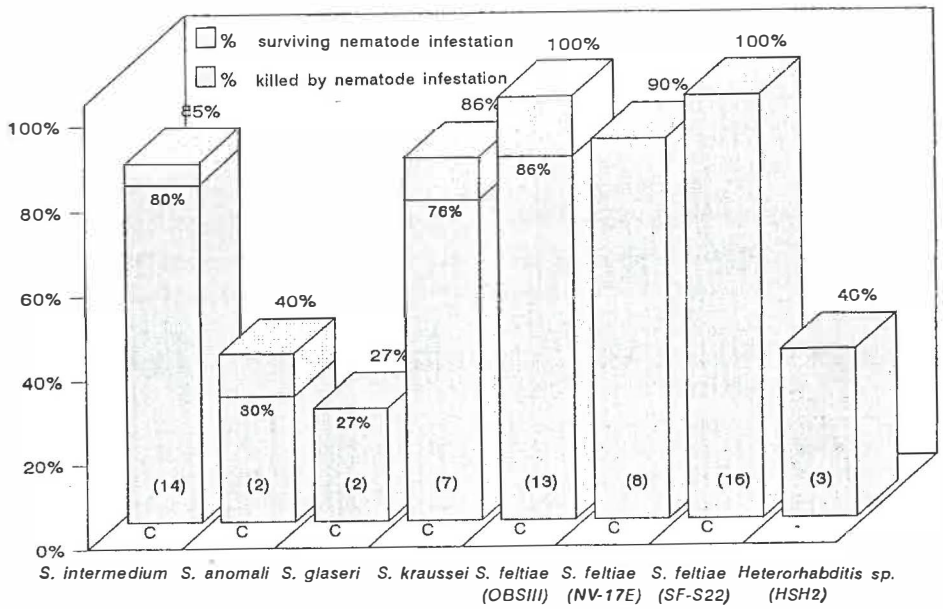

Fig. 2: Infestation and mortality of *T. oleracea* (L2) after application of 100 DJs/larva of different strains/species of *Steinernema* and *Heterorhabditis* (20 insects/nematode strain). Number in brackets are mean number of nematodes/larva. C = encapsulated nematodes observed.

T. oleracea (Fig. 2) proved to be more susceptible than *T. paludosa* (Fig. 1) to all strains, except *S. anomali* and *Heterorhabditis* sp. (HSH2). Maggot larvae never encapsulated invading DJs, while *Tipula* spp. were able to encapsulate DJs of all *Steinernema* strains. Heterorhabditids were never encapsulated. Some leatherjackets survived nematode infestation by the encapsulation of the penetrating DJs. The number of invaded nematodes which escaped encapsulation was significantly correlated to the mortality for *T. oleracea* (t-test: $r = 0.88$, $df = 5$, $p < 0.05$). For *T. paludosa* no correlation could be confirmed at the same level of significance ($r = 0.58$, $df = 5$, $p < 0.05$).

4. Conclusions

S. feltiae was the most effective species to both, *Tipula* spp. and *D. radicum* and thus may represent the most suitable species for the control of leatherjackets and the L3 stage of the cabbage root maggot. However, no strain was superior for the control of the L1 of *D. radicum*. The L1 is the only free-living stage that can easily be reached by entomopathogenic nematodes, since maggots inside the roots are barely effected by nematodes (Bracken, 1990). As there is no difference in susceptibility of the first instar *D. radicum* to different nematode species/strains (also found in young instars of *Tipula*

spp. [Peters & Ehlers, submitted)], chances for improvement of the nematode control potential for use in *D. radicum* biocontrol by selection of strains are poor. In contrast, susceptibility of *Tipula* spp. differs considerably according to the nematode strain used. Thus an improvement by selection among different strains and even within a population of one strain may be more promising for biocontrol of *Tipula* spp..

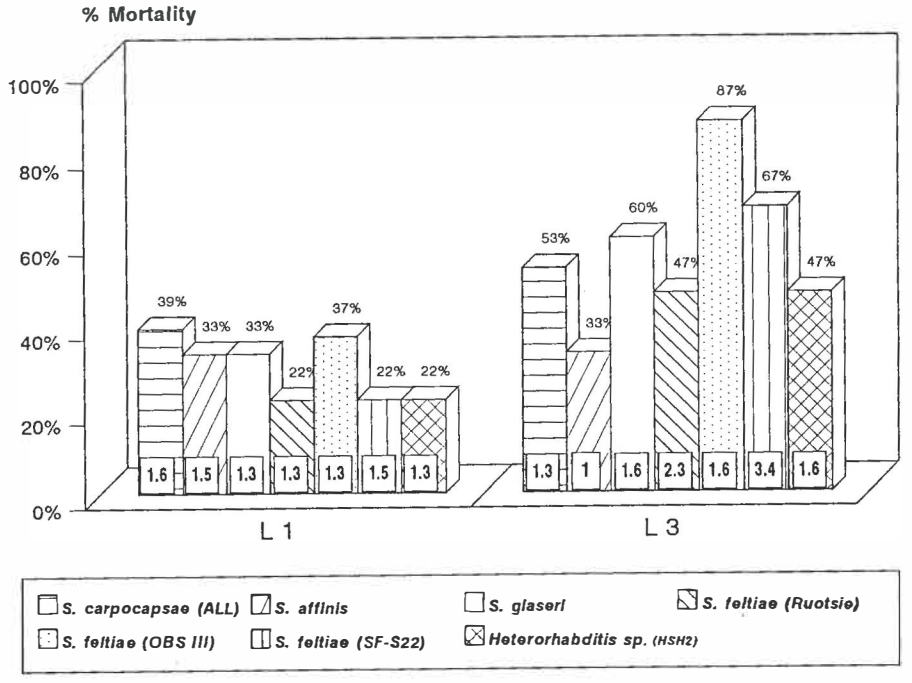

Fig. 3: Mortality of *D. radicum* (L1 and L3) after application of 20 DJs/larva of different strains/species of *Steinernema* and *Heterorhabditis* (20 first and third instars/nematode strain). Numbers on the bars represent mean number of nematodes/larva.

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CONTROL POTENTIAL OF A NATURALLY OCCURRING *STEINERNEMA FELTIAE* POPULATION

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Summary

In a strawberry field in Schleswig-Holstein (Germany) infested with larvae of the weevil *Phyllobius urticae* (Curculionidae, Otiorhynchinae) a high level of natural infection with an indigenous population of the entomopathogenic nematode *Steinernema feltiae* was found. In 16,2% of the insects (N = 179) collected on September 23, 1992 the nematode *S. feltiae* was found. 7,3% died due to infection with *Entomophtora* sp. and from one larva *Metarhizium* sp. was isolated. *S. feltiae* also was the main mortality factor on October 1 and 16, although soil temperatures had dropped below 12°C after October 1. No larvae were infected with two released nematode strains (*Heterorhabditis* sp. HSH and *Steinernema carpocapsae*), applied at 500,000 dauer juveniles (DJs)/m^2 on October 1. In laboratory assays mortality of *P. urticae* after application of 10 DJs of the indigenous *S. feltiae* was 25% at 20°C and 4% at 10°C, while *Heterorhabditis* sp. (HSH) and *S. carpocapsae* caused lower mortality (13% and 8%, respectively) at 20°C and did not kill any larvae at 10°C. Mean population density of the indigenous nematode was 70 DJs/500ml soil sampled monthly from September 1992 to July 1993 and exceeded the density of the released species. The relatively high control potential of this *S. feltiae* population to *P. urticae* may be attributed to an increase in population density by propagation on the weevil larvae, which could have resulted in an increased pathogenicity by means of natural selection.

1. Introduction

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are geographically widespread and are regularly found in forest and farmland soils (e.g. Hominick & Briscoe, 1990). Nevertheless, reports on the interaction of naturally occurring populations of *Steinernema* spp. or *Heterorhabditis* spp. with insect populations are rare (Mracek, 1988; Georgis & Hague, 1981; Raulston *et al.*, 1992). Observations on these interactions contribute to the understanding of the ecology of entomopathogenic nematodes and may also provide efficient strains against certain insect pests.

2. Material and Methods

Larvae of the weevil *Phyllobius urticae* were collected on a strawberry field in Schleswig-Holstein (Germany) on Sep. 23, Oct. 1 and Oct. 16, 1992 and kept at 20°C for 5

days before mortality was assessed. Larvae were dissected and the pathogen was identified by microscopical examination. After the second sampling on Oct. 1, each of two nematode strains, *Heterorhabditis* sp. (HSH) and *S. carpocapsae*, was released on 4 plots of 54 m². Half a million DJs/m² were sprayed in 20 cm bands on the strawberry plants, which were irrigated with 1l/m². Soil temperature was recorded every two hours in 5, 10 and 20cm soil depth with a data-logger system.

For the monthly estimation of the DJ population density soil samples were taken over a period of 10 months from two plots, one treated with *S. carpocapsae* and one treated with *Heterorhabditis* sp. HSH. 7 soil cores of 30 cm depth and 1.8 cm ϕ were randomly taken in a 2 m section of a strawberry lane of 20 cm in breadth. Nematodes were extracted from 500 ml of the soil sample by the flotation and the Baermann technique and entomopathogenic DJs were identified and counted.

Pathogenicity of the indigenous and the two applicated nematode species against *P. urticae* was tested in laboratory assays at 20 and 10°C. Single weevil larvae were put in plastic vials (1.5cm ϕ) and covered with moist (15% water) silica sand (grain size 0.2-0.4 mm). 24 larvae were used for each nematode species and treated with 10 DJs/larva. DJs of *Heterorhabditis* sp. and *S. carpocapsae* were commercial products. The DJs of *S. feltiae* were obtained from infected weevil larvae using a White trap. Prior to application, DJs of all species were stored in tap water at 6°C for at least 2 days. Larvae were exposed to nematodes for 5 days and mortality of larvae incubated at 20°C was assessed. Larvae exposed at 10°C were washed and incubated at 20°C for another 5 days prior to mortality assessment. Dead larvae were dissected and the penetrated nematodes were counted.

3. Results

Strawberry plants were damaged by the high densitiy of *P. urticae* larvae (mean: 4 larvae/plant, n = 60 plants). A high percentage (24.1%) of the larvae collected on Sep. 23 were dead and the indigenous entomopathogenic nematode *S. feltiae* represented the most important mortality factor (16.2 % larvae infected). The rest of the dead larvae revealed infections with entomopathogenic fungi (7.3% *Entomophtora* spp., 0.6% *Metarhizium*). Due to falling soil temperatures (<12°C) infections with fungi and nematodes decreased in larvae sampled on Oct. 1 and 16. *S. feltiae* always was the main mortality factor (Fig. 1). Coinfection with nematodes and fungi was never observed. None of the applied nematode species had infected a weevil larvae on Oct. 16, which must also be attributed to the decrease in soil temperature after Oct. 1. The laboratory assays showed, that the indigenous *S. feltiae* was the only species that infected weevil larvae at 10°C and was also most pathogenic at 20°C (Tab. 1).

Population density of the indigenous nematode *S. feltiae* (mean of DJs recovered in both, the *S. carpocapsae* and the *Heterorhabditis* sp. plot) varied considerably over the 10 month period when compared with the DJ density of the two released species. With two exceptions DJ density of *S. feltiae* always exceeded the density of the released species (Fig. 2).

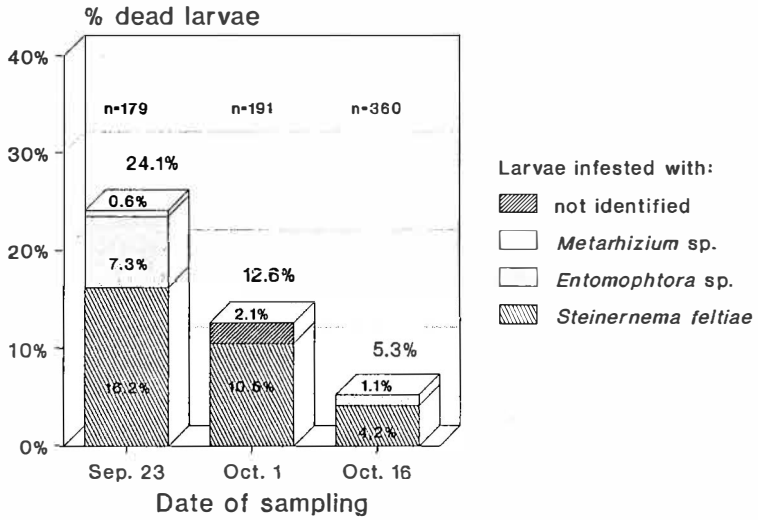

Fig. 1: Pathogens isolated from dead *Phyllobius urticae* larvae collected on a strawberry field in Schleswig-Holstein in September/October 1992.

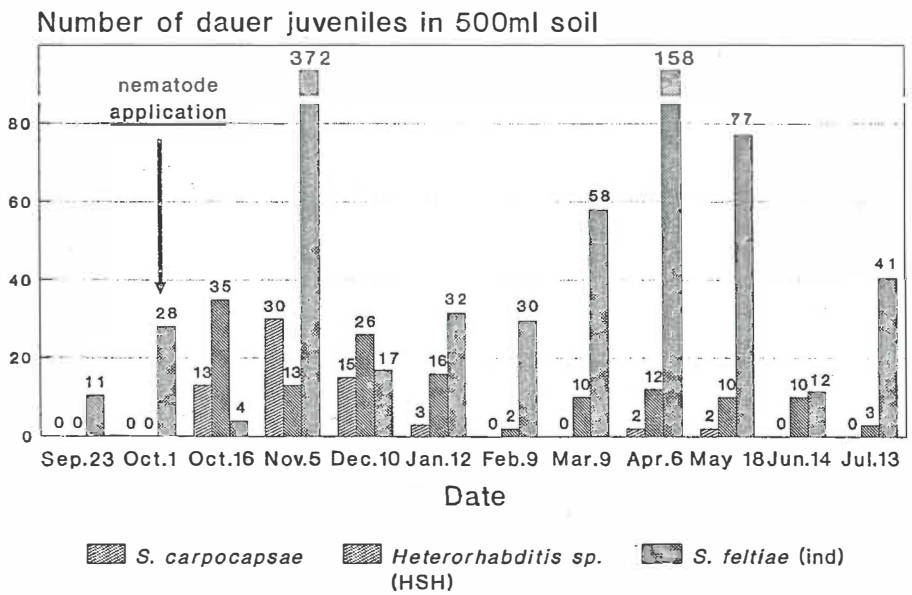

Fig. 2: Density of dauer juveniles (DJs) in soil samples taken on two plots in a strawberry field in Schleswig-Holstein. *Steinernema carpocapsae* and *Heterorhabditis* sp. were sampled on plots where the respective species was applied. Numbers of *S. feltiae* represent the mean DJ density in both plots treated with *S. carpocapsae* and *Heterorhabditis* sp.

Table 1: Mortality of *Phyllobius urticae* after application of 10 dauer juveniles of the indigenous *Steinernema feltiae* and the released nematodes (*Heterorhabditis* sp. (HSH) and *S. carpocapsae*) in moist sand.

Nematode species	20 °C		10 °C	
	% Mortality (n = 24)	Mean number of nematodes in larva	% Mortality (n = 24)	Mean number of nematodes in larva
Heterorhabditis sp. (HSH)	13	1.3	0	-
S. carpocapsae	8	1	0	-
S. feltiae	25	3.8	4	1

4. Conclusions

Although the indigenous *S. feltiae* population infested 16.2% of the larvae in September it did not prevent plant damage during the previous summer. This may be explained by a relatively low population density of *S. feltiae* in spring which increased by propagation on the weevil larvae during the summer. During this propagation the pathogenicity of this *S. feltiae* population to *P. urticae* may have increased by means of natural selection. The variation in DJ density of *S. feltiae* may be explained by its highly aggregated distribution in contrast to the released species, which were evenly distributed by the application. Neither *S. carpocapsae* nor *Heterorhabditis* spp. (HSH) did control the weevil larvae due to soil temperatures < 12°C. However, *Heterorhabditis* spp. was successfully established. In contrast to *Otiorhynchus sulcatus* weevils of *P. urticae* occur earlier (April). Due to low soil temperatures in autumn and early spring, nematode application can only be successful in summer (July, August), which emphasizes the necessity of early pest population monitoring.

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GREENHOUSE AND FIELD CONTROL OF BLACK VINE WEEVIL *Otiorhyncus sulcatus* F., WITH *Steinernema carpocapsae* AND *Heterorhabditis* sp.

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Nematodes of the genera *Heterorhabditis* and *Steinernema* are obligate parasities of insects, mutualistically associated with entomopathogenic bacteria of the genus *Xenorhabdus* which play an important role in killing the host (Poinar and Thomas, 1966). There are several reports on control of soil pests in glasshouses and in open fields (Klein, 1990).

In Belgium, the black vine weevil is an important and major pest of glasshouse and outdoor ornamentals. The real damage is caused by larvae which feed on roots, stems and other subsoil parts. Biocontrol of this weevil with nematodes gained momentum and the present investigation was taken up to study the pathogenicity of *S. carpocapsae* W.(strain 25) and *Heterorhabditis* sp.(HF85) on black vine weevil larvae.

MATERIALS AND METHODS

Two commercial preparations of nematode species, *Steinernema carpocapsae* S25 (Exhibit) and *Heterorhabditis* sp HF85 (Optimaaltijes) are used for the experiments.

In greenhouse experiment there were five treatments with 1000, 3000, 10000, 30000 nematodes per 250 ml container pot and control. Different doses of nematodes were applied with 10ml pipette. The mortality of larvae was counted at 10, 15 and 20 days after inoculation seperately. The dead larvae were kept on White's trap for collection of infective juveniles and adults.

In open field experiment where the soil was clay loam, the *Cyclamen* plants were planted at 0.5 X 0.5 mts, later introduced five big larvae of black vine weevil near the root zone. After two days different doses of nematodes of the genera *Heterorhabditis* and *Steinernema* (0, 1000, 10000, and 100000 nematodes) were introduced into the soil. At 15 and 20 days after inoculation the mortality of the larvae was counted. During experiment period the weather parameters like temperature and soil temperature were recorded and their effect on the infectivity of nematodes were studied.

RESULTS

The results of the greenhouse experiment are given in Table 1. As the nematode dose is increased the mortality percent is increased in both the cases at different observation days. The infectivity of Heterorhabditis sp. (HF 85 Strain) is increased from 70 to 95% from 1000 to 30000 nematodes dose after 10 days. This increasing trend is reinforced after 15 and 20 days of infection and reaches 100% mortality. A dose of 1000 nematodes per pot gave 100% mortality after 15 andf 20 days of

Table 1. Mortality of black vine weevil larvae exposed to *Steinernema carpocapsae* W.(S25) and *Heterorhabditis* sp. (HF85) at 20°C in greenhouse

Dose IJ/250ml pot.	Mean Percent Mortality					
	S. carpocapsae (S25)			*Heterorhabditis* sp. (HF85)		
	10days	15days	20days	10days	15days	20days
1000	35	50	50	70	95	85
3000	75	70	65	75	95	90
10000	85	80	75	85	100	100
30000	95	80	95	95	100	100
Control	-	-	-	-	-	-

IJ = Infective juveniles

Table 2. Average percent mortality of black vine weevil larvae and pupation in the field after 15 and 20 days of infection with two nematode species

Treatment	S.carpocapsae (S25)				Heterorhabditis sp. (HF85)			
	Mortality		Pupation		Mortality		Pupation	
	15	20	15	20	15	20	15	20
Control	0	0	80	80	0	0	35	80
T1	15	0	40	25	10	10	30	30
T2	20	0	65	60	40	10	20	60
T3	20	10	80	80	30	10	30	30

T1 = 1000 infective juveniles/plant
T2 = 10000 "
T3 = 100000 "
(Average of four replications)

infection. All the infected larvae turn red and large numbers of infective juveniles and few adults were recovered.

In case of *S.carpocapsae* W., as the nematode doses are increased the mortality of larvae were increased. This is true at 10, 15 and 20 days after inoculation. The minimum percent mortality was 35 after infection with 1000 infective juveniles, where as it was 80-95% after infection with 30000 infective juveniles per plant between 10 to 20 days. For the data analysis Response Surface Methodology (Khuri and Cornell, 1987) was used.

RESPONSE SURFACE METHOD

In Response Surface Methodology, mortality is expressed as a function of nematode doses and contact days. The complete model can be expressed as in form of the following formula:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{\substack{j=2 \\ i<j}}^{k} \beta_{ij} X_i X_j + \varepsilon$$

Where X_i (i = 1, 2) represent the linear terms, X^2_i (i = 1, 2) represent the quadratic terms and $X_i X_j$ (i= j; 1,2) the interaction terms B_o, B_i (i=1, 2) B_{ij}(i=j=1, 2) are the parameters to be estimated and " ℰ" is a random error. For this estimation "least suares" are used.

As several terms do not contribute to the responsible varaible, a restricted model is proposed. These restricted models are as follows:

Y (%mortality) = 48.32 + 4.53*doses - 0.06*days - 0.10*doses^2

(*S. carpocapsae,* F = 21.56, Prob. = 10^{-4})

Y (%mortality) = -16.03 + 1.37*doses + 13.25 days - 0.4*days^2

(*Heterorhabditis* sp., F = 11.11, Prob. = 0.0032)

The formulae are graphically represented in Fig. 1 & 2. In both *S. carpocapsae* and *Heterorhabditis* sp. the contact time and doses are linearly related to the mortality. The doses in *S. carpocapsae* however, have greater effect on the mortality of larvae of the black vine weevil because the coefficient of doses is much greater than one of the contact time. In *Heterorhabditis* sp. the contact time has greater effect than the doses (coefficients). The interpretation of the graphs suggests that after a contact period of 10 days, a 100% control of the black vine weevil is achieved with about 7000 *S. carpocapsae* infective juveniles. To obtain the same results with *Heterorhabditis* sp. about 18000 infective juveniles are needed. The 100% isomortality line of *Heterorhabditis* sp. never reaches values lower than 5000 infective juveniles. To obtain a 100% kill of the black vine weevil low doses of *S. carpocapsae* require a contact period higher than 18 days.

The results of the filed experiment are shown in Table 2. During the experimental period (May 93), the daily average air and soil temperature(-5cm) were recorded and shown in Fig 3. The mortality of the black vine weevil due to one of the

Fig. 1 Mortality of black vine weevil larvae in function of time and doses of *Heterorhabditis* sp.

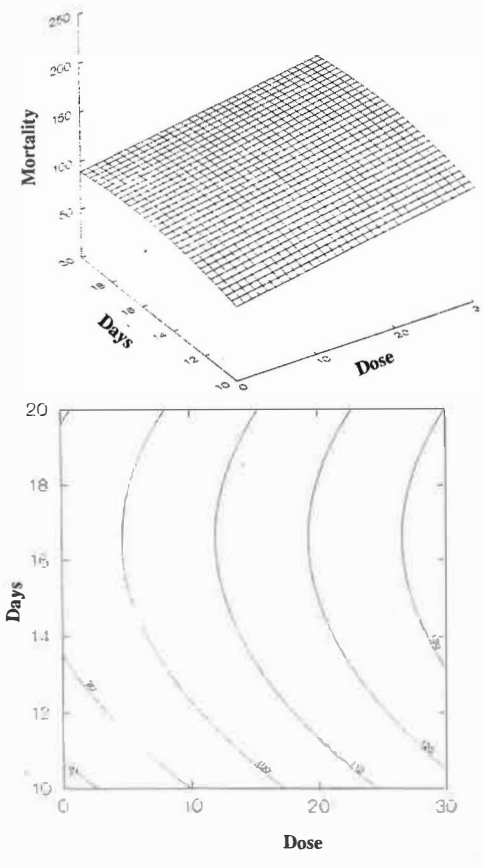


Fig. 2 Mortality of black vine weevil larvae in function of time and doses of *Steinernema carpocapsae*

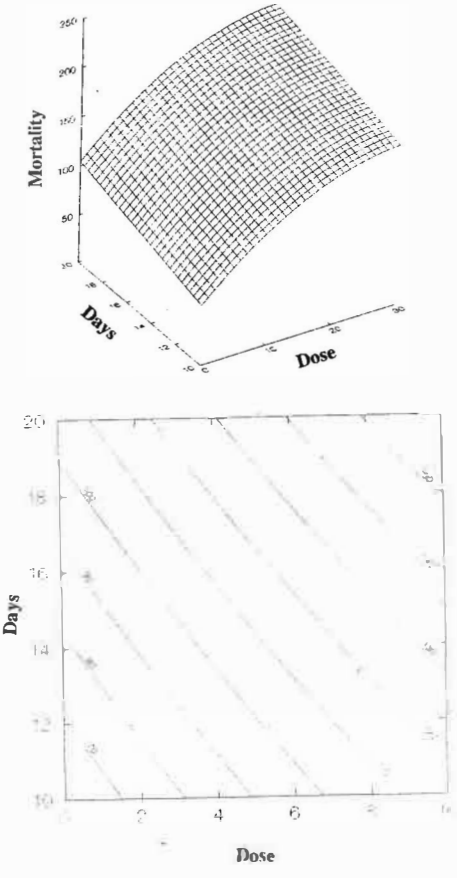


Fig. 3 Daily average temperature and Soil temperature below 5 cm depth.

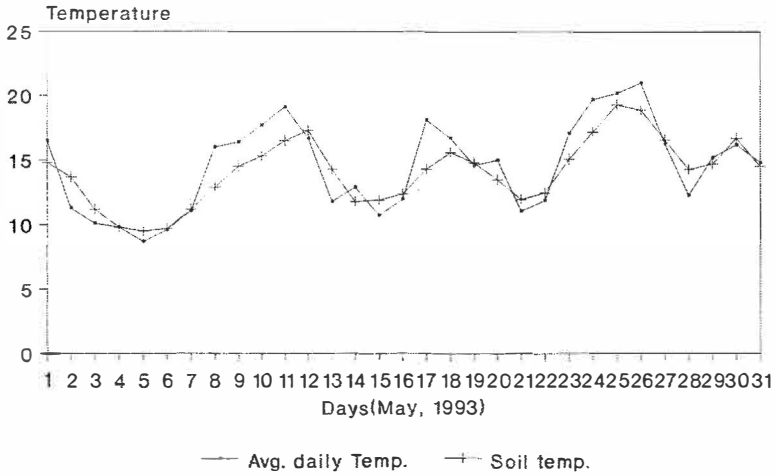
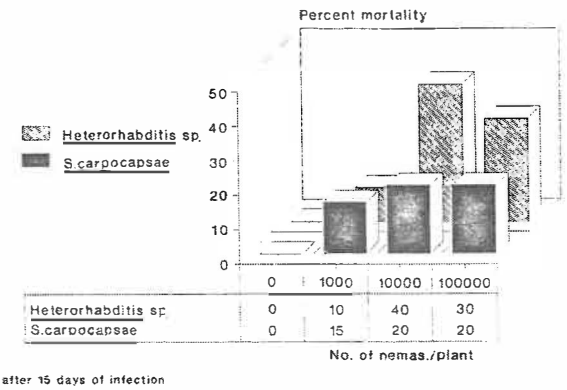
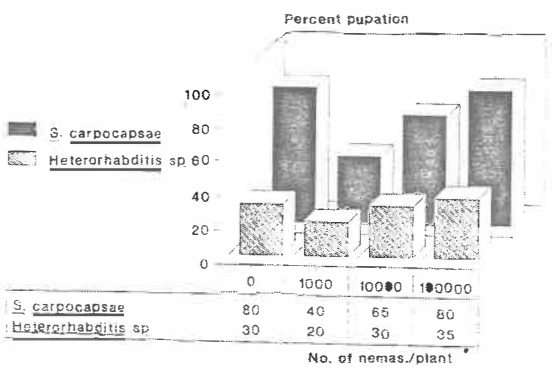


Fig. 4 A. Average percent mortality of black vine weevil larvae in the field



after 15 days of infection

Fig. 4 B. Average percent pupation of black vine weevil in the field.



nematode infections was low compared to the greenhouse experiment. The infection dose had no influence in case of *S. carpocapsae* (S25) the mortality after 15 days was 20% at both the infection levels 10,000 and 100,000 infective juveniles per plant. After 20 days only 10% mortality was noticied at higher dose (Fig.4A). In all the treatments 40-80% pupation of black vine weevil larvae was noticied.

In case of *Heterorhabdits* sp. the mortality was high compared to *S. carpocapsae*. After 15 days it was 10-40% and 10% after 20 days. In this case also the pupation was high from 20-35% in all the treatments after 15 and 30-80% after 20 days(Fig 4.B).

DISCUSSION AND CONCLUSION

The two strains of nematode species tested sucessfully controlled the larvae of black vine weevil in a climate room experiment at constant temperature of 20°C. A minimum dose of 1000 infective juveniles of *Heterorhabditis* sp. can effectively control black vine weevil (Table 1).

It is known that the pathogenicity of heterorhabditid and steinernematid nematodes to insects vary with species and strains (Bedding et al., 1983; Molyneux et al., 1983). Our results confirm the results of Van Tol (1993). This author proved that *H. bacteriophora* (HI) from Italy and *S. carpocapsae* (S25) from USA are generally inferior to the North West European strains of *Heterorhabditis* sp. (UK 211, HS 11, HF 85).

The results of field experiment indicate that the black vine weevil can be controlled with these two nematodes. However, this control was not complete. The reason for this low mortality can be the low soil temperature; it was 10-15°C during the experiment (Fig.3). Poor results were also reported in British Columbia with *H. heliothidis* NC19 below 13-14°C (Rutherford et al., 1987) and in the Netherlands (Dolmans, 1983). In New Zeeland, Garnham and McNab (1990) reported that the average soil temperature(-5cm) during the course of trial was 14.9°C and 21.3°C which was found good for *H. bacteriophora* activity.

In both nematode treatments the pupation of the black vine weevil was as high as 25-80% (Fig.4B). This agrees with the findings of Smith (1932) who reported that *O. sulcatus* larvae grow and develop at temperatures as low as 3°C and pupate at temperatures of around 10°C. The possible reasons for low mortality are the low soil temperature that may limit the nematodes to find the larvae in the soil, the large soil volume in which the infective juveniles had a less chance to find host insect and the natural soil micro-habitants antogonistic to nematodes.

Effective control of black vine weevil both in the field and greenhouses is of much concern to farmers. Few strains of *Heterorhabdits* sp. and *S. carpocapsae* are highly effective in greenhouse container culture at constant temperatures. However, weevil control in field conditions has its own limitations. Further research is needed on this line.

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Lipid Content of Insect Parasitic Nematodes

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Introduction

For commercial purposes it is important that insect parasitic nematodes can be stored over a long period of time without a loss in numbers, and that the nematodes maintain their efficacy during storage. Currently available formulations, such as the clay based formulation used by AGC, can allow *Steinernema feltiae* UK 76 and *Heterorhabditis megidis* UK 211 to be stored for up to several months without losing viability or a reduction in efficacy. The shelf-life can be extended by storage at 5°C but further improvements, particularly during storage at ambient temperature, are desirable.

In the absence of a host, non-feeding nematodes use their stored food reserves (Tiilikkala, 1992; Vänninen, 1990). For plant parasitic nematodes a positive correlation was found between the depletion of food reserves and the loss of infectivity (Tiilikkala, 1992; Van Gundy et al., 1967). The depletion of food reserves, such as lipids, in insect parasitic nematodes during storage might result in restricted shelf life of the nematodes and could be responsible for the loss of efficacy after prolonged storage. Experiments using nematodes stored in the clay based formulation were set up to test this hypothesis.

Material and methods

S. feltiae UK 76 was cultured monoxenically in shake flasks containing a liquid medium comprising homogenized offal, vegetable oil and yeast extract. After a culture time of 16 - 19 days the nematodes were harvested and cleaned. The excess water was removed, the nematode paste mixed with clay and the formulated product stored at 22°C. Sampling was done biweekly.

Efficacy was tested by means of a bioassay. 6400 infective juveniles in 10ml of water, 10 lettuce discs (⊕1.5cm) and 50 *Tenebrio molitor* larvae were added to 110g of moist peat (50% w/w) in a 325ml plastic pot. The pots were kept at 22° C for four days. After four days the number of dead and live *T.molitor* larvae were assessed.

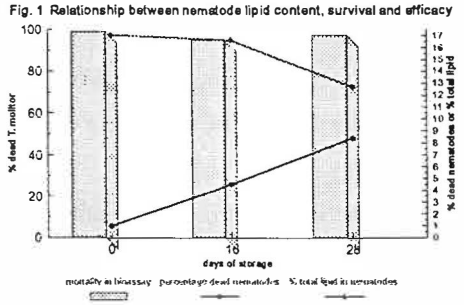
Nematodes were analysed for lipid content by gas chromatography.

Results

The results are presented in Figs. 1 to 3.

Initially, *S.feltiae* contained 17.0% dry weight total lipid which declined to 12.7% over the four week storage period (Fig. 1). The decline in lipid content was accompanied by only a 9% loss in viable nematodes and no measurable loss in efficacy(Fig. 1).

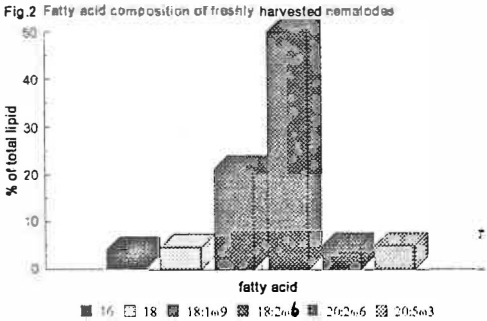
Fig. 1 Relationship between nematode lipid content, survival and efficacy



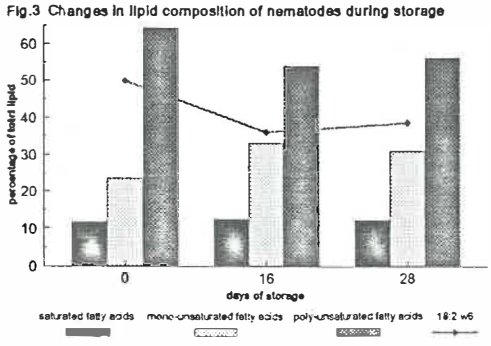
The major fatty acids present in freshly harvested nematodes were oleic (18:1ω9) and linoleic (18:2ω9) acids, which accounted for 50.0% and 21.2% of the total lipids respectively (Fig. 2). Only four other fatty acids were present at levels above 4% of total lipids (Fig. 2).

The decline in total lipid content of *S.feltiae* during storage was caused mainly by loss of oleic acid which declined from 50.0% to 38.8% of the total lipids (Fig. 3). This was reflected in a decline in the proportion of total polyunsaturated fatty acids relative to those of saturated and monounsaturated fatty acids (Fig 3.).

Fig.2 Fatty acid composition of freshly harvested nematodes



The results presented here show a decline in total lipid content for *S.feltiae* over a four week storage period. This did not effect performance in the bioassays. Vänninen (1990) found that the lipid content of two insect parasitic nematodes declined over a period of 1.5 months storage in polyurethane foam. In her experiments *H. bacteriophora* showed sharper decline in lipid content than *S.feltiae* . For *H. bacteriophora* this resulted in a reduction in infectivity towards *T. molitor*. Unpublished data show that *S. feltiae* and *H. megidis* stored in the clay formulation maintained their efficacy even after eight weeks of storage. The difference between the results of Vänninen (1990) and the present results might be explained by the choice of carrier material for the experiments. Nematodes in the clay based formulation are less mobile than nematodes stored in water or polyurethane (Bedding, 1988).



When lipid levels fall below 65% of the original level, the plant parasitic nematode *Globodera rostochiensis* is less mobile and the ability to invade a host is reduced (Tiilikkala, 1992). In this study, the reduction in lipid content for *S. feltiae* was 25.3% of the initial level. This did not result in a measurable decline in infectivity.

The lipid analyses of the nematodes show that total lipid represent about 20% of the dry weight of freshly harvested nematodes. The main fatty acids present were linoleic and oleic acid. This pattern is normal for free living nematodes (Krusberg, 1971; Sivaplan and Jenkins, 1966). The results in this study differ from those found by Selvan et al. (1993) who cultured their insect parasitic nematodes *in vivo* on *Galleria mellonella,* whereas the nematodes used for the present storage experiments were cultured *in vitro* on a liquid medium containing vegetable oil which has a high proportion of linoleic and oleic acids.

Acknowledgement

We would like to thank Dr Steve Mudge and John East of the School of Ocean Sciences, Bangor, Wales, for the lipid analyses of the nematodes. This research was funded by a training mobility grant from the EC programme ECLAIR no. PL151.

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Use of antiserum to discriminate between *Photorhabdus luminescens* (*Xenorhabdus luminescens*) strains and form variants.

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Photorhabdus luminescens, recently separated from the genus *Xenorhabdus* (8,24), are insect pathogenic bacteria symbiotically associated with nematodes of the genus *Heterorhabditis* (19,24). The infective dauerlarvae of the nematode carry the bacterial symbiont in the intestine. The nematode penetrates an insect host, moves into the haemocoel, and releases the bacterium. Subsequently, the bacterium starts multiplying and kills the host, helped by excretion products of the nematode that repress the immune system of the insect (14). *P. luminescens* further produces antibiotics to inhibit growth of other micro-organisms in the insect cadaver and provides nutrients utilized by the nematodes (2,24).

Colony morphology of *P. luminescens* isolates can be highly variable. Two extreme colony forms are characterized as phase 1 and phase 2 or, respectively, primary and secondary form (1,4,6,13). The primary form can be isolated from the infective dauerlarvae and often converts into the secondary form when cultured *in vitro*. The primary form has the ability to produce antibiotics and a pigment, can absorb dye from agar media and is luminescent. The secondary form has lost these abilities and does not support growth of the nematode as well as the primary form (1,4,7,9). Reversion from secondary to primary form has not yet been detected in any *P. luminescens* strain (4). Several intermediate colony forms, showing at least some properties of the primary form, have been described (13,16).

P. luminescens isolates can be separated into groups by DNA homology (5,10,15,22,23), RFLP (5,13) and fatty acid analyses (17). However, these methods do not discriminate between form variants within isolates (5,13,22). Frackman *et al.* (12) compared the *lux* genes of a luminescent primary form and a non-luminescent secondary form and found no differences. This gives reason to believe that it may be difficult to detect form variants at DNA level. Form variants do differ in their protein contents, as is shown in SDS-PAGE protein patterns (7,11,13). In this paper it is shown that it is possible to discriminate between different strains and form variants of *P. luminescens* using serological techniques.

MATERIALS AND METHODS

Bacterial isolates. Table 1 lists the sources of the nematodes from which the bacterial strains were isolated. Bacteria were isolated directly from infective stage nematodes (1,13), or from the haemocoel of waxmoth larvae (*Galleria mellonella*) infected by nematodes. Bacteria were grown on nutrient agar (0.8% Lab Lemco Broth, Oxoid; 1.5% agar) and

incubated in the dark at 25°C. Red or orange luminescent colonies were selected. These isolates were subcultured every 3 or 4 days on nutrient agar. Six form variants of strain PE were used: PE-red, PE-pink, PE-yellow, PE-white, PE-GB and PE-P2. The first four were described by Gerritsen *et al.* (13). PE-red was the primary form of this strain, none of the other variants was a truly secondary form. PE-pink and PE-yellow had some of the characteristics of a primary form and some of a secondary form. PE-white had most characteristics of a secondary form, but had small colonies and small cells and shifts back to PE-red constantly. PE-P2 and PE-GB were isolated as described by Gerritsen *et al.* (13). PE-P2 was a pink pigmented strain, but a different color pink than PE-pink, and it had the same characteristics as PE-red (13). PE-GB had the same characteristics as PE-red except for its pigmentation, which was pale yellow, and it was more luminescent. Both variants were stable during subculturing.

Most other *P. luminescens* strains had small colony variants like PE-white (13) but only a few of them were tested in double diffusion test.

Antiserum production. Antiserum 9226 was produced in rabbits against live, whole cells of PE-red. Bacterial cells were grown on nutrient agar plates for 4 days at 25°C, suspended in 0.85% NaCl and washed three times by centrifugation and resuspension. Final concentration was adjusted to 5.10^8 cells per ml. For initial injection, 1 ml of cell suspension was emulsified with an equal volume of Freund's incomplete adjuvant (Sigma) and injected subcutaneously in the neck region of the rabbit. After 3 weeks a series of intravenous injections of 0.5, 1.0 and 1,5 ml of cell suspension without adjuvant were given at 2 to 3 day intervals. Rabbits were bled from the marginal ear vein 3 weeks after the final injection and at 1 week intervals thereafter up to 3 bleedings.

Absorption of antiserum. Part of the second bleeding of antiserum 9226 was absorbed with a bacterial suspension of either PE-white or Pbac (thereafter called antiserum 9226-W and 9226-B respectively). Bacterial cells were grown on nutrient agar plates for 4 days at 25°C and harvested in 3 ml PBS (0.8% NaCl, 0.27% $HNa_2PO_4.12\ H_2O$, 0.04% $H_2NaPO_4.12\ H_2O$. pH7.2) to a final concentration of approximately 10^9-10^{10} cells per ml. 0.5 ml of bacterial suspension was added to 2 ml of antiserum and incubated at 37°C for 30 min. The agglutinates were removed by centrifugation. This procedure was repeated until agglutination could no longer be observed (e.i. 3 times). The degree of absorption was checked by double diffusion.

Immunodiffusion. Agar plates for Ouchterlony double diffusion tests (20) were prepared with 15 ml of 1% Difco purified agar, 0.85% NaCl and 0.02% sodium azide; the medium was dispensed into plastic Petri dishes (9 cm diameter). Wells, 3 mm in diameter and 4 mm apart, were cut in sets of six peripheral wells surrounding a centre well; 5 sets were cut in each plate. Bacterial cells were grown on a nutrient agar plate for 4 days at 25°C and harvested in 0.5 ml distilled water (cell concentration $\approx 10^9$-10^{10} cells per ml). The centre wells were filled with the second bleeding of antiserum 9226, 1:1 diluted with glycerol, or with undiluted antiserum after cross-absorption. The surrounding cells were filled with bacterial suspensions. Pre-immune serum and a heterologous bacterium, *Enterobacter* sp., served as negative controls. The plates were incubated at 25°C for 2-4 days and examined with a stereomicroscope with darkfield illumination for precipitin bands.

RESULTS

Reaction of bacteria in double diffusion test. Figure 1 shows the results of the double diffusion test. PE-red developed four precipitin bands (Fig. 1, well 1); one thick band, close to the well with bacteria, two bands close together in the middle, and one weak band near the middle well. This last band was broad and vague, running out to the middle well. The two bands in the middle were not always separated clearly.

The different form variants of PE had a different precipitin pattern (Fig. 1, wells 1-5,22,23). All six variants had the first bright band. Except for PE-white (well 3) they all had the last broad band. PE-white and PE-yellow (well 4) did not have the two middle bands. One or both of the middle bands of PE-pink (well 2), PE-P2 (well 22), and PE-GB (well 23) differed from the bands of PE-red and from each other, but they all fused with the bands of PE-red without spur formation.

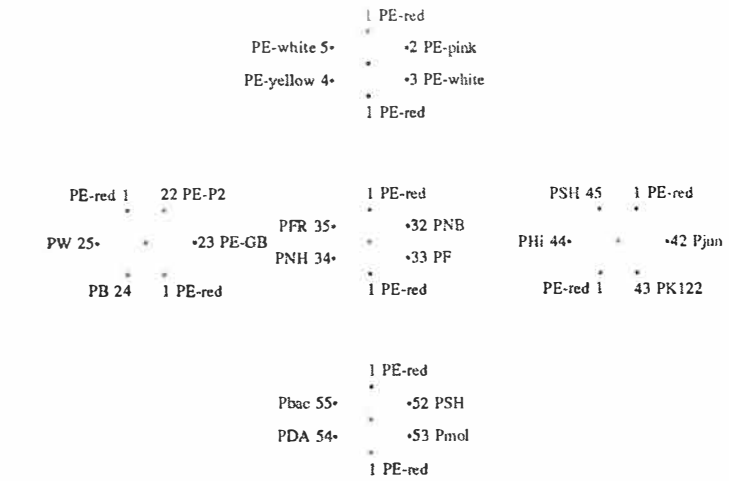
Most *P. luminescens* strains tested gave a cross-reaction with the antiserum (Fig 1). Only two strains, PH1 and PH2, did not react with the antiserum (results not shown). PHi (well 44) only gave a very weak reaction. The precipitin patterns of PB (well 24) and PW (well 25) were very similar to the pattern of PE-red. The small colony variants of PW and PB, showed the same precipitin pattern as PE-white (results not shown). The small colony variants of PSH1, PFR and PK122 did not react with the antiserum. PFR and PNH had similar patterns, which differed from the pattern of PE-red. All other strains had different precipitin patterns (Fig. 1, Table 1).

Figure 2 shows the double diffusion test with antiserum after cross-absorption. Pbac and PE-white did not develop a precipitin band in reaction with the antiserum after cross-absorption with Pbac (9226-B) and PE-white (9226-W), respectively. PE-red lost most of the first bright band in reaction with 9226-W. When PE-red reacted with 9226-B the second and fourth band of the precipitin patterns were vague.

TABLE 1. Origin and double diffusion pattern of *P. luminescens* strains used in this study.

Bacterial strain	Associated nematode strain or species	Original place of isolation		D.D. pattern[a] (well nr. in Fig. 1)
PE	NLH-E87.3	Eindhoven	The Netherlands	A (1)
PB	NLH-B87.1	Bergeyk	The Netherlands	A (24)
PW	NLH-W79	Wageningen	The Netherlands	A (25)
PFR	NLH-FR86	Friesland	The Netherlands	B (35)
PNH	NLH-NH-87.1	N-Holland	The Netherlands	B (34)
PL	NLH-L81	Limburg	The Netherlands	C
PF	NLH-F85	Flevoland	The Netherlands	D (33)
PNB	NLH-NB87	N-Brabant	The Netherlands	E (32)
PJun	NLH-Jun91	Papendal	The Netherlands	F (42)
PHi	NLH-Hi93	Hilversum	The Netherlands	G (44)
PH1	NLH-H92.1	Eindhoven	The Netherlands	-
PH2	NLH-H92.2	Eindhoven	The Netherlands	-
PSH	DH-SH-1	Kiel	Germany	H (45.52)
PDA	DH-DA1	Darmstad	Germany	I (54)
Pbac	*H. bacteriophora*	Brecon	S-Australia	I (55)
Pmol	CISH-Mol	Moldavia	CIS	J (53)
PK122	IRH-K122		Ireland	K (43)

[a] pattern in double diffusion test with antiserum 9226
strains with equal characters have a similar precipitin pattern
- = no reaction with the antiserum



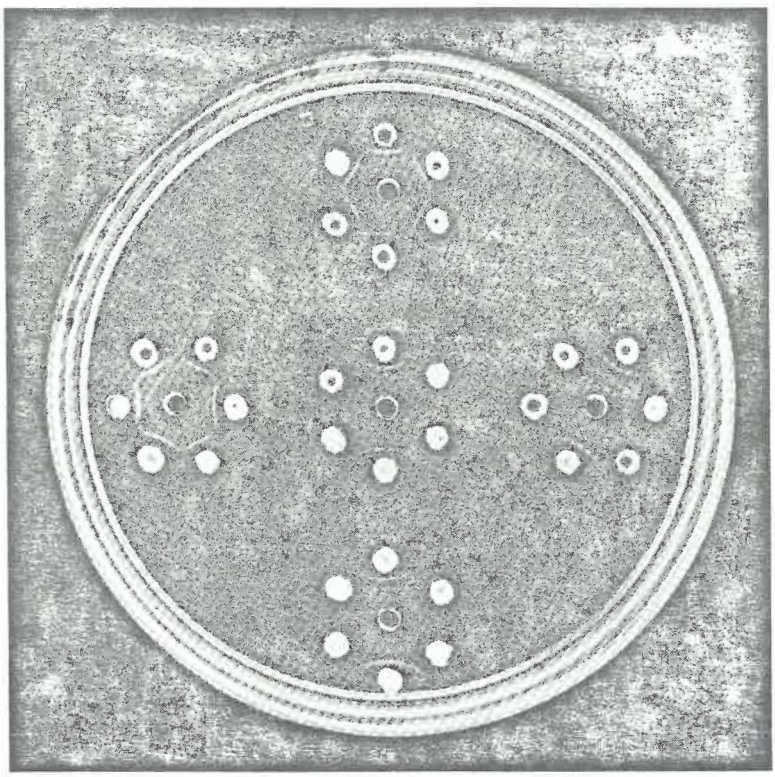


FIGURE 1. Ouchterlony double diffusion patterns depicting reaction of antiserum 9226 with different *P. luminescens* strains and form variants.

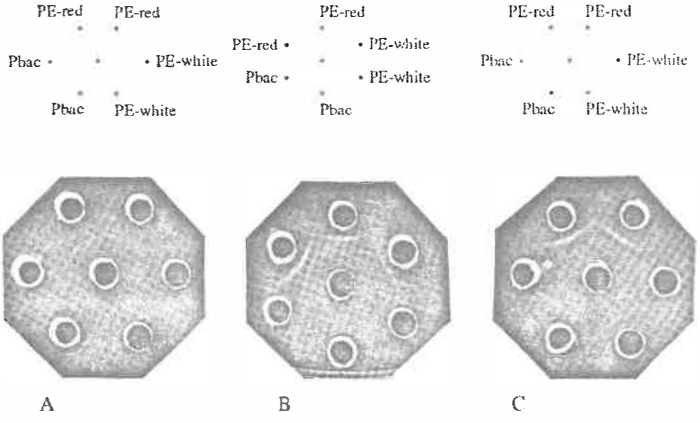


FIGURE 2. Ouchterlony double diffusion patterns depicting reaction of *P. luminescens* strains PE-red, PE-white and Pbac with A: antiserum 9226-W, after cross absorption with PE-white, B: antiserum 9226, C: antiserum 9226-B, after cross absorption with Pbac.

DISCUSSION

The polyclonal antiserum 9226, produced against *P. luminescens* strain PE, contains antibodies against different antigens. In the double diffusion test the four bands of PE-red each represent a different antibody-antigen complex. All form variants of PE react with the antiserum, but they all have a different pattern. Some bands are identical which means that the antigens are identical (reaction of identity). The bands that differ from the pattern of PE-red show that the epitopes (antigenic site on a molecule) are located on antigens (molecules) with a different diffusion speed (reaction of partial-identity). These differences make it possible to discriminate between form variants. Also between strains there are several differences. Most strains have a specific pattern. Only strains PW and PB are serologically similar to PE. All strains react with the antiserum, even strains that belong to genetically different groups than PE, such as Pbac, PDA, Pmol and PK122 (23). This shows that serological groups of *P. luminescens* do not coincide with genetical groups distinguished by DNA probes (5,13,15,22,23). Although the antiserum reacts with almost all bacteria, it shows a different reaction to each bacterium in the double diffusion test. Even bacteria, isolated from nematodes that originate from the same area (PE, PH1 and PH2) show a different reaction. Whether these nematodes actually carry different symbionts or that these symbionts only have minor differences in their antigenic region is not clear. The differences might even be caused by subculturing (PE was subcultured for 3 years, while PH1 and PH2 were isolated recently). These differences in serological reaction are reproducible, and therefore make it possible to produce strain or variant specific antisera, which can be very useful for research and detection purposes.

One way of making a more specific antiserum, is by cross-absorption. PE-red has epitopes that are not present in other bacteria. By cross-absorption with for example PE-

white all antibodies that react with PE-white are removed and only antibodies that react with epitopes specific for PE-red are left. Thus, it is possible to make the antiserum more specific for PE-red. For example, the antiserum 9226-W can used to detect PE-red cells in a PE-white culture, which is unstable and shifts to PE-red very easily (13). This can be used in mass-rearing of nematodes, in which mixed cultures of primary and secondary cells are to be avoided, because the secondary cells do not support nematode growth as well as primary cells (9).

Alternatively, monoclonal antibodies (antibodies that reacts to one epitope only) could be produced to distinguish strains or form variants. Differences observed in the double-diffusion-pattern show that it might be possible to make these specific monoclonal antibodies.

The availability of variant specific antisera offers new ways for research on *Photorhabdus* and *Xenorhabdus* bacteria. Serological techniques can be used to study the mechanism and function of fase variation in *Photorhabdus* and *Xenorhabdus* spp..

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Control of mushroom flies: dispersal and persistence of nematodes in mushroom compost.

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Summary

The dispersal and persistence of the Dutch Heterorabditid *NLH-F85* (Nematoda: Heterorhabditidae) and the UK strain *Steinernema feltiae* (Nematoda: Steinernematidae) were tested in columns filled with mushroom compost and casing layer at the top. The dispersal of both species was largely restricted to the upper compost layer. *NLH-F85* was able to persist longer than *S. feltiae*.

Introduction

In Dutch mushroom culture the mushroom phorid *Megaselia halterata* (Hood) (Diptera: Phoridae) is a primary pest. Although the compost can sustain large numbers of larvae (Hussey, 1959) the phorid has to be controlled as the adults easily transmit diseases such as *Verticillium fungicola* (White, 1981).

The phorid *M. halterata* is highly attracted to the full grown compost and flies enter the mushroom houses when doors are opened during the filling of the mushroom beds with full grown compost. The control of the phorid *M. halterata* with commercially produced nematodes is currently investigated. Preliminary research revealed that nematodes failed to obtain a substantial reduction of the larval population in compost. The unsuitability of the compost environment is one possible reason of nematode failure. Indeed, Tomalak & Lipa (1991) showed that in casing material much higher parasitization rates are obtained than in compost. But, since phorid larvae are primarily confined to the compost, a nematode species that is able to survive and act in a compost environment should be looked for. To elucidate the behaviour of nematodes in compost the dispersal and persistence of nematodes in columns filled with compost was studied. The strain *NLH-F85* was chosen as it is known to have good dispersal capacities (Westerman & Godthelp, 1991). The species *S. feltiae* is very successful in controlling the mushroom sciarid *Lycoriella auripila* when applied at the casing (Grewal & Richardson, 1993). Although *S. feltiae* is probably less effective when applied at full grown compost, *S. feltiae* seems to be a likely candidate for the control of the mushroom phorid in compost.

Materials and Methods

Cylinders with a height of 27 cm and a diameter of 7,2 cm were used to simulate a mushroom bed. Cylinders were filled with 3 equal layers of full grown compost until a height of 20 cm. In the upper compost layer 3 waxmoth larvae were placed. At the compost 4000

nematodes were applied in 2 ml water. A casing layer was applied on top of the compost, three hours later. Cylinders were placed in the dark at a temperature of 25°C. After a period of 1, 3 and 6 days the layers in the columns were taken apart. Nematodes were extracted from each layer with the Seinhorst's spray mist method (s'Jacob & Bezooijen, 1983) and the nematodes were counted. The efficacy of this method was evaluated by extracting the nematodes directly after application at compost and casing material. This method resulted in a recovery of 93% of all nematodes of *NLH-F85* from the compost and 91% from the casing material. For *S. feltiae* the efficacy was 90% and 82%, respectively. In further calculations the numbers of nematodes were not adjusted for the efficacy of the extraction method, as the recovery rates were high and consistent.
Infected waxmoth larvae were dissected after 7 days and adult nematodes were counted. These numbers were added to the numbers of nematodes retrieved from the upper compost layer. Three replications were performed at different days, with different batches of full grown compost and fresh batches of the Dutch strain *Heterorhabditis F85* produced by "De Groene Vlieg", the Netherlands and *Steinernema feltiae* produced by MBI-AGC, United Kingdom.

Results

The percentage of nematodes recovered from the whole columns significantly decreased in time (fig 1a). At all days, however, significantly lower numbers of *S. feltiae* than numbers of *NLH-F85* were recovered.

Fig 1b shows that in the upper compost layer exceedingly more nematodes were recovered from the hosts than from the compost in time. Most nematodes located and penetrated the hosts between 1 and 3 days.

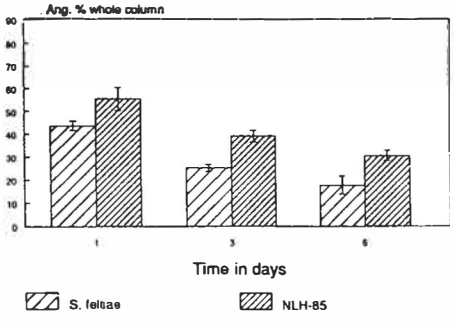

Fig. 1a

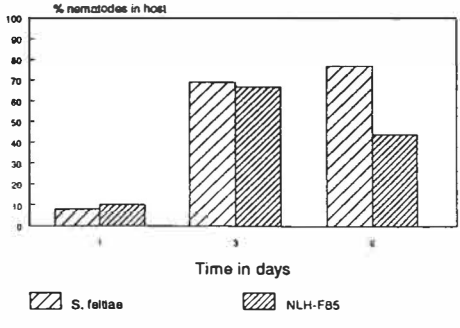

Fig.2b

Fig. 1a. Percentage of *S. feltiae* and *NLH-F85* recovered from the whole columns. LSD(0.05)= 8.9. An angular transformation has been applied to the percentages of nematodes recovered to obtain stabilization of the variance.
Fig. 1b. Percentage of *S. feltiae* and *NLH-F85* recovered from dissected waxmoth larvae. Percentage was based on the total number of nematodes recovered from the upper compost layer and hosts.

In columns, both with and without hosts, significantly higher numbers of nematodes were recovered from the upper compost layer and the casing layer than from the two lower compost layers (fig 2). In columns with hosts present (fig 2), the number of nematodes of both species recovered from the upper compost layer does not significantly decrease in time. In columns without hosts present, however, numbers of nematodes in the upper compost layer do significantly decreased in time.

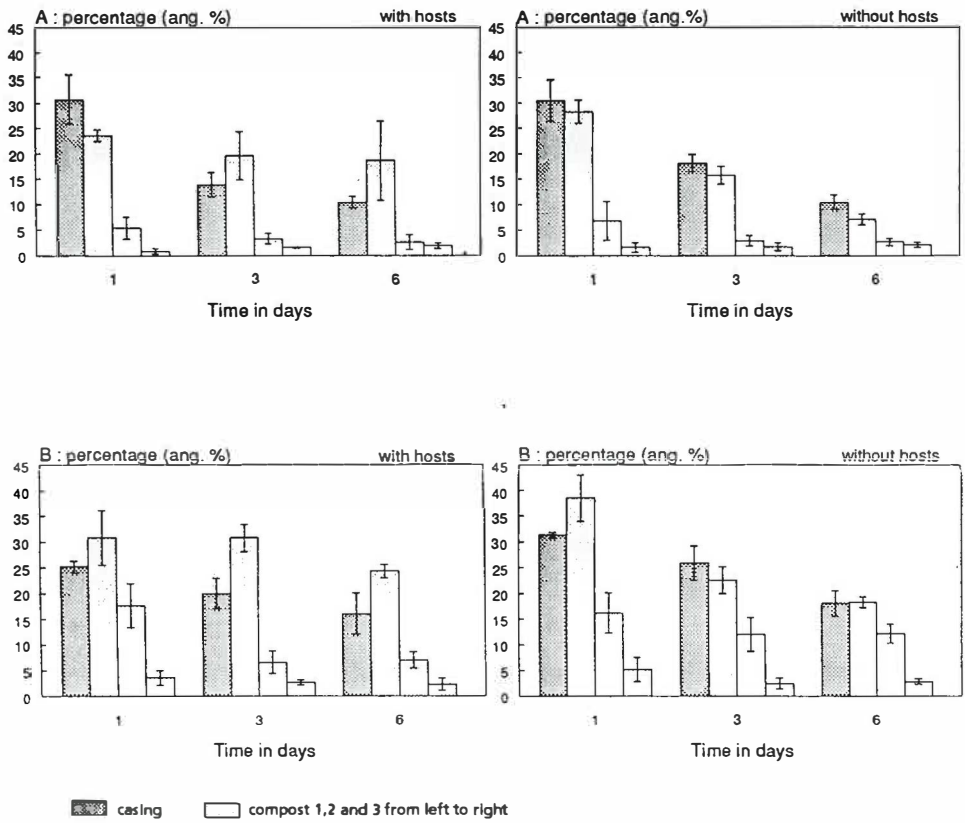

Fig. 2. Percentages of *S. feltiae* and *NLH-F85* recovered separately from the casing layer and three compost layers.
A = *S. feltiae*. LSD(0.01) within days = 10.2 when comparing means between layers of the same day. For all other comparisons: LSD(0.01) between days = 10.56.
B = *NLH-F85*. LSD(0.01) within days = 9.8. LSD(0.01) between days = 9.3.

Discussion

When applied at the compost the dispersal of both nematode species is largely restricted to the upper compost layer. As larvae of *M. halterata* occur at all layers of the compost (personal observation), the application of nematodes at the compost is not suitable for obtaining a major reduction of the population of *M. halterata.* Mixing of the nematodes with the full grown compost during filling of the mushroom beds will probably not suffice either. During the first 6 days only eggs and first larval stages are present which are too small to be penetrated by the nematodes. These experiments show that the numbers of active nematodes are reduced to a low percentage after 6 days. So, when mixed with the compost during filling of the beds, the nematodes do not persist long enough to parasitize larvae.
A further decrease of nematodes after 6 days is expected. After a decline of nematodes during 14 days, Grewal & Richardson (1993) found a slight increase of *S. feltiae* in the casing layer which was attributed to the recycling of nematodes in the infected larvae of the sciarid *L. auripila.* In mushroom beds infected by *M. halterata*, such an increase is not likely, due to the small size of the larvae, only allowing very few females to mature and reproduce.

In columns with hosts present, the numbers of nematodes in the upper compost layer do not significantly decrease in time, whereas in columns without hosts numbers do decrease significantly. Although not significant, the numbers of *NLH-F85* (fig 2b) in all other layers are almost always higher in columns without hosts present than in columns with hosts. This effect is less evident with *S. feltiae.* As numbers of nematodes in the upper compost layer are composed of nematodes extracted from the compost and nematodes dissected from the waxmoth larvae, part of the accumulation in the upper compost layer can be attributed to a better survival in hosts than in compost. This effect will be strongest in columns taken apart after 6 days. Indeed, fig 1b shows that a high percentage of the nematodes is recovered from the hosts and not from the compost.

In Dutch mushroom culture the use of full grown compost will be a standard procedure by 1995. The mushroom phorids easily invade the cells during filling of the compost. Therefore, the first generation develops synchronously. Control of the phorids is aimed at the larvae of the first generation. These experiments show that the short activity range of the tested nematodes in both space as time explains the unability of nematodes to obtain a substantial reduction of the larval population. Further research is required to find species of nematodes that persist longer in compost.

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BIOLOGICAL CONTROL OF INSECTS IN FORESTRY NURSERIES WITH ENTOMOPATHOGENIC NEMATODES

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These results represent one part of the COST 813 - Action "Diseases and Disorders in Forestry Nurseries".

On the basis of an interview among foresters and producers a toplist of the most dangerous insect pests in Swiss forestry nurseries was created. The highest score in plant damage got the cockchafer *Melolontha melolontha*, followed in decreasing order by the Black vine weevil *Otiorrhynchus sulcatus* and the summer chafer *Amphimallon solstitialis*.

In Switzerland *M. melolontha* goes through a 3 year life cycle. Serious losses of nursery plants are caused by all instar larvae feeding on the root system. The main damage occurs in the year following the flight of the adult insects.

The main experiments were focused on controlling the larvae of *M. melolontha* with different nematode strains of *Steinernema*- and *Heterorhabditis* - species. Several biotests in laboratory and field trials were established with 3rd instar larvae. The efficacies of the insect mortality caused by infections with the chosen isolates of nematodes varied between 0% and 57% under laboratory conditions (15°C, 70% RH) and 0% to 36% in field trials, respectively.

These preliminary results show a promising potential of insect parasitic nematodes controlling the larvae of *M. melolontha*. But with the nematode isolates tested in these experiments, the mortality rate of the insect larvae infected by nematodes is not successful enough to promote nematodes as a biocontrol agent yet. Under laboratory conditions tests seem to confirm, that the younger instar larvae are more susceptible to nematodes. It would be of great interest to repeat these experiments in fields infected with 1st and 2nd instar larvae.

DYNAMICS AND EFFECT ON INFECTIVITY OF ENDOGENOUS LIPID RESERVES IN TWO AGING *STEINERNEMA* SP.

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The emphasis of the poster is on the method used for relative quantification of lipid reserves of Steinernematid infective juveniles. The software application "Densi" of the Olympus Image Analyser Cue-2 (GALAI PRODUCTION Ltd.) is employed to measure the relative proportion of lipids in individual specimens of *Steinernema feltiae* (Finnish strain SFS—22) and *S. carpocapsae* (Swedish Umeå strain) stained with Oil-Red O and preserved on microscope slides. After the initial setting of grey levels to be taken as lipids in the image, the method is an objective way of determining the ratio of area covered by lipids to the total area of the body of the juveniles. It is essential to have a microscope of good precision to allow for the objectivity of the method.

Due to the unexpectedly good persistence of the nematodes in the polymer gel used for storing the nematodes in +20 °C, no comparisons on the effect of lipid amount on infectivity of the two species are available so far. After six months, the mortality of the nematodes is less than 10%, and the relative area covered by lipids has declined only from 90% to 65-70%. No decline in infectivity has been recorded yet, measured in a bioassay consisting of 9 cm high sand cylinders with *Tenebrio molitor* larvae as bait insects. In pots of peat and mineral soil stored in 20 °C the lipid ratio of the juveniles is declining more rapidly, indicating higher rates of metabolism of the nematodes in soil than on inert polymer gel.

5. Meeting of the subgroup entomopathogenic fungi.

Entomopathogenic fungi associated with cocoons of nettle caterpillars (Lepidoptera, Limacodidae) in oil palm plantations in Sumatra

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Summary

In North Sumatra, a *Cordyceps* species, closely related to *Cordyceps militaris*, was regularly found at epizootic levels on cocoons of *Setothosea asigna*, the most important defoliating pest of oil palm in the region. Other nettle caterpillar species were also subject to attacks by the fungus.

The development of the anamorph (a *Verticillium* type) at the surface of infected cocoons was studied in experimental conditions. The influence of the temperature and relative humidity was demonstrated.

Two supplementary Deuteromycetes, a *Verticillium* species different from the anamorph, and a *Paecilomyces* species, occurred on a few of the laboratory studied specimens. The former fungus formed on coremia of the anamorph, the latter on cocoons without coremia. These two fungi, and the anamorph were isolated; they were growing on simple media. The *in vitro* produced conidia were pathogenic towards *Galleria mellonella* and *Spodoptera littoralis* caterpillars, *Paecilomyces* sp. being the most virulent fungus.

Introduction

Given their more or less regular outbreaks, nettle caterpillars (Lepidoptera, Limacodidae) are severe pests of oil palm plantations in South-East Asia. Chemical control is difficult, especially because efficient application of pesticides can be a problem in this type of culture. Alternative measures are therefore investigated since several years. In contrast to the special attention paid to application of viruses (Entwistle, 1987; Desmier de Chenon et al., 1988), fungi parasitizing limacodids were much less studied. From the annotated list given by Evans (1987), *Cordyceps* spp. (Ascomycotina, Clavicipitales) appeared to be the most commonly fungi found on these insects, their ability to provoke epizootics in limacodid populations being known since observations made by Schneider (1940) in Indonesia. A few authors (see Evans, 1987) successfully tested the efficacy of application of *in vivo* fungal material in controlling limacodid pests, but there was no continuation of these biological control attempts.

A few years ago, high percentages of infection by a *Cordyceps* species were observed in limacodid populations in North Sumatra, offering us the opportunity to initiate an in-depth study of this pathogen. Furthermore, other fungi were found associated with the *Cordyceps* species, and then investigated.

Data on the occurrence of the fungi associated with cocoons of nettle caterpillars and their taxonomic position

In the Medan area (North Sumatra), a *Cordyceps* species was regularly found parasitizing pupae of *Setothosea asigna*, the most important pest of oil palm in the region (Mariau et al., 1991), and also pupae of *Setora nitens* and *Darna bradleyi*. The populations of the former species, which pupates in the soil, were subject to the heaviest attacks, the infection level ranging from several % to 84 %, according mainly to the time of the year, the topographic situation of the plots and the type of soil. Highest infection rates occurred in February. Infected pupae are more often found in lower parts of plantations. It seems that mulching and weed cover could favour the fungus by providing suitable humidity conditions at the soil surface.

The fungal species is closely related to *Cordyceps militaris*, but there are some differences, especially in the size of perithecia and asci.

The growth of the anamorph of *Cordyceps* aff. *militaris* (a *Verticillium* type), which was rarely found in the field, was almost always achieved (more than 95 %) on infected pupae brought to the laboratory, by using a process originated by Papierok et Charpentié (1982).

The development of two supplementary Deuteromycetes was also observed. A *Verticillium* species, different from the anamorph, occurred on the coremia of the anamorph formed on 2 % of the studied specimens, whereas a *Paecilomyces* species appeared on cocoons without coremia (2 % of the studied specimens as well). In contrast to the former fungus, which was occasionnally observed in the field, *Paecilomyces* sp. was so far no noticed outside the laboratory.

Data on the influence of climatic factors on the development and survival of *Cordyceps* aff. *militaris*

The influence of light, and to a lesser extent, of temperature on the development of the teleomorph on mummified pupae of *Setothosea asigna* was demonstrated in out of doors conditions in Sumatra (Desmier de Chenon *et al.*, 1990). Especially, daylight (3900-4300 lux) is almost inhibiting development of stromata on the sclerotium, but seems to be essential for the formation of perithecia.

The development and survival of the anamorph were studied on infected pupae of *S. asigna* and *Setora nitens* in laboratory conditions in Paris. The freshly collected specimens, with no external fungal growth, were sent from Sumatra by a rapid air transportation.

A saturated or a nearly saturated atmosphere are essential to the growth and fructification of the anamorph at the surface of infected pupae; this development if favoured by the presence of free water. The influence of temperature was clearly demonstrated. Stromata are twice longer at 26° C than at 18° C. At 26° C, the fungus is keeping growing at least for 3 months; at 18° C, the sclerotia were able to survive for 9 months when kept at 60-70 % RH.

Occasional observations of infected pupae of *Darna bradleyi* allowed us to observe, in one case, the formation of stromata after a 16 months storage at 18° C and 60-70 % RH.

In the laboratory conditions, the fungus appeared to almost exclusively growth and develop as anamorph. In very few occasions (5 on 1000 cocoons studied), we observed fructifications of the teleomorph on coremia of the anamorph growing on cocoons of *Setora nitens*.

Data on the entomopathogenicity of the fungi associated with limacodid cocoons.

Infection experiments with ascospores produced from naturally infected pupae were successfully carried out in Sumatra using *Setothosea thosea* caterpillars as hosts. Procedure and results will be detailed elsewhere (B. Papierok and R. Desmier de Chenon, in preparation).

Since the anamorph and the two supplementary Deuteromycetes were isolated in pure culture, we tested these strains for infectivity for caterpillars of *Galleria mellonella* (Lepidoptera, Pyralidae) and *Spodoptera littoralis* (Lepidoptera, Noctuidae). Conidia produced an solid media (yeast extract agar or Sabouraud dextrose agar supplemented with egg yolk and milk) formed the inoculum; the entire caterpillars were brought into contact with conidia suspension in Petri dishes.

The three fungal species were able to infect the two insects tested. *Paecilomyces* sp. appeared the most virulent strain, with the fastest action. Most of the infected caterpillars were died and covered with fungal fructifications after 4 days at 20° C. The incubation period of the anamorph of *Cordyceps* aff. *militaris* was about 12 days at 20° C; the fungus sporulated on about 50 % of the cadavers of the two lepidopteran species. Conidiation of *Verticillium* sp. was noticed on only a few of the cadavers of *Galleria mellonella*, whereas there was no sporulation of this fungus on the cadavers of *Spodoptera littoralis*.

Preliminary injection experiments of fungal material produced in a yeast extract liquid medium were carried out with *Galleria mellonella*. Only *Cordyceps* aff. *militaris* and *Paecilomyces* sp. were tested. As in the case of inoculum from solid cultures, the later fungus appeared more virulent than the former.

Discussion and conclusion

On several accounts, *Cordyceps* aff. *militaris* is an interesting fungus to work with. From the applied point of view, which was actually not the matter of this contribution, the fungus proved to be, in North Sumatra, an effective natural enemy of several limacodid pest of oil palm, especially *Setothosea asigna*, the most important one. Moreover, *C.* aff. *militaris* appeared a promising component of integrated control programmes of oil palm plantations (B. Papierok, R. Desmier de Chenon, J.-M. Freulard & W.P. Suwandi, in preparation). On the other hand, the mycologist is confronted with the problem of interrelations between this species, both as teleamorph and anamorph, ant the two other, more or less associated, Deuteromycetes. From field and laboratory data, *Verticillium* sp. seemed to be an opportunist fungus, but the actual role, and potential, of *Paecilomyces* needs to be explained.

Finally, the fungus/host insect model *Cordyceps* sp./limacodids is revealed a good material for, among others, a comprehensive study of factors governing the growth of the fungus as teleomorph or anamorph.

Résumé

Des épizooties dues à une espèce de *Cordyceps* proche de *Cordyceps militaris* ont été régulièrement observées dans les populations de cocons de *Setothosea asigna*, le défoliateur le plus important des plantations de palmiers à huile du Nord Sumatra. Le champignon s'attaque également à d'autres limacodides.

Le développement de l'anamorphe (un *Verticillium*) à la surface des cocons infectés a été étudié en conditions expérimentales, soulignant l'influence de la température et de la lumière.

Deux autres Deutéromycètes, une espèce de *Verticillium* différente de l'anamorphe et une espèce de *Paecilomyces*, ont été observées sur une petite partie des cocons étudiés au laboratoire. L'espèce de *Verticillium* est apparue sur les corémies de l'anamorphe, tandis que *Paecilomyces* sp. l'a été sur des cocons ne portant pas de corémies. Ces deux champignons ont été mis en culture, de même que l'anamorphe. Ils se développent sur des milieux simples. Les conidies produites *in vitro* se sont révélées pathogènes à l'égard des chenilles de *Galleria mellonella* et de *Spodoptera littoralis*, *Paecilomyces* sp. étant le champignon le plus virulent.

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Chitin synthetases in the protoplastic Entomophthorales.

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Summary.

Two chitin synthetase-like fragments, EaCHS1 and EaCHS2 of about 600 bp have been amplified from total genomic DNA of *Entomophaga aulicae*. Their deduced aminoacid sequences were compared with the other fungal chitin synthetases deposited in the protein database SWISS-PROT. An unweighted pairwise comparison shows that both the two *E.aulicae* fragments belong to class II, and that they cluster most with each other. Except for the two chitin synthetases of *Rhizopus oligosporus*, another Zygomycetes, *E. aulicae* is the only species having two chitin synthetases in the same class.

Introduction.

In the early infection stage *Entomophaga aulicae* proliferates in its hosts in a protoplast form characterized by the complete absence of a wall. Protoplasts are not recognized by the insect immune defense system. In contrast the walled form, hyphal bodies, elicit a strong defense reaction. The wall of the protoplastic Entomophthorales is mainly composed of 1,3-ß-glucan and chitin which represent 55-67% and 15-30% of the wall respectively (Latgé & Beauvais, 1987). Glucan synthetase and chitin synthetase (CHS) are two membrane bound enzymes responsible for their synthesis.

Two CHS zymogens from the yeast, *Saccharomyces cerevisiae* (Bulawa et al., 1986; Valdivieso et al., 1991), one from *Candida albicans* (Au-Young & Robbins, 1990) and one from *Neurospora crassa* (Yarden & Yanofsky, 1991) have been isolated. Comparision of sequences of these genes revealed regions of highly conserved amino acid sequences in all three genes (Bowen et al., 1992).

By the help of PCR-primers designated from such homologous regions, we amplified two distinct CHS-like fragments from genomic DNA of *E. aulicae*. After sequencing, their amino acid sequences were compared to the other fungal CHSs deposited in the protein data base SWISS-PROT.

Matherials and methods.

Entomophaga aulicae (isolate 2896) was isolated from *Choristoneura fumiferana* by Drion Boucias (Entomology and Nematology Department, University of Florida, Florida, USA).

Total genomic DNA of *E. aulicae* was prepared from hyphal bodies cultured in liquid medium (3 % glucose, 1 % yeast) and extracted by the method of Girardin et al. (1993).

PCR amplification was done using a Hybaid OmniGene Temperature Cycler and the "*GeneAmp* PCR Core Reagents" (Perkin Elmer Cetus).

Primers and temperature cycles were done according to Bowen et al. (1992). The PCR product was digested with *Hind* III and *Xho* I, and purified on a 2% low melting agarose gel using the "Prep-A-Gene" DNA Purification Matrix Kit (Bio-Rad). The PCR fragment was inserted into a *Hind* III, *Xho* I digested bluescript SK^+-plasmid and sequenced by the method of Sanger et al. (1977).

The SK, Reverse, and -40 primers and the [α-^{35}S]dATP (Amersham) were used in the sequence reactions which were performed with the "Sequenase Version 2.0" DNA Sequencing Kit (United States Biochemichal).

Computer analysis of the sequences was done using the "PileUp" program from the GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, Wisconsin).

Results and discussion.

Two CHS-like fragments of about 600 bp designated EaCHS1 and EaCHS2 have been isolated from *E. aulicae* mycelial DNA. Their basepair composition and deduced amino acid sequences can be seen in fig. 1.

The amino acid sequences from the two *E. aulicae* fragments were compared to the other fungal CHS's deposited in the protein database SWISS-PROT by the unweighted pair-group method using arithmetic averages (UPGMA) (Sneath & Sokal, 1973). This method compares the sequences two by two creating a similarity score between each possible pair of sequences. These gave the final optimal alignment of the sequences (data not shown) and the corresponding dendrogram (fig. 2). The names of fungi and the abbreviates of their respective chitin synthetases are shown in table 1.

Table 1. Taxonomic affinities of fungal species and chitin synthetase gene designations.

Species	Affinity	Gene designations
Saccharomyces cerevisiae	Ascomycetes	CHS1, CHS2
Schizosaccharomyces pombe		SpCHS1
Emericella (Aspergillus) nidulans		AdCHS1, AdCHS2
Aspergillus niger		AnCHS1, AnCHS2
Ajellomyces (Blastomyces) dermatitidis		BdCHS1, BdCHS2
Ajellomyces (Histoplasma) capsulatas		HcCHS1, HcCHS2, HcCHS3
Neurospora crassa		NcCHS1, NcCHS2, NcCHS3
Ustilago maydis	Basidiomycetes	UmCHS1, UmCHS2
Schizophyllum commune		ScCHS1
Candida albicans	Fungi Imperfecti	CaCHS1, CaCHS2
Exophiala jeanselmei		EjCHS1, EjCHS2, EjCHS3
Phaeococcomyces exophialae		PeCHS1, PeCHS2,
Rhinocladiella atrovirens		RaCHS1, RACHS2,
Wangiella dermatitidis		WdCHS1, WdCHS2, WdCHS3
Xylohypha bantiana		XbCHS1, XbCHS2
Entomophaga aulicae	Zygomycetes	EaCHS1, EaCHS2
Rhizopus oligosporus		RoCHS1, RoCHS2

Taxonomic affinities are modified from Dixon & Fromtling (1991).

As it can be easily seen the amino acid sequences fall into three distinct classes as described by Bowen et al. (1992). Three genes fall out of the classification, the two CHS genes from *Saccharomyces cerevisiae* CHS1 and CHS2, and CHS2 from *Ustilago maydis* (fig. 2). Both *E. aulicae* fragments belong to class II.

A

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1    GAG AAC TTA TTC ACC AAA ACT ATG ACT TCA GTC ATG AAG AAT GTG GCG CAT CTA TGC CGT CGT AAT CGC
1    E  N  L  F  T  K  T  M  T  S  V  M  K  N  V  A  H  L  C  R  R  B  R

70   TCG CGT ACT TGG GGA GAT AAC GGA TGG CAG AAG GTG GTG GTC TGT ATC GTA TCG GAC GGT CGA CTC AAA
24   S  R  T  W  G  D  N  G  W  Q  K  V  V  V  C  I  V  S  D  G  R  L  K

139  ATC AAC AAG CGA GTA CTG ACC GTG CTG GCT GCG ATG GGG GTT TAC CAG GAT GGG ATT GCT CAG TAT GCC
47   I  N  K  R  V  L  T  V  L  A  A  M  G  V  Y  Q  D  G  I  A  Q  Y  A

208  GTC AAC GGG AAG CCC GTG ACG GGC CAT ATT TAC GAG TAT ACC ACC CAG ATT GTA GTG GAT ACC GAT CTG
70   V  N  G  K  P  V  T  G  H  I  Y  E  Y  T  T  Q  I  V  V  D  T  D  L

277  AAT ATC CGC GGA CGT GAT GCC GGG CTC GTC CCA GTA CAA ACA ATC TTT TGC CTC AAG GAG AAG AAT GCC
93   N  I  R  G  R  D  A  G  L  V  P  V  Q  T  I  F  C  L  K  E  K  N  A

346  AAG AAA TTG AAT TCG CAT CGC TGG TTC TTT AAC GCC TTT GGC CCC CTC CTC AAC CCT AAC GTG TGC GTC
106  K  K  L  N  S  H  R  W  F  F  N  A  F  G  P  L  L  N  P  N  V  C  V

415  CTT ATC GAT GTG GGT ACT CGG CCC AGT GGG ACC TCC ATC TAT CAC TTA TGG AAG GCC TTT GAT CGT AAT
129  L  I  D  V  G  T  R  P  S  G  T  S  I  Y  H  L  W  K  A  F  D  R  N

484  CCA ACC TTG GGT GGA GCC TGT GGC GAG ATT TAC GCT GAG TTG GGC AAG GGA TGG CAA AAG CTC ATC AAC
151  P  T  L  G  G  A  C  G  E  I  Y  A  E  L  G  K  G  W  Q  K  L  I  N

553  CCT CTC GTG GCA ACC
174  P  L  V  A  T
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B

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1    GAA CAC CTA TTC GTC AAG ACC ATG AGG GCT GTA ATG AAG AAC GTG ACC CAT CTT TGC AGT CGC AAC CGC
1    E  H  L  F  V  K  T  M  R  A  V  M  K  N  V  T  H  L  C  S  R  N  R

70   TCT CGA ATA TGG GGA GAC CAA GGT TGG GAG AAA GTG GTT GTT TGT ATT GTT GCT GAT GGG CGT ACA AAA
24   S  R  I  W  G  D  Q  G  W  E  K  V  V  V  C  I  V  A  D  G  R  T  K

139  ATC CAT CCA CTC ACT TTG AAA GTA CTT GCC GCC ATG GGG ATT TAT CAA GAC AAT GTC TCG CAG ACA TCA
47   I  H  P  L  T  L  K  V  L  A  A  M  G  I  Y  Q  D  N  V  S  Q  T  S

208  GTC AAC GGA AAC CCT GTA ACG GCA CAC ATT TAT GAA TAC ACA AGT CAA GTT TAT GAT GGA TTC GGA TTT
70   V  N  G  N  P  V  T  A  H  I  Y  E  Y  T  S  Q  V  Y  D  G  F  G  F

277  AAA AGT CAG AGC ATC TCA GGA GAA ACT GTA CCT ATT CAG ACC ATC TTT TGT CTT AAA GAG AAG AAT GCA
93   K  S  Q  S  I  S  G  E  T  V  P  I  Q  T  I  F  C  L  K  E  K  N  A

346  AAG AAG CTA AAT TCT CAT AGG TGG TTC TTT AAT CGG TTT GGC CCT GTT CTT TCA CCG AAT GTC TGT GTC
106  K  K  L  N  S  H  R  W  F  F  N  R  F  G  P  V  L  S  P  N  V  C  V

415  CTT AAT GAT GTT GGA ACC AAA CCC ACT CCG ACC TCA ATT TAT CAT TTA TGG AAA GCG TTC GAT CGC AAT
129  L  N  D  V  G  T  K  P  T  P  T  S  I  Y  H  L  W  K  A  F  D  R  N

484  CCC GAT CTC GGA GGA GCA TGT GGA GAG ATA TAC GCT GAA TGG GGG AAG GGT GGC GTC AAG TTG ATC AAC
151  P  D  L  G  G  A  C  G  E  I  Y  A  E  W  G  K  G  G  V  K  L  I  N

553  CCT CTA GTG GCA GCC
174  P  L  V  A  A
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Fig 1. The basepair composition and the aminoacid sequences of **A:** EaCHS1 and **B:** EaCHS2.

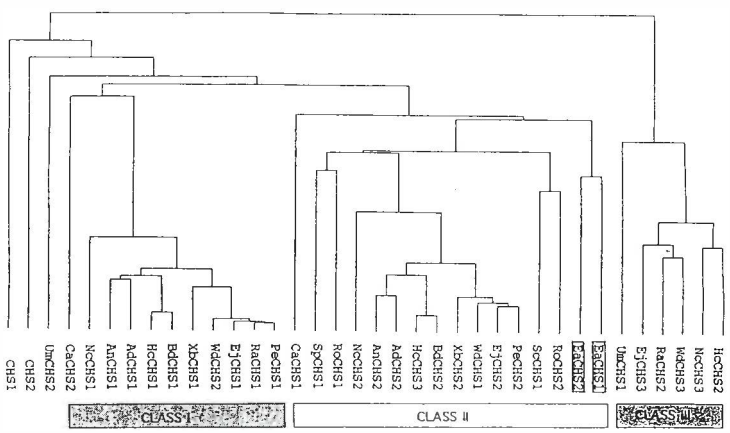

Fig. 2. UPGMA dendrogram showing three distinct chitin synthetase classes. [] *E. aulicae* PCR chitin synthetase fragments

The EaCHS genes cluster most with each other and are also very close to CHS2 from *Rhizopus oligosporus*, the only other Zygomycetes wich have CHSs deposited in the data-base. However, the dendrogram shows not the phylogenetic relationship between the sequences since the distance matriz have not been calculated.

Until now the two Zygomycetes are the only species having more than one chitin synthetase gene in the same class. The role of these genes in the fungal chitin formation have still to be elucidated.

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DEVELOPMENT OF A NEW PROCEDURE FOR THE MASS PRODUCTION OF CONIDIA OF *METARHIZIUM FLAVOVIRIDE*.

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Summary

A two phase system for the production of aerial conidia of *Metarhizium flavoviride* has been developed. The first phase involves the production of fungal biomass in a simple liquid medium. The product of the liquid fermentation is then absorbed onto cellulose cloths on which conidiation occurs. The yield of conidia can be increased by the adjustment of the nutrient ratio in the liquid medium and further increased by the addition of supplementary sucrose in the second phase.

Introduction

Many different methods have been described for the mass production of entomopathogenic fungi. The choice of method depends both on growth characteristics and the type of formulation in which the infective propagules will ultimately be applied.

Work on oil formulations carried out by Bateman *et al* (1993) showed that conidia of Deuteromycete fungi are more infective to the host insect when applied in oil formulations than those in water and this effect is particularly pronounced at low relative humidities. The hydrophobic properties of aerially produced conidia of many Deuteromycetes, enable these fungi to be easily suspended in oil formulations. However, hydrophobicity predisposes them to aerial production on a solid or semi-solid medium.

The IIBC/IITA/DFPV collaborative programme for the control of Locusts and Grasshoppers has the aim of developing an effective mycoinsecticide based on an oil formulation of conidia of an entomopathogenic fungus (currently *M. flavoviride*). Currently, production of conidia is via a two phase system in which the fungus is first grown in a liquid, shake flask phase, followed by conidiation on rice. This system is simple and does not require high technology equipment. It also makes use of substrates which are localy available in West African countries. However, disadvantages of this system, are that the rice is not fully utilised by the fungus during

growth and conidiation, thus resulting in waste of resources. The system is also inefficient in the use of space, which during the 1993 field season, was the major factor limiting production.

The following method is being developed as a possible alternative to the rice system. Biomass is produced in liquid shake flasks and then absorbed onto the surface of cellulose cloths.

Materials and Methods

M. flavoviride IMI 330189ss was used throughout these studies. Liquid medium was prepared using brewers' yeast (BY) obtained from the Brewery Beninoise, Cotonou, Republic of Benin (SOBEBRA) and sucrose (SUC). BY/SUC liquid medium was prepared at the following concentrations (g/l): 20g BY, 20 SUC; 20 BY, 30 SUC; 30 BY, 20 SUC and 30 BY, 30 SUC for initial experiments and thereafter, 30 BY, 30 SUC was used as standard. 75ml of the liquid medium were distributed into 250ml conical flasks and after autoclaving was inoculated with 1 ml of a 6.6×10^6 conidia/ml suspension of *M. flavoviride*. Liquid cultures were incubated for 48 hours at 150 rpm and 24°C.

Cellulose fibre absorbent cloths were cut into rectangles measuring 19 X 16 cm and were autoclaved for 30 min prior to use. In some experiments, the cloths were pre-treated by the addition of 20 mls of sucrose or other carbohydrate solution containing between 1 and 10% w/v sucrose. Pre-treated cloths were allowed to air dry before autoclaving.

Cloths were inoculated by the addition of liquid biomass to the saturation point of the cloth and were suspended in specially constructed stainless steel boxes. Humidity was maintained inside the box by the addition of a reservoir of sterile water in the bottom of the tanks. Each box was designed to hold 9 cloths and was covered with a glass top. The boxes were incubated at between 25 and 30°C for a period of 6-11 days.

On removal from the boxes, conidia were harvested from the cloths by stomaching with 0.05% Tween in water in a 'Colworth S Stomacher 400'. Conidial yields were assessed using an improved Neubauer haemacytometer.

Results

Fig.1. shows the effect of altering the ratio of nutrients in the liquid medium in the first phase on the subsequent production of conidia on cellulose cloth. The highest level of production of conidia/cm^2 of cellulose cloth was achieved in the treatment containing 30 g/l of both brewers' yeast and sucrose (30/30 BY/SUC). Lowest conidial yields were harvested from cloths inoculated

Fig.1

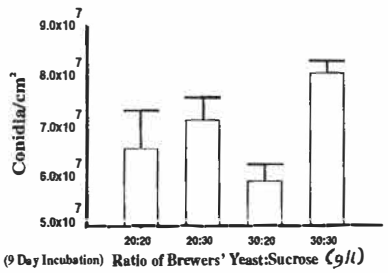


Fig.2

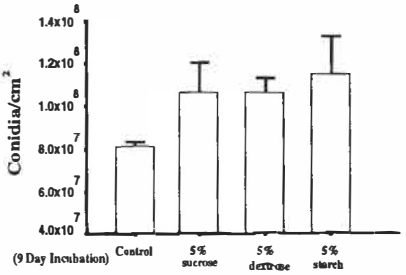


Fig.3

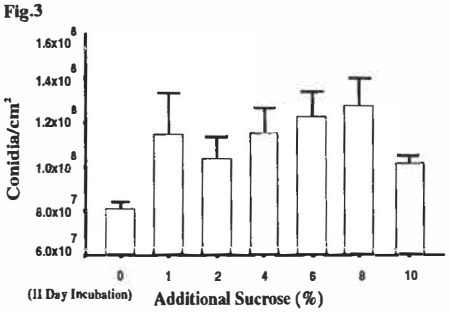
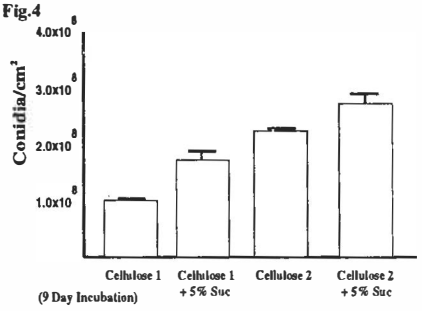


Fig.4



Production of conidia by *M.flavoviride* on absorbant cellulose cloth. Fig.1 Adjustment of ratio of BY:SUC in liquid medium. Fig.2 Addition of supplementary carbon sources to cloth. Fig.3 Addition of increasing levels of sucrose. Fig.4 Use of cellulose of two absorbancies. Cellulose 1=0.16ml/cm^2 cloth; cellulose 2=0.23ml/cm^2 cloth Bars=SE $p<0.05$

with the 30,20 BY/SUC medium. Both treatments containing the lower level of 20g/l BY gave low conidial yeilds at both 20 and 30 g/l SUC.

Cellulose cloths pre-treated with 20 mls of a 5% solution of sucrose, dextrose or starch all resulted in significantly increased production of conidia from approximately $8x10^7$ conidia/cm^2 to $>1x10^8$ conidia/cm^2 (Fig.2.). Where a range of sucrose concentrations was used for pre-treating the cloths, all treatments in the range 1-8% sucrose caused a significant and equal increase in conidial yeild (Fig 3). However, a significant reduction in this yield is apparent in the highest, 10% sucrose treatment.

Yields obtained from cellulose cloths of different absorbancy are shown in Fig 4. This illustrates the significant increase in production of conidia by *M. flavoviride* on cellulose cloth of higher absorption capacity. The addition of supplementary sucrose further increased yield to approximately $3x10^8$ conidia/cm^2.

Discussion

The series of experiments described above was designed to assess the potential efficacy of cellulose cloths as a

physical support for the production of aerial conidia of *M. flavoviride*. Initial investigations into the basic nutritional requirements of the fungus in this system indicated that a relatively high level of both nitrogen and carbon sources was required. The results presented in Fig. 1 suggest that nitrogen in the form of dried brewers' yeast is inhibitory to sporulation when supplied in a higher proportion w/w than that of sucrose. This effect has also been shown to occur in *M. flavoviride* during conidiation in liquid culture (Jenkins & Prior, 1993).

Investigations into the effect of supplementary carbon on the production of conidia in the second phase, indicated that sucrose, dextrose and starch all boosted the total yield of conidia to the same extent. Moreover, the addition of only 20mls of a 1% solution of sucrose is sufficient to boost conidia production to a higher level and further increases in the supply of sucrose have no further contributary effect.

A further increase in the production of conidia/cloth was obtained when cellulose of an increased absorption capacity was used. An increase of $>1x10^8$ conidia/cm^3 of cloth was observed on slightly thicker cellulose, boosting production of conidia to as much as $3x10^8$ conidia/cm^2 with the use of supplementary sucrose.

Further development of this technique is still required in order to achieve a fully optimised mass production system. Economically, production of conidia on cloths such as cellulose has a number of advantages over the system currently employed with rice. With respect to production of conidia/cubic meter of incubation space, the cloth system will currently give an output of $2.8x10^{14}$ conidia/cm^3, whereas the same area occupied by the rice system will produce only $3.6x10^{13}$ conidia. In addition to this, a two phase production system on cloth does not involve the use of a valuable food product. Cloth is re-usable, thus reducing the generation of large amounts of waste. Once the system is fully optimised, it may be possible to ensure the complete utilisation of all nutrients supplied.

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VIRULENCE AND PERSISTANCE OF *CONIDIOBOLUS CORONATUS* AND *CONIDIOBOLUS* SP. IN GLASSHOUSE POPULATIONS OF *BEMISIA TABACI*

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INTRODUCTION

The tobacco whitefly, *Bemisia tabaci* (Gennadius) is a serious pest of economically important field and glasshouse crops in Israel and other countries with subtropical climates. Biological control agents against this pest become ever more important because of its ability to develop resistance against many classes of chemical insecticides.

Deuteromycete fungi such as *Verticillium lecanii*, *Aschersonia aleyrodis* and *Paecilomyces fumosoroseus* are known as pathogens with epizootic potential against this pest. Spontaneous outbreaks of *V. lecanii* have been observed in glasshouse populations of *B. tabaci* in Israel during 1991-1992 (Gindin, in Ben-Ze'ev, 1993). The only members of the Entomophthorales recorded on *B. tabaci* are *Erynia radicans* on cotton, in Tchad (Papierok, 1987) and Israel (Ben-Ze'ev et al., 1988) and *Conidiobolus coronatus* on cotton in a glasshouse (Gindin, in Ben-Ze'ev, 1993).

In the course of a study concerned with natural outbreaks of *V. lecanii* in glasshouse populations of *B. tabaci* reared on cotton, we isolated several times *Conidiobolus coronatus* and what appeared to be *Conidiobolus thromboides*. A new study was initiated, aiming to determine the efficacy of these two species against glasshouse populations of *B. tabaci*, to compare their relative virulences and their survival abilities in the glasshouse environment. A detailed paper on this study is in preparation (for *Phytoparasitica*).

MATERIALS AND METHODS

Isolates of *Conidiobolus* spp. from dead *B. tabaci* were obtained on MYA medium (2% malt extract, 0.1% yeast extract, 2% agar, 100mg/l ampicillin), following which single-conidium isolates were secured. Cultivation was on MYA without ampicillin.

Conidial doses for inoculation experiments were determined as follows: Petri dishes with MYA were showered for 2-3 hours with conidia from uniformly spread pieces of sporulating colonies. The conidial fields obtained on MYA developed into uniformly sporulating colonies within 18 hrs (for *C. coronatus*) or 24 hrs (for *Conidiobolus* sp.). Incubation was at room temperature (23-25 °C) under constant fluorescent illumination. Preliminary observations on cultures of both species showed that the intensities of conidial showers (measured and averaged as conidia/mm^2/min) tended to increase during the first 18 hours from the onset of sporulation for *C. coronatus* and 24 hr for *Conidiobolus* sp., and stabilized for the next 18-24 hr for *C.coronatus* and for 24-48 hr for *Conidiobolus* sp. Ca. 30 uniform colonies which achieved stable sporulation were inverted over clean Petri dish covers for periods of 5 minutes, the covers were replaced and the numbers of conidia/mm^2 were calculated by averaging 10 microscope fields/cover. Colonies with similar averages vere grouped and used for different sporulation intensities. Conidial

doses for inoculations were calculated with the help of the formula: $D = T(I_1 + I_2)/2$; D= dose (conidia/ mm^2), T= time of inoculation (minutes), I= intensity (dose/minute) measured immediately before (I_1) and immediately after inoculation (I_2).

Infestation of fresh cotton plants with adult whiteflies for one day, followed by complete removal, resulted in synchronized populations of larvae which were used for inoculation experiments. Leaf sections with such synchronic populations of larvae or ready-to-hatch pupae were placed on moist filter paper and exposed to calculated conidial showers. Hatching adults were infected by contact with conidia on pupal cuticles and on the leaf surface. Dead individuals were removed daily for examination and to prevent interference from secondary inoculum.

Survivability of both *Conidiobolus* species on the surface of cotton leaves was assayed in the absence of insect hosts: a) Cotton leaves free of insects were treated with conidial doses of 50-60 conidia/mm^2 in Petri dishes lined with moist filter paper. Treated leaves were incubated in an illuminated incubator (white, fluorescent light) at 24±1 °C; Small pieces of inoculated leaves were cut and briefly touched to the surface of MYA + Ampicillin in Petri dishes. This was done at intervals of 7 days, starting 1 week after inoculation, to assess the survival of conidia on leaves. The Petri dishes were incubated in darkness at 20-23 °C for 2-3 days and examined for *Conidiobolus* before becoming covered by saprophytes.

b) Potted cotton plants with 10±1 leaves were used for the glasshouse assay. Individual (attached) leaves were inoculated with *Conidiobolus* conidia ejected from inverted Petri dishes, 5-6 leaves per plant were inoculated. Each *Conidiobolus* species was used to inoculate 12-13 leaves. Inoculated plants were kept in a glass box with cloth covered openings, to protect the rest of the glasshouse from contamination with entomogenous fungi. Conidial doses ranged between 50-200 conidia/mm^2. The glasshouse was temperature controlled (20-23 °C at night, 24-27 °C daytime), humidity was measured twice daily at 7.30-8.00 AM and at 14.30-15.00 PM during the three weeks duration of the experiment. Conidial viability assay was carried out as the one in Petri dishes (a), but was monitored twice weekly.

Fatty acids profiles were obtained by gas-chromatography (Hewlett Packard GC 5890 Series II connected to an HP Vectra personal computer) using the "Microbial Identification System" software and Operating Manual, Version 4, Appendix B: Fungi Library Culture Techniques [Microbial ID, Inc. (MIDI)].

RESULTS AND DISCUSSION

1. Identification -- Conidiobolus coronatus was identified by its characteristic primary conidia, production of microconidia and transformation of some primary conidia into villose spores (Prasertphon, 1963) and others into loricoconidia (Weiser and Batko, 1966). Primary conidia dimensions were L x D 22.1-51.1 x 15.2-34.5µm, $\bar{x}$=31.5 x21.6µm, s.d.=6.36 x 4.1µm, n=51 conidia. The other *Conidiobolus* was initially misidentified as *C. thromboides*. Conidial measurements of four isolates from culture averaged 16.5-17.7 x 12.3-12.8 µm, which did not fit any of the published species of entomopathogenic *Conidiobolus*. This species did not produce microconidia or capilliconidia and therefore belongs in subgenus *Conidiobolus* (Ben-Ze'ev and Kenneth, 1982). Primary conidia resporulate to give secondary ones of type Ia (Ben-Ze'ev and Kenneth, 1982) or transform into loricoconidia. Fatty acids analysis followed by cluster analysis shows the 4 isolates to be a rather tight cluster, apparently one strain, different from both *C. thromboides* and *C. corona-*

tus, but related to them at the genus level. Comparison with the fatty acids profiles of several species of *Conidiobolus* subgen. *Conidiobolus* with small conidia is in progress, in order to establish the taxonomic position of this strain.

2. Natural Occurence and Symptoms -- Both *Conidiobolus* species occured naturally on *B. tabaci* in our glasshouses during June to October. None of them achieved epizootic levels and in hundreds of cadavers investigated, infection levels were limited, between <1% - 3% for *C. coronatus* and to <2% for *Conidiobolus* sp. A number of cadavers with double infection were observed. The symptoms caused by both species, observed in artificially inoculated individuals, were similar. Diseased adults were sluggish, could not fly and their abdomens were dark beige in contrast to the bright yellow of healthy individuals. After death, the dark crimson pigment of the eyes spread and colored most of the head. In diseased larvae the abdomens had an opaque, beige colour, in contrast to the transparent, bright honey abdomens of healthy ones. *C.coronatus* produced conidia from 70-80% of adults killed. The rest were found to contain hyphal bodies. Resting spores were not found. *Conidiobolus* sp. produced resting spores in ca. 60% of killed adults, ca. 20% produced conidia and the rest had hyphal bodies only.

3. Pathogenicity Assays -- Microscopic observations revealed that eggs were not infected even by very high doses of conidia (>500 con/mm^2) of either species. Larvae hatched similarly from treated and untreated eggs.

Second instar larvae are apparently quite resistant to both species of *Conidiobolus*. Very high doses of *Conidiobolus* sp. conidia killed <1% larvae. *C. coronatus* was somewhat more virulent, killing 0.4% larveae at doses of ca. 74 con/mm^2, 1.7% at 460 con/mm^2 and 4.6% at ca. 950 con/mm^2.

The difference in virulence between the two species becomes even larger when adult *B. tabaci* are infected (Tables 1 and 2). Adults are markedly more susceptible than larvae. *C. coronatus* can kill close to 95% of adult populations at the relatively low dose of 60 con/mm^2, while *Conidiobolus* sp. is much less effective (Table 2). It is possible that the initial doses of *C. coronatus* conidia increase by producing microconidia, while the other species cannot do so.

TABLE 1. Dose-related virulence of *Conidiobolus coronatus* to *Bemisia tabaci* hatching adults and pupae (one of two similar repeats).

Dose (conidia/ mm^2)	No. pupae treated (total)	No. adults hatched	No. adults mycosed	No. pupae mycosed	% mortality adults/ hatched adults	% mortality pupae + adults/ total
0.0 control	147	132	0	0	0.0	0.0
1.2	144	138	25	2	17.4	18.7
2.3	148	135	43	3	29.1	31.1
6.0	149	130	95	16	73.1	74.0
11.8	192	157	134	33	85.4	87.0
28.0	185	144	128	36	88.9	88.6
60.0	176	145	136	30	93.8	94.3

4. Persistance Assays -- Conidia of *C. coronatus* discharged on 10 detached cotton leaves kept in moist Petri dishes remained viable on all 10 leaves for 42 days and were still viable on 4 leaves after 70 days. Those of

Conidiobolus sp. maintained viability on all 10 leaves for 14 days, were still viable on 6 leaves after 21 days and showed no viability after 28 days.

TABLE 2. Dose-related virulence of *Conidiobolus* sp. to *Bemisia tabaci* pupae and hatching adults (one of two similar repeats).

Dose (conidia/ mm^2)	No. pupae treated (total)	No. adults hatched	No. adults mycosed	No. pupae mycosed	% mortality adults/ hatched adults	% mortality pupae + adults/ total
0.0 control	120	99	0	0	0.0	0.0
3.0	131	101	0	3	0.0	2.3
5.5	105	90	3	2	3.3	4.8
10.9	103	92	7	3	7.6	9.7
30.3	92	77	7	6	9.1	6.5
54.6	122	86	17	11	18.6	14.1
109.6	119	97	22	9	22.6	22.9
210.0	98	84	20	8	23.8	28.6

On cotton plants in glasshouse environment the viability of both species was much shorter, 10 days for *C. coronatus* and surprisingly longer, 17 days, for *Conidiobolus* sp. As in both species primary conidia can transform into resting conidia (loricoconidia), obviously *Conidiobolus* sp. is either more resistant to low humidity or more efficient in production of loricoconidia.

Of the two *Conidiobolus* species found to be entomopathogens of *Bemisia tabaci*, *C. coronatus* appears to be more promissing as a biocontrol agent, with high mortality obtained at rather low conidial dosage. The other *Conidiobolus* sp. should be further investigated as it showed remarkable survivability in glasshouse conditions. Isolates with improved virulence should be looked for.

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PHYSICAL PROPERTIES AND ATOMISATION OF ULV FORMULATIONS OF MYCO-INSECTICIDES

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Abstract
Oil-based suspensions of *Metarhizium* conidia have shown high infectivity to locusts and grasshoppers in both laboratory and field studies. ULV suspension (SU) formulations have been prepared which should be similar in use to ULs and have the following characteristics:
i. Low toxicity to humans and other animals; low phytotoxicity.
ii. Stability: it should be possible to store formulations (ideally for more than 1 year) without loss of activity. Suspensions should re-homogenize easily after shaking and not "cake" in the bottom of the bottle.
iii. Low viscosity: an ability to flow satisfactorily through existing ULV equipment. Low viscosity oils often have a high viscosity index: they are less affected by changes in ambient temperature. These properties are especially important for hand-held sprayers which do not have a pump; they rely on a gravity feed mechanism with a restrictor and an air bleed to control formulation flow.
iv. Evaporation retardation: at least some of the mixture should have a low volatility, so that small droplets maintain sufficient size to impact on the target.
v. Satisfactory atomisation characteristics with standard ULV spray equipment, with a substantial proportion of spray droplets within a size band appropriate for the target (e.g. >80% of the total volume between 50-100μm).

Introduction: Controlled Droplet Application (CDA)
Because of the concerns expressed about the widespread use of chemical pesticides in the last desert locust plague, a collaborative research programme to investigate the biological control of locusts and grasshoppers was set-up. Symmons (1992) gives an over-view of the logistics of locust control. Large areas of often difficult terrain must be rapidly treated so Ultra Low Volume (ULV) techniques are the only practical means of applying pesticides. Although ULV can be defined as the application of less than 5 l of formulation per hectare, operators often aim to apply < 1 l/ha in locust control campaigns. There is therefore a need to maximise the effectiveness of very small volumes of formulation by minimising waste, and this is achieved by rigorously controlling the size of spray droplets using rotary nozzles.

Controlled Droplet Application (CDA), with its emphasis on droplet size, is not strictly synonymous with ULV. When he coined the term in 1975 E.J. Bals stated that "The efficiency of a spraying machine is inversely proportional to the range of droplets it emits, whilst the suitability for a specific problem depends on the actual size of the droplets emitted". The droplet size spectra of all formulations prepared by the project are checked for appropriate atomisation with standard rotary nozzles using a 'Malvern' particle size analyser. *Fig. 1.* shows the droplet size spectrum of an oil-based formulation atomised with a Micron 'X10' nozzle (which is used in the vehicle mounted 'Ulvamast' sprayer for acridid control). In the illustration 81% of the volume of droplets are found within a 60-120μm size band. Rotary atomisation of insecticides usually involves ligament disintegration, in which principal and "satellite" droplets are formed. Although these satellites may be more numerous than the principal droplets, they only account for a small proportion of the volume. The testing of blank oils usually presents a "worst case", and the addition of fungus conidia normally results in a narrowing of the droplet size spectrum. This effect has been shown to be common with particulate suspensions (Bateman, 1989).

Formulation and Application Strategies
Considerations about the formulation and application of myco-insecticides must be closely linked to those of production and storage. The toxicity of all formulating media to conidia is assessed empirically. We have been examined three strategies:-

1. The preparation of ULV suspension (SU) formulations that remain in suspension or re-suspend with minimal agitation, and require no mixing or measuring by the spray operator.

Conidia of Deuteromycete fungi such as *Metarhizium* and *Beauveria* have lipophilic cell walls and easily suspend in oils. The normal approach that has been taken to date has been to suspend conidia for spraying in mixtures consisting a viscous oil (either vegetable or mineral in origin) and light "solvents": such as paraffinic oils. This concept dates from some of the original work on preparation of ULV formulations (ULs) containing dissolved chemical ingredients (Coutts and Parish, 1967). In the case of organic chemical pesticides, solvents (and co-solvents) are used to dissolve the active ingredient however this is obviously not

required for the suspension of conidia. The use of mixtures containing paraffins is useful - at least in research programmes - since the viscosity of the formulations containing different spore concentrations can be adjusted by changing the proportion of solvent. Addition of viscous oil has several advantages including: limiting the effects of evaporation (see below), retardation of spore settling, improved atomisation and possible improvements to the persistence of droplets on plants and the transfer of the active ingredient to target insects.

2. The preparation of oil miscible concentrates for easy transportation and storage, for dilution to the required concentration near to the time of spraying.

Conidia usually have a higher density than the formulating oil and settle out into a concentrated mass that can be difficult to re-suspend in quantity. Oil miscible flowable concentrates (OF) of chemical pesticides have been prepared for dilution with organic liquids, however these may involve sophisticated formulation techniques. Oil dispersible powders (OP) containing high proportions of conidia probably constitute a more accessible technology, and when appropriately packaged these are easily stored and transported.

3. The use of water-based ULV ("WULV") techniques: for spraying certain grasshopper and other pest species in the humid tropics and cooler climatic zones.

Although the use oil-based formulating media can be highly efficacious, the high price of commercial ULV formulations has prompted the use of conventional formulations (e.g. wettable powders) mixed with water. These are atomised with spinning disc sprayers at very low volumes of application - and therefore high work rates - in comparison with hydraulic spraying techniques. In order to make this more effective:

i. Adjuvants such as molasses (Gledhill, 1975) or emulsified oils (Wodageneh and Matthews, 1981) can be added to the mixture to limit the effects of evaporation.
ii. Modifications can be made to the spraying equipment so that droplet size is enlarged (to a band say 75-150μm) and the atomiser is manufactured to a higher specification (to cope with increased volume application rates of 5-15 l/ha): Bateman (1989), Clayton (1992).

Industrial mycologists prefer to culture myco-pathogens in a single phase liquid fermentation process. Two current lines of research have demonstrated the feasibility of preparing hydrophilic blastospores (Kleespies and Zimmerman, 1992) and submerged conidia (Jenkins and Prior, 1993) in such a process. Aqueous suspensions of both types of spore have been shown to be infective, and "WULV" may prove to be a useful method of application.

Limiting the Effects of Evaporation

ULV sprays consist of small droplets which have large surface areas relative to their volume. If the formulation were to consist entirely of volatile ingredients these droplets would rapidly be reduced to a tiny, dry nucleus of active ingredients. Unless the air velocity is high, very small droplets ($<40\mu$m diameter) may fail to impact, even on small target surfaces. The presence of a non-evaporative component will limit the diminution in the droplet size. *Fig 2.* shows the theoretical relationship between the final droplet diameter after maximum evaporation and the non-volatile content of the formulation. If the non-evaporative proportion is 12.5% a droplet will not diminish to more than 1/2 of its original diameter, although a higher proportion is normally used in practice. A droplet "IIBC 003": containing a mixture of equal proportions of 'Ondina' (a non-evaporative mineral oil) and 'Shellsol T' (an isoparaffin) will not reduce to less than 79% of its original diameter (e.g. a 75μm droplet leaving the sprayer will not evaporate to less than 60μm).

Viscosity and flow rate

Low viscosity is especially important for hand-held sprayers which do not have a pump; they rely on a gravity feed through a restrictor and an air bleed mechanism to control formulation flow. Formulations should have a high viscosity index, so that the effects of temperature on flow rates are minimised. In practice, temperature has little effect on low viscosity oil-based formulations, and practically no effect on "WULV" mixtures. Unfortunately there are no clear international standards for UL formulations, and pesticide companies formulate "for the market". In West Africa most spraying is for cotton pest control, and most products appear to be approximately 7.0 cSt - or typically $\approx$8 cP (J. Clayton, pers. comm.). A convenient method for checking viscosity is by using standard cups such as the BS1733 'B2' flow cup (BSI, 1955); the efflux time for 7 cSt formulations is approximately 43 seconds. Since its mechanism is based on gravity feed through a restrictor, this is essentially similar to flow through hand-held spinning disc sprayers and has proved to be a reliable method of conveying viscosity specifications between the research laboratory and the field.

If settling does occur suspensions should re-homogenize easily after shaking and not "cake" in the bottom of the container. Ground-based ULV spraying machines practically never have specific agitation mechanisms: this occurs only by shaking of the reservoir during operation. The rate of sedimentation of suspended particles is directly proportional to their size and density relative to the formulating liquid. Conidia often grow from their phialides in long chains, and these must be split up. Specifications for good wettable powders (Matthews, 1992) include:

i. All particles should pass through a 44μm mesh screen
ii. A sample of suspended WP is prepared, and kept undisturbed in a graduated cylinder. Liquid withdrawn from halfway down the cylinder after 30 minutes should contain at least 50% of the original pesticide concentration.

Stability

Amongst the greatest research challenges has been the achievement of adequate shelf life and storage stability at high temperatures. Although not as stable as chemical insecticides, the results of small sample experiments have shown that, provided moisture is removed, formulations can be stored at 17°C for more than 18 months without serious loss of conidial viability (Moore, D., Bateman, R., Carey, M. and Prior, C., In preparation)). Short term storage at high temperatures (up to 55°C) is also possible if the water content is minimised. Particular care will be needed to maintain quality control of the formulation processes.

Toxicity

The emphasis on formulating for very low mammalian toxicity is a relatively new concept, and the FAO list of locust insecticides still includes ULV formulations that are highly-moderately hazardous; e.g. bendiocarb 20% UL with an LD_{50} of approximately 200 mg/kg. *Metarhizium spp.* are presently classified in the UK in risk group 1 ("most unlikely to cause human disease"). Formulations are currently undergoing mammalian toxicity tests, to EEC, and EPA standards, at an independent laboratory. Fungi have been studied for many years, and the principal risk (assuming adequate quality controls in production) appears to be from allergic reactions in certain people. Formulations are checked for phytotoxicity by spraying enlarged droplets onto test plants (Coutts, 1974) before carrying out field trials.

There have been no reports of adverse environmental consequences from widespread use of entomopathogenic fungi (Greathead & Prior, 1990). The programme is collaborating with organisations such a LOCUSTOX (sponsored by FAO) in order to assess the impact of formulations of *M. flavoviride* on non target organisms. There are virtually no records of fungi in this genus infecting non-arthropod hosts, and none of infection in vertebrates.

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Fig. 1. Droplet size analysis of an 'Ulvamast' X10 nozzle (6000 RPM, IIBC 003 blank, 750 ml/min)

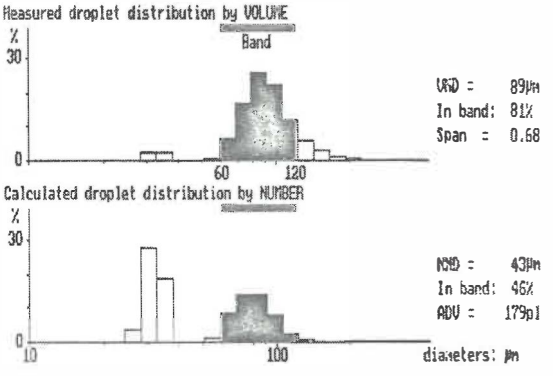
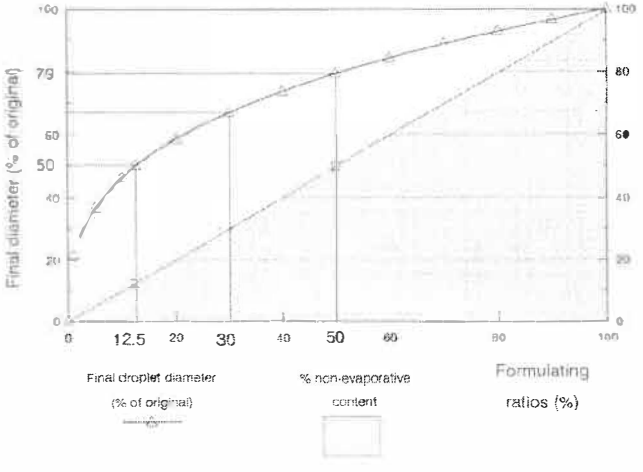


Fig. 2. Spray formulations: minimum final droplet diameter after complete evaporation of the volatile component, with various mixing ratios



Side effects of pesticides on insect pathogenic fungi: Some remarks and a proposition

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Summary

Two tests with two groups of insect pathogenic fungi are proposed to check the side effects of pesticides. They follow the principle that side effects of pesticides applied on the soil or into the soil should be tested on soil pathogens like *Beauveria bassiana* or *Metarhizium anisopliae*, whereas pesticides applied on plants should be tested on epigaeic fungi like Entomophthorales. The proposed tests are designed to stress the effects of a pesticide on the host-pathogen interaction and not on the effects on the pathogen alone.

1. Introduction

During the past few years the IOBC/WPRS working group "Pesticide and beneficial organisms" has developed standardised methods to test side effects of pesticides on beneficial organisms. These tests include the insect pathogenic fungi *Verticillium lecanii* (TUSET, 1985), *Beauveria bassiana* and *Metarhizium anisopliae* (HOKKANEN & KOTILUOTO, 1992). Whereas the tests including beneficial arthropods are accepted by the plant protection industry there is some resistance to accept those including entomopathogenic fungi.

The objections are partly fundamental denying any practical importance of insect pathogenic fungi (an information deficiency), but concern mainly the testing procedure which is said to be too complicated and too far away from reality, especially the tests on agar and the tests with fungi which are unlikely to come ever in contact with the pesticide (ZOBRIST, pers. comm.). I think we should consider and examine these objections in order to develop a testing procedure which is simple but nevertheless allows effective evaluation of the side effects of the pesticides on insect pathogenic fungi.

2. Testing procedure

a. General principles and test organisms.

Testing procedures usually include the following parameters which are used in sequence: growth (diameter of colonies, colonised surface), spore production (speed, amount), viability of spores (rate and speed of germination) and virulence of spores. (e.g. HOKKANEN & KOTILUOTO, 1992, KELLER et al., 1993). Influences on virulence can be measured in at least two different ways: Starting with spores produced under the influence of a pesticide or starting with a fungus growing and producing spores under the influence of a pesticide. The latter procedure combines growth, spore production, viability and virulence of spores and makes separate tests superfluous at least for practical purposes.

Pesticides can either be applied on plants, on the soil or into the soil. Consequently their effect should be measured either on a soil pathogen-pest interaction or on an epigaeic pathogen-pest interaction. The most frequent entomopathogenic soil fungi are *Metarhizium anisopliae* and *Beauveria bassiana* (e.g. KLEESPIES et al., 1989), which are widely distributed and common. Typical epigaeic pathogens are Entomophthorales. These fungi should therefore be used to test side effects of pesticides.

b. Proposed procedure for entomopathogenic soil fungi

At the laboratory level the proposed method is identical with the standard method developed by HOKKANEN & KOTILUOTO (1992) (Tab. 1). In the semi-field and field test the proposed methodis based on the use of fungus kernels. These are autoclaved, peeled barley kernels colonised with mycelium of the fungus to be tested. They were originally developed to control larvae of the May beetle, *Melolontha melolontha* L. with *B. brongniartii*,

but are also suitable to produce *M. anisopliae* (AREGGER, 1992; KELLER, 1993). At present *B. brongniartii*-kernels are used to check soils for antifungal effects (KELLER & JACOBER, unpubl.).

Tab. 1: Comparison of the standard method (HOKKANEN & KOTILUOTO, 1992) with the proposed method

Standard method	Proposed method
Laboratory level	
Tests on agar with pesticides growth production of conidia viability virulence (bioassay)	Tests on agar with pesticides growth production of conidia viability virulence (bioassay)
semi-field level	
Mix conidia with standard soil Apply pesticide Incubate Determine viable spores per unit of soil	Mix pesticide to standard soil Add fungus kernels Bioassay
field level	
Like semifield tests but in natural soil (field)	Place fungus kernels in soil of treated fields Bioassay
Remarks	
Bioassay only at the laboratory level. Semi-field and field tests base only on the viability of spores and neglect their virulence (host-pathogen interaction) which, however, should be the aim of such tests.	Semi-field and field tests base on bioassays. Homogenisation with a given volume of natural soil followed by a modified *Galleria* bait method (ZIMMERMANN, 1986) is proposed.

c. Proposed procedure for Entomophthorales

Entomophthorales are typical and common pathogens of epigaeic pest insects. One of the most common species is the aphid pathogen *Erynia neoaphidis*, with which it is proposed therefore to test side effects of pesticides applied on plants.

The proposed laboratory procedure consists of two tests: The first is a study of the influence of the pesticide on the sporulation capacity as described by KELLER & SCHWEIZER (1991). Dead aphids infected with *E. neoaphidis* which is not yet sporulating are treated with pesticides. Subsequently sporulation is initiated and the number of conidia projected per unit of time is determined. The second test is to estimate the virulence of the conidia produced under the influence of pesticides. This test is yet to be defined but can be adopted from previously described methods (e.g. BELLINI et al., 1992; OGER & LATTEUR, 1985; PAPIEROK, 1982).

A semi-field test for fungicides and herbicides may consist of aphids on potted plants. Plants or leaves with a single infected aphid attached by rhizoids and the fungus not yet sporulating are treated with a pesticide at the recommended rate. Subsequently young, healthy aphids are added and the plants are placed in a humid chamber to initiate sporulation. Infection rates among the added aphids are determined during 5 days at 20-22°C. This test may replace the second laboratory test.

For a field test I propose the following procedure: Plots in fields with naturally occurring epizootics are treated with pesticides. Samples of living aphids are taken 1-2 days and 7-10 days after treatment in treated and untreated plots. The aphids are reared in the laboratory for 4 days at 20-22°C and the infection rates are compared.

3. Discussion

Methods to test the side effects of pesticides on entomopathogenic fungi, which should become part of the registration process, must be accepted and followed by the plant protection industry. They therefore should be simple but nevertheless allow a reliable judgement of the effects on the "beneficial capacity" of these fungi. The proposed tests can fulfill these requirements.

Two methods are proposed following the principle that the side effects should be examined on those fungi exposed to the highest risk to be affected by the pesticides, e.g. (1) *B. bassiana* or *M. anisopliae* for pesticides applied on or into the soil, and (2) *E. neoaphidis* for pesticides applied on plants.

Two new elements are included in the first method, the fungus-kernels and the bait method with a suitable host insect. These two elements simplify the test procedure and allow an estimation of the virulence of the fungus grown and sporulating under the influence of the pesticide. They are easier to use than the isolation of the fungus from the soil, which allows only an estimation of the viability of the spores or of the fungal material. However, this method needs to be evaluated further before its adoption can be discussed.

The method using Entomophthorales as test organisms is a new one although most elements have been used in other test procedures. Therefore it must first be carefully tested and adapted where necessary.

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TREHALASES PRODUCED BY THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* AND THEIR POTENTIAL ROLE IN PARASITISM

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Summary
Metarhizium anisopliae produces in culture an exocellular and an endocellular trehalase which have somewhat different properties. Both enzymes have been purified to homogeneity. Trehalose is the major blood sugar in insects and therefore fungal trehalases may play an important part in pathogenesis. Competition between fungal parasite and insect host for trehalose may be particularly important in locust-*Metarhizium* interactions. Flight activity of desert locusts mycosed with *Metarhizium flavoviride* is reduced with respect to controls. This is in part at least due to reduced blood trehalose levels in mycosed insects since an injected trehalose supplement boosted flight performance of infected insects.

1. Introduction

The study of mechanisms of fungal pathogenesis in insects is still in its infancy. We know something of how entomopathogenic fungi invade their hosts using cuticle-degrading enzymes (see Charnley and St Leger, 1990). We also know a little about how eg *Metarhizium anisopliae* overcomes host defences with the aid of cyclic peptide toxins, the destruxins (Huxham *et al.*, 1989). However, we know precious little about what happens in between, namely what impact a fungus has on its host during the development of disease. The present work is aimed at shedding light on how *Metarhizium* spp. derive nutrients from their host during early stages of infection when the fungus is restricted to the blood. Since trehalose is the major blood sugar in most insects including the desert locust, *Schistocerca gregaria*, we started by looking for fungal enzymes that degrade trehalose. In addition we looked at the effect of mycosis on flight capability of desert locusts. Since these insects are known to use trehalose for fuel at the onset of flight, competition for trehalose between insect and fungus may impede flight.

2. Materials and Methods

Metarhizium anisopliae (isolate ME1) and *M. flavoviride* (isolate 330189) were maintained on 1/4 strength Sabouraud's dextrose agar and Molisch's agar respectively. Trehalases of *M. anisopliae* were purified from liquid cultures grown on Sabouraud's dextrose broth or 1% bacteriological peptone, 1% trehalose and basal salts, using either a sepharose-Q anion exchange column or

preparative isoelectric focussing (Rotofor). Trehalase activity was determined in terms of glucose released from trehalose using a glucose oxidase test kit (Sigma). The effect of mycosis on flight was determined using equal numbers of male and female adult desert locusts, *Schistocerca gregaria*, at 20-23d post fledging. They were inoculated under the pronotum with 2μl of cotton seed oil containing 4 x 10^7 ml^{-1} conidia of M. *flavovoride*. Controls received cotton seed oil only. Experimental insects were maintained at 30% RH and 30°C. Flight capability of experimental insects was determined in a wind tunnel where insects were suspended by a wire loop around the neck facing an air stream of 4m/sec. Insects were recorded as flying or nonflying at intervals during a 5h flight period and awarded a score, which was the number of positive (flying) observations expressed as a % of the maximum possible. In an experiment to determine the effect of a carbohydrate supplement on flight capability, 3d after inoculation, insects were flown for 1 h in the wind tunnel. The insects were then injected with 25μl of either saline (Maddrell and Klunsuwan, 1973) or saline containing 6mg trehalose, and then flown for a further 1h in the wind tunnel. A cam corder was used to film the insects and the amount of time spent in flight by each insect, before and after the injections, was determined from the video. Blood samples (5μl) were taken from a hole made in the arthrodial membrane at the base of a hind leg. A crystal of phenylthiourea was added to prevent phenoloxidase activity, then the sample was centrifuged to remove blood cells and fungal hyphae. The sugar concentration in these supernatants was determined using the anthrone method (Roe, 1955).

3. Results and Discussion

M. anisopliae (ME1) produced trehalase in culture. Assays performed on culture filtrates and mycelial homogenates revealled that there were two types; an exocellular form which was secreted into the medium and an endocellular form which was cell bound. Both enzymes were purified to homogeneity and the properties of the enzymes are given in table 1.

Often the first overt symptom of mycosis is reduced feeding. With locusts infected with *M. flavoviride* the difference in food consumption between controls and experimentals becomes significant 3 days after infection (Seyoum, Moore and Charnley, unpubl). This is just 24h after the fungus breaches the integument. One might expect that the insect at this time would enter a state of semi starvation as its food consumption declines; doubly so because the fungus begins to take its share of the insect's resources. 3d of starvation does not affect blood sugar concentration, but over a similar time period mycosis causes a significant drop in blood sugar levels (Seyoum, Moore and Charnley, upubl). Trehalose constitutes >90% of blood sugar. Given the importance of trehalose as a metabolic fuel at the onset of flight (Robinson and Goldsworthy, 1976), it seemed possible that reduced blood sugar might interfere with flight capability of mycosed locusts. Certainly mycosed insects showed reduced flight capability in the

wind tunnel (Fig 1). The difference between mycosed insects and controls was significant 3 days after inoculation.

An injected supplement of trehalose significantly increased the flight time of mycosed insects in comparison with mycosed insects injected with saline alone (Fig. 2). Neither saline nor trehalose injections affected the flight performance of control insects. Therefore reduced trehalose reserves in the locust may at least contribute to the poor flight performance of the mycosed insects.

Table 1; Properties of exocellular and endocellular trehalases produced by *Metarhizium anisopliae*

	exocellular	endocellular
Molecular weight,kDa	30	80
pI	4.35	4.23
pH optimum	6.5	6.5
Km, mM	10.5	10.5
Inhibitors (% inhibition):		
2mM ascorbic acid	60%	66%
2mM silver nitrate	96%	90%
2mM lead acetate	15%	9%
0.05mM validoxylamine	100%	98%
0.05mM trehazoline	98%	94%

Fig. 1; Flight performance of locusts infected with *Metarhizium flavoviride*

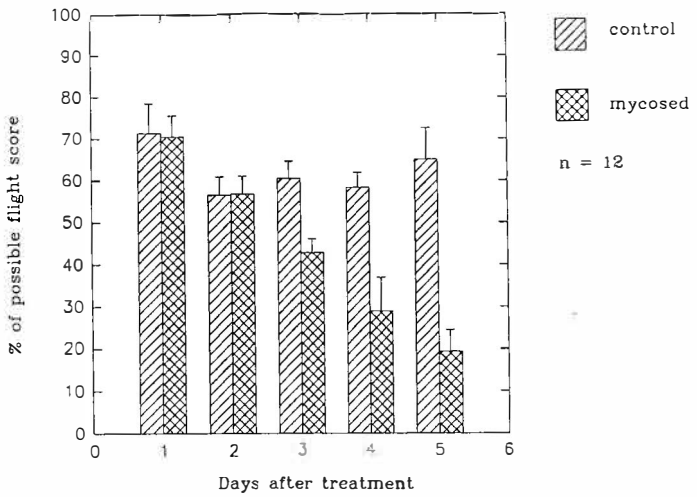
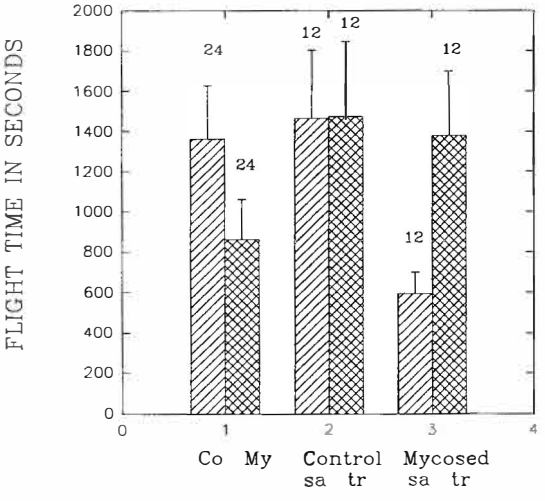


Fig. 2; Flight performance of locusts 3d after inoculation with *Metarhizium flavoviride*

1 = 1st flight (1h)
2,3 = 2nd flight (1h)
Co = control
My = mycosed
sa = saline injected
tr = trehalose injected



4. Conclusions

M. anisopliae produces trehalase enzymes in culture. These enzymes may help the fungus acquire nutrition from the blood of its host. Certainly the blood sugar concentration in locusts infected with the related fungus *M. flavoviride* is significantly reduced. In addition infected locusts show reduced flight capability which can be significantly improved by injection with trehalose.

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Utilization of entomogenous fungus *Paecilomyces fumosoroseus* against sweetpotato whitefly, *Bemisia tabaci*.

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SUMMARY

Entomogenous fungus ***Paecilomyces fumosoroseus*** was used as a main component of an IPM program against sweetpotato whitefly ***Bemisia tabaci*** on Poinsettia and Hibiscus grown in a commercial greenhouse. An identical structure of IPM program was used for both host plants. The first part of IPM program was focused on cuttings and young plants being grown in propagation area. The second part of an IPM program was designed to protect both host plants after displacement from a propagation area into the production greenhouse. The number of ***B. tabaci*** adults on plants have been assessed weekly within the whole period of experiment and additional treatments with ***P. fumosoroseus*** were used when the mean number of adults exceeded an average of one adult of ***B. tabaci*** per plant. The control groups of plants were grown under the same conditions, but the standard insecticidal program was used to protect those plants against sweetpotato whitefly. The final quality of plants being protected with IPM program based on ***P. fumosoroseus*** was fully comparable, with the quality of plants being protected with insecticides only. However, to suppress the population density of ***B. tabaci*** equally, 18 treatments with various insecticides were needed to protect Poinsettia and 21 insecticidal treatments were needed to protect Hibiscus, during the same growing period.

Introduction

Sweetpotato whitefly ***Bemisia tabaci*** (Homoptera; Aleyrodidae) is considered a major pest of field and greenhouse crops worldwide, including over 500 ornamental host plants (Greathead 1986). Chemical control of ***B. tabaci*** by conventional chemical means has became more difficult in recent years. This is forcing researchers to look for alternative methods, particularly for means of biological control. In addition to predators and parasitoids, several species of deuteromycetes are known to be associated with whiteflies, including **B. tabaci** (Osborne et al. 1990; Osborne, Landa 1992). Until now, ***Aschersonia aleyrodis*** and ***Verticillium lecanii*** are the most frequently studied fungal pathogens of whiteflies (Fransen 1990; Hall 1985; Landa 1984). Recently, an isolate of ***Paecilomyces fumosoroseus*** (Wize) Brown & Smith, highly virulent to the sweetpotato whitefly and a broad range of other pests has been isolated in Florida (Osborne 1990). This strain (***PFR 97*** - Apopka, Florida, ATCC 20874) is currently being evaluated in the laboratory and under greenhouse conditions. The fungal biopreparation based on ***PFR 97*** has been developed by **W.R.Grace & Co.- Conn.,USA**, which is currently tested for utilization in biological control against ***B. tabaci***. This study presents recent data being obtained, when ***PFR 97*** was used as a main component of an IPM program for the protection of poinsettia (*Euphorbia*

pulcherrima) and hibiscus (*Hibiscus rosa-sinensis)* in a commercial greenhouse.

Materials and Methods

All experiments were conducted in a commercial greenhouse (Nelson & Sons, Ltd., Apopka, Florida) with full respect to current growing technology. Two hundred-fifty cuttings of poinsettia have been obtained from stock plants, stuck into pots (1 cutting per 1 pot) and kept in misting propagation area. When rooted (28 days), pots were displaced on tables in production greenhouse where plants were grown for the rest of the growing cycle (additional 72 days). Similarly, two thousands cuttings were cut out from shoots of stock hibiscus plants and then stuck into pots (8 cuttings per 1 pot) and placed in the same propagation area for 44 days. When rooted, pots with plants were displaced on tables in the production greenhouse for the rest of the growing cycle (an additional 102 days).

P. fumosoroseus has been applied as a suspension of conidia. The conidia were obtained from PFR-alginate pellets after activated under controlled conditions (Osborne et al., - in press). Dry alginate pellets with incorporated biomass of ***PFR 97*** were dispersed over a wet bottom of a plastic box forming a single continuous layer, moistened with a sterile water (100 ml of water per 100 g of prills) and kept in a growing chamber (25°C, 24 hrs light). The fungus grew over an entire surface of prills and fully sporulated within 7 days. The conidia were harvested by soaking of activated prills in to 0.05% Tween 80. The concentration of conidia was determined using an Improved Neubauer hemocytometer and properly adjusted to the final concentration of **1.0×10^7 conidia per 1 ml** before application. The same concentration of conidial suspension was used for all treatments (e.g., dipping of cuttings, drenching of pots, foliar treatments).

Two groups (each consisting of 250 pots) have been established for both host plants using an identical growing system as briefly described above. However, two different programs were used to protect the plants against the sweetpotato whitefly within a whole growing cycle. One group of plants has been protected based on a **conventional chemical program** (1-2 routine application per week, rotation of insecticides e.g. Azatin, Nicotine Sulphate, Orthene, Plant Fume, Tame, Thiodan). A second group of plants has been protected using an **IPM program** based on ***P. fumosoroseus***, the structure of which was as follows:

Part 1 of IPM - Mandatory preventative protection of cuttings in propagation area
- dipping of cuttings into conidial suspension of ***PFR 97*** before stuck in pots
- drenching of pots with conidial suspension of ***PFR 97*** (100 ml per 1 pot)

Part 2 of IPM - Protection of plants in production greenhouse
A. Mandatory foliar treatments with PFR 97
- 1st. treatment after plants displaced from propagation area
- 2nd. treatment 5 to 7 days after first one
- 3rd. treatment 5 to 7 days after second one

B. Additional treatments with PFR 97 and other activities
- scouting for ***B. tabaci*** adults weekly to determine mean number of adults per 1 plant
- an additional treatment(s) with ***PFR 97*** if threshold level - **1 adult per plant** was achieved

The principal evaluation of all treatments (Hibiscus and/or Poinsettia, Chemical control vers. IPM) was based on determination of 1) alive, and 2) PFR-infected adults of ***B. tabaci*** on the plants. Twenty-five plants (10 %) have been assessed weekly, and the total amount of both categories of adults has been recorded. Furthermore, all immature stages of ***B. tabaci*** have been

Table 1. Final status of all immature stages of *B. tabaci* on Hibiscus before displacement in to production area (Mean ± STDV number of immature stages per 1 leaf).

Assessed category	IPM program with PFR 97	Chemical control
alive imunatures	0.38 ± 0.236	0.40 ± 0.143
dead immatures	1.35 ± 0.422	0.78 ± 0.255
PFR infected immatures	2.10 ± 0.672	0.03 ± 0.012
Emerged scales	0.85 ± 0.232	0.90 ± 0.378

Table 2. Final status of all immature stages of *B.tabaci* on Hibiscus at the end of experiment (Mean ± STDV number of immature stages per 1 leaf)

Assessed category	IPM program with PFR 97	Chemical control
alive immatures	0.32 ± 0.236	0.10 ± 0.132
dead immatures	5.35 ± 0.422	0.88 ± 0.116
PFR infected immatures	0.98 ± 0.672	0.06 ± 0.043
Emerged scales	0.76 ± 0.232	0.26 ± 0.241

assessed on both groups of Hibiscus (chemical control vers. IPM) when plants were moved into the production greenhouse (the end of Part A of IPM) and at the end of experiment.

Results and Discussion

Poinsettia

The adults of sweetpotato whitefly were recorded on plants in both treatments within the whole period of experiment (Figure 1). Mean number of alive adults on plants being protected with insecticides only did not exceed the average of 0.45 adults per plant. However, a total of eighteen insecticidal treatments has been needed to suppress the population of whitefly on such a low level (6 treatments on plants in propagation area - 28 days, and 12 treatments on plants in production greenhouse - 72 days). The records on presence of alive adults of sweetpotato whitefly on plants which were protected with an IPM demonstrate that fully sufficient control of pest has also been ensured. The highest amount of adults was recorded after plants where displaced from the propagation area into the production greenhouse (max. average of 0.96 adults per plant). However, three mandatory foliar treatments with ***PFR 97*** did induce a significant decrease of adults and at the end of experiment the pest had been almost eradicated (0.08 adults per 1 plant), when only one additional treatment with ***PFR 97*** was needed. The occurrence and presence of PFR-infected adults fully correlated with dates of treatments (Figure 1).

Hibiscus

The total of 22 insecticidal treatments ensured almost an eradication of sweetpotato whitefly on hibiscuses which were protected with insecticides only (8 treatments on plants in propagation area - 44 days, 14 treatments on plants in production greenhouse - 102 days). The mean number of alive adults of ***B. tabaci*** did not exceed an average of 0.08 adults per plant and at the end of experiment the pest was eradicated totally (Figure 2). The presence of alive adults on plants being protected with an IPM was also strongly reduced within the whole period of experiment (146 days). Three mandatory treatments with ***PFR 97*** (production greenhouse) caused a significant decrease of alive adults on plants, followed by an increase of PFR-infected adults. The mean number of alive adults did not exceed an average of 0.45 adults per plant. Beside three mandatory treatments, only one additional treatment with ***PFR 97*** was done on week 8, after scouting showed an increase of alive adults. This additional treatment was immediately followed by an increased presence of PFR-infected adults and there were no alive adults recorded until the end of the experiment (Figure 2). The final status of all immature stages was assessed at the end of propagation and when growing cycle was completed. The data indicates that both, either an intensive chemical control program or IPM based on ***PFR 97*** resulted in to strong suppression of all immature stages of ***B. tabaci***. The number of alive immatures was significantly lower than the number or PFR-infected. Furthermore, a few immatures only completed development up to the adult stage (see Tables 1 & 2).

The final market quality of all poinsettia and hibiscus plants has been assessed as very good when evaluated at the end of experiment in both programs. However, the IPM based on ***PFR 97*** ensured comparable and fully sufficient control against sweetpotato whitefly with significantly less intensity of treatments and insecticides were totally excluded.

Figure 1. Mean number of B. tabaci adults on Poinsettia in production greenhouse.

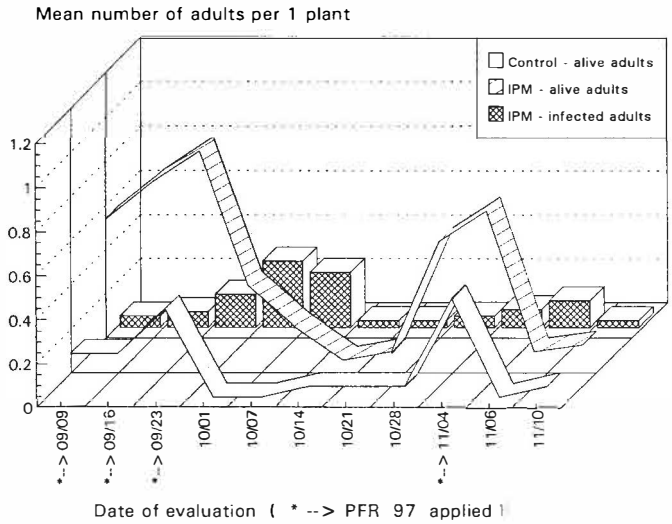
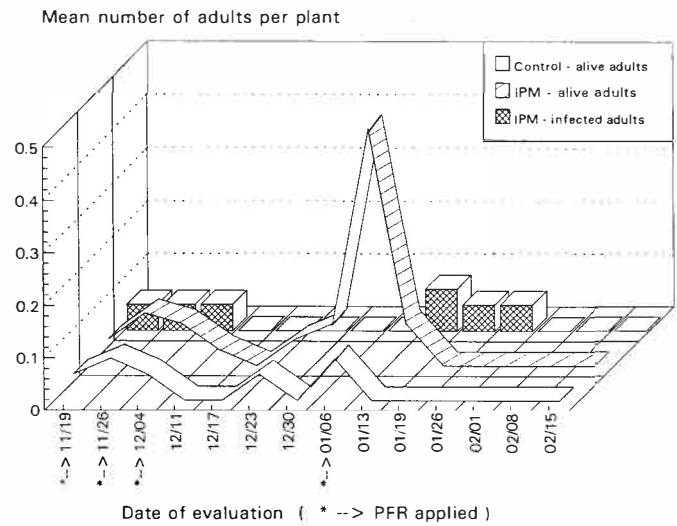


Figure 2. Mean number of B. tabaci adults on Hibiscus in production greenhouse



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SUBGROUP "FUNGI"

Report of the general discussion, Wednesday 8th September

by Bernard PAPIEROK, Convenor of the Subgroup

The discussion was just following the presentation by Siegfried KELLER, of his contribution on side effects of pesticides on entomo- pathogenic fungi. This topic was therefore discussed at first. Then B. PAPIEROK gave a short overview of the meeting and after that, tackled several supplementary points.

Entomopathogenic fungi and the testing of side effects of pesticides on beneficial organisms

The purpose of the communication by S. KELLER was to attract the attention of the audience on the fact that only soil entomopathogenic fungi, like *Beauveria bassiana* or *Metarhizium anisopliae*, are until now taken into account in the proposed methods to test side effects of pesticides on beneficial organisms. Entomophthorales, which are epigaeic fungi, were simply ignored, although they are known to play a significant role as natural enemies of important pests in our regions, as aphids for instance. These naturally occurring fungi and pesticides are likely to come into contact and therefore, Entomophthorales should be used to test side effects of pesticides. On the other hand, methods should also be designed to study the effects of a pesticide on the host-pathogen interaction, which implies bioassays.

Given these considerations, S. KELLER proposed two procedures for testing side effects of pesticide on entomopathogenic fungi, one for the soil fungi *(M. anisopliae* and *B. bassiana),* and one for the Entomophthorale *Erynia neoaphidis*, one of the most common species of this group. Tests were designed at the laboratory, semi-field and field levels.

After a wide exchange of views, devoted mainly in the beginning to the "conceptual" aspects of the question (ecological concerns of scientists working on entomopathogenic fungi, interweavings with the policy of IOBC...), the audience agreed to stress the necessity to consider Entomophthorales when testing side effects of pesticides, and to inform the IOBC/WPRS Working Group "Pesticides and beneficial organisms" of its position. Furthermore, S. KELLER undertook to send for comments, remarks, and approval, the text of his communication to the members of the Subgroup.

What about the present meeting?

The Subgroup convenor pointed out the diversity of the contributions devoted to entomopathogenic fungi during these two days. Oral communications and posters covered a very wide field of research (natural occurrence, production and formulation, applications, basic studies of the mode of action of toxins, biochemistry and molecular genetics...). Obviously the "human" size of the Working Group, and a fortiori of the Subgroup, allowed the people to develop friendly and fruitful exchanges. Moreover, and interestingly enough, entomopathogenic fungi were shown a new or an increased interest in Southern and Central Europa (Spain, Greece, Austria). Concerning the Working Group on the whole, the confrontation of the experience of entomomycologists and entomonematologists in the field of mechanisms of resistance and insect immunity should appear promising.

Then B. PAPIEROK observed that, this time, there were no contribution by people from Rothamsted (Great Britain) and from INRA (France). Marc ROUGIER (INRA, La Minière) was then asked to give a general survey of the work recently done by Jacques FARGUES and his colleagues on the analytical study of effects of climatic factors on the survival of fungal propagules. Furthermore, he informed the audience of the move of this team to Montpellier.

Databases on entomopathogenic fungi

Israel BEN-ZE'EV published in 1993 the check-list of fungi found on insects or mites in Israel, updated through 1992 (*in Phytoparasitica*, **21**, 213-237). Reprints are very likely still available. Compilations of this type should be set up for other countries. Such a work is, of course, an uneasy venture for places where entomopathogenic fungi were collected by numerous scientists for a long time. On the other hand, in the case of countries like Spain or Greece for instance, where these microorganisms were studied for a short while, authors are advised to establish check-lists from the very beginning.

Forest insects

Until now, fungal pathogens of forest insects appeared to be rather poorly taken into account within the Subgroup. The participation of Rudolf WEGENSTEINER (Vienna, Austria) to the meeting had therefore to be underlined, all the more so because outbreaks of *Lymantria dispar* are expected in Central Europa for next years. An intensification of the collaboration between scientists of the concerned countries for the search of pathogens, would be appropriate. As regards the fungi, especially Entomophthorales, one should keep in mind the situation observed since a few years in North America.

Formulation of fungal preparations to be used in biological control

The possibility of applying the oil formulation suitable for ULV applications developed by the IIBC/IITA/DFPV collaborative research programme in the case of *Metarhizium flavoviride* for locusts, to fungi in other situations was discussed. The feasibility should be studied in each case, given the fungus, the pest and the crop involved. R.P. BATEMAN stated that the Programme would willingly consider any prospect of collaboration.

New methods for characterization of fungal species and strains

New methods for characterization of entomopathogenic fungi, involving for instance study of RNA sequences, analysis of allelic variations of allozyme coding genes, random amplified polymorphic DNA fingerprintings, were already tested. However there is much need for in-depth studies in this field. I.S. BEN-ZE'EV gave the example of his current work on the identification of isolates of *Conidiobolus* spp. (Entomophthorales) by a computer analysis of fatty acids profiles.

Workshop on Entomophthorales

Following the meeting, a workshop on Entomophthorales was organized by S. KELLER. 21 participants attended first the collection tour in Katzensee, around the lake, in the vicinity of Zürich and then, the practical work in the ETH-Institute of Entomology. The weather looked favourable to fungi, and indeed, Entomophthorales were found infecting various insects. Even a Deuteromycete of the genus *Hirsutella* was observed on an Hymenoptera. The success of the collection tour would

have not been complete without a female of the tick *Ixodes ricinus* caught by B. PAPIEROK. Maybe it should be more appropriate to say that this arthropod, which is known at this stage to feed mostly on mammals like stags or boars, caught him!

Practical work in the laboratory included preparation of the material, isolation and identification. A very useful manual was kindly provided by the organizer.

6. Posters on entomopathogenic fungi.

Purification and partial characterisation of a fungal protease inhibitor in the haemolymph of the tobacco hornworm, *Manduca sexta*

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Summary

We have purified and partially characterised a protease inhibitor from the haemolymph of the tobacco hornworm *Manduca sexta*. It has a molecular weight 18.2 kDa. and a pI of 4.5. It inhibited the activity of several serine proteases against synthetic substrates. Most notably it inhibited the chymoelastase enzyme, Pr1, produced by the entomopathogenic fungus *Metarhizium anisopliae* and the moulting fluid protease, MFP1, isolated from *M. sexta*, by 100 %. Furthermore, the hydrolysis of [^{3}H]-cuticle by Pr1 was halted by preincubation with the inhibitor. These results suggest possible roles for the inhibitor in regulating cuticle degradation at the moult and contributing to host defence against pathogenic fungi.

1. Introduction

Protease inhibitors have been implicated in the control of activity of proteolytic enzymes from a wide variety of sources, including insects (Travis and Salveson, 1983) and protease inhibitors specific for trypsin and/or chymotrypsin have been found in the blood and/or cuticle of a number of insects (see Eguchi, 1993). Particular interest has focussed on the role of these inhibitors in the control of phenoloxidase activity. But the inhibitors may also help regulate cuticle-degradation at the moult and contribute to host defence against fungal parasites. The last named, such as *Metarhizium anisopliae*, invade their hosts through the exoskeleton with the aid of enzymes. The cuticle-degrading protease Pr1, a chymoelastase, has a major role in this process.

Eguchi *et al*. (1993) have purified an inhibitor from the haemolymph of the silkworm, *Bombyx mori*. This inhibitor has a high affinity for fungal proteases produced by *Aspergillus melleus* and *Beauveria bassiana*, the latter being particularly pathogenic to *B. mori*. Furthermore, the inhibitor was shown to suppress the germination of conidia and germ tube development of *B. bassiana*.

Protease inhibitors have been previously purified from *M. sexta* larvae. Ramesh *et al*. (1988) found two Kunitz-type trypsin inhibitors with molecular weights of 14 and 8 kDa whilst Kanost (1990) found four high molecular weight serine protease inhibitors (serpins) of 46-48 kDa. However, the activity of these inhibitors

against fungal proteases was not tested. We have isolated and partially characterised a protease inhibitor from the blood of the tobacco hornworm, *M. sexta*. This inhibitor is distinct from those described by Ramesh et al. (1988) and Kanost (1990) and it has particular activity against Pr1 from *M. anisopliae*.

2. Materials and Methods

Larvae of *M. sexta* were reared on artificial diet (Samuels *et al.*,1993) and maintained at 25^{o}C with a photoperiod of 18 h light and 6 h dark. Fifth instar larvae were used for all experiments. Larvae were anaesthetised on ice and the haemolymph was collected by severing the rear horn and bleeding into 2 ml ice cold 20 mM Tris containing 0.05% phenythiourea (to prevent coagulation and melanisation respectively). The blood was centrifuged to remove haemocytes (13000rpm, 5 min, 4^{o}C) and the supernatant was mixed with an equal volume of carbon tetrachloride. This mixture was centrifuged (8000 rpm, 10 min, 4^{o}C) and the supernatant mixed with 30% (v/v) ammonium sulphate and left on ice for 1 h. The suspension was centrifuged (8000 rpm, 20 min, 4^{o}C) and the supernatant dialysed against distilled water overnight. The dialysate was loaded onto a Q-sepharose column. Fractions showing inhibitory activity were pooled and dialysed against distilled water overnight and concentrated. The sample was applied to a 300-C_{18}-dynamax reversed phase column on an high performance liquid chromatography system and eluted with a linear gradient of 0-60% acetonitrile.

Protease inhibitory activity was determined spectrophotometrically in a solution containing the synthetic substrate Succ-ala-ala-pro-phe-*p*-nitroaniline. Ten μl of protease (0.1 mg/ml) was incubated for 30 min at room temperature with 100 ul of test sample. The reaction mixture was made up to 950 ul with 0.1 M Tris-HCl buffer (pH 8.0) and the reaction was initiated by the addition of 50 μl 1 mM substrate. The absorbance of the mixture at 410 nm after 30 secs was recorded. The percentage inhibitory activity was calculated from the expression % = $(1-A_I/A_0)$ x 100, where A_I and A_0 represent the absorbances in the presence and absence of inhibitor respectively.

3. Results and Discussion

Preliminary experiments showed that the protease inhibitory activity was in the plasma but not in the blood cells. Using the protocol described in the materials and methods section the inhibitor was purified to homogeneity as demonstrated on SDS-polyacrylamide electrophoresis. A 12 % yield of the protein was obtained with a purification of 102-fold. The inhibitor has a molecular weight of 18.2 kDa and a pI of 4.5. These values are distinct from the Kunitz-type inhibitors (14 and 8 kDa molecular weight; 5.7 and 7.1 pI respectively) (Ramesh *et al*, 1988) and the serpins (46-48 kDa molecular weight; 4.4-4.8 pI) (Kanost, 1980).

Partially pure protease inhibitor was tested against a variety of enzymes (see Table 1). It inhibited chymotrypsin and trypsin by 84.5 and 67.3 % respectively. Interestingly, there was 100 % inhibition of Pr1 and a recently purified moulting fluid protease (MFP-1; Samuels *et al.*,1993). This indicates that the inhibitor may have a dual function in regulating hydrolysis of cuticle during the moulting process as well as contributing to insect defence reactions against pathogenic fungi. There was no inhibitory activity towards the trypsin-like protease, Pr2, also produced by *M. anisopliae*. This enzyme has little cuticle-degrading activity and its function in pathogenesisis yet to be determined. When the protease inhibitor was pre-incubated with Pr1 for 30 min and then added to a [^{3}H]-cuticle extract, then hydrolysis of the cuticle was prevented; there was a 100 % inhibition of enzyme activity (see Table 2). The mechanism of inhibition is not known and further investigations into the properties and function of this protein are underway in our laboratory.

4. Acknowledgements

This work was supported by a CASE-studentship awarded to J. Gillespie by SERC and the International Institute of Biological Control.

Table 1; The effect of *M. sexta* protease inhibitor on the activity of a variety of proteases

Protease[a]	Synthetic substrate[b]	Percentage inhibition[c]
Chymotrypsin	Succinyl-Ala-Ala-Pro-Phe-pNA	84.5
Pr1	Succinyl-Ala-Ala-Pro-Phe-pNA	100
Proteinase K	Succinyl-Ala-Ala-Pro-Phe-pNA	0
Trypsin	Benzoyl-Phe-Val-Arg-pNA	67.3
MFP-1[d]	Benzoyl-Phe-Val-Arg-pNA	100
Pr2	Benzoyl-Phe-Val-Arg-pNA	0
Subtilisin	Benzooxycarbonyl-Ala-Ala-Leu-pNA	0
Papain	Benzoyl-Arg-pNA	0

[a] Each enzyme was dissolved in 0.1 M Tris-HCl buffer (pH 8.0) at a concentration of 0.1 mg/ml

[b] Each substrate used was at 1 mM concentration

[c] Values given are the mean of 4 replicates. Standard error was less than 10%

[d] Moulting fluid protease (Samuels *et al.*,1993)

Table 2; The effect of *M. sexta* protease inhibitor on the proteolytic activity of the fungal protease, Pr1, on [^{3}H]-cuticle.

Sample	DPM[a]	Percentage inhibition
Buffer	168.5	N.A.
Buffer + Inhibitor	169.4	N.A.
Buffer + Pr1	1157.7	0
Buffer + Pr1 + inhibitor	135.7	100

[a] Disintigrations per minute; Values given are the mean of 7 replicates.Labelled cuticle was washed with buffer for 1 h prior to use. The inhibitor and Pr1 were preincubated together for 30 min. The treatments were mixed on a rotator for 5 h before being centrifuged. 100 µl of supernatant was removed and assayed for radioactivity.

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ELECTRON MICROSCOPE INVESTIGATIONS ON THE ULTRASTRUCTURE OF BLASTOSPORES AND CONIDIA OF *METARHIZIUM ANISOPLIAE*

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Summary

The ultrastructure of blastospores and conidia of the entomopathogenic fungus *Metarhizium anisopliae* was studied and compared. Conidia are growing on the surface of the host and on solid growth media, while blastospores are generated in the insect or in liquid media. Contrary to conidia, blastospores are in an active physiological state and thus are able to germinate after 2-3 hours, while conidia need 12-24 hours to initiate this process. Blastospores contain numerous mitochondria, plenty of ribosomes and much endoplasmic reticulum, while these organelles are only scarcely present in mature conidia. In blastospores many fat-vacuoles and oil globules are formed, often beeing associated with primitive dictyosomes; in mature conidia two big fat-vacuoles can be observed at the spore poles. The plasmalemma of blastospores is closely adjacent to the inner spore wall, often displaying two ore more layers as well as lomasomes. The wall of blastospores is covered by a thick granular mucous layer which increases their resistance to high temperatures and low relative humidities. Contrary to conidia, the accumulation of various organelles in blastospores reflects the metabolic activity of this spore-type.

Introduction

In the frame of the project "Biological-Integrated Control of Locusts", funded by the Federal Ministry for Economic Cooperation (BMZ) and coordinated by the German Agency for Technical Cooperation (GTZ), the ultrastructure of blastospores and conidia of the entomopathogenic fungus *Metarhizium anisopliae* was studied and compared. Although Zacharuk (1970 a, b, 1973) investigated the ultrastructure of conidia of *M. anisopliae*, so far there are no comparable studies on blastospores of this species. Attention was paid to differences in the ultrastructure of blastospores and conidia which may contribute to a better understanding of their physiology and behaviour to environmental conditions, such as period of germination, high temperatures, low relative humidities or UV-light.

Materials and methods

Blastospores were produced in Erlenmeyer-flasks using a liquid medium after Adámek (1963). They have been grown for 72 hours at 25°C on a rotary shaker (180 rpm). Conidia were grown on malt extract agar (malt extract 3%; mycological peptone 0,5%; agar (technical grade) 1,5%) for 10-14 days at 25°C.

Blastospores and conidia of three strains of *M. anisopliae* have been fixed with various concentrations of glutaraldehyde and osmiumtetroxyde. Embedding was done in Epon or Methacrylate. Informations about methods for preparation were taken from Hayat (1970) and Plattner & Zingsheim (1987).

Ultrathin sections were cut with an LKB-Ultrotome III and studied with a Zeiss-902 electron microscope.

Results

The wall of conidia consists of a primary spore wall (550-650 Å), a secondary spore wall (500-600 Å), and an outer thin spore wall membrane (50-150 Å). The plasmalemma (30-50 Å) is closely adjacent to the inner side of the wall and sometimes invaginated and folded in young conidia but not in older ones. One nucleus is usually located in the middle of the spore. Fat-vacuoles and oil globules are numerous in young conidia, often being associated with primitive dictyosomes. In mature conidia, two big fat-vacuols can be observed at the spore poles. Only a few mitochondria, mostly two, are present in the vicinity of the nucleus. Endoplasmic reticulum and ribosomes are scarcely found.

Contrary to conidia, blastospores are in an active physiological state, and thus are able to germinate after 2-3 hours, while conidia need 12-24 hours to initiate this process. Blastospores have a thick primary (850-950 Å) and a thin secondary spore wall (100-150 Å). Instead of the spore wall membrane surrounding conidia, the blastospores are covered by a granular mucous substance. The plasmalemma, closely adjacent to the inner spore wall, often displays two or more layers as well as numerous digitate folds, membrane whorles and/or lomasomes. Mostly the nucleus is also positioned in the middle of the blastospore. Many fat-vacuoles and oil globules are formed, often being associated with primitive dictyosomes. Numerous mitochondria are distributed all over the endoplasm. Plenty of ribosomes and much endoplasmic reticulum enable the blastospores to germinate and multiply within a short time.

Discussion

The differences in the cell wall of blastospores and conidia obviously depend on their mode of growing. Conidia, growing on the surface of hosts or media, develop a thick, folded, electron dense secondary spore wall that protects from desiccation. In contrast, the secondary spore wall of blastospores growing in liquid medium is much thinner. Apparently the granular mucous substance covering the blastospores is responsible for their higher resistance to extreme temperatures in connection with low relative humidity as well as to UV-light, as compared to conidia.

The numerous digitate folds and membrane whorles of the plasmalemma of blastospores indicate increased metabolism. The vesicular and lamellar structures of the plasmalemma, known as lomasomes (Moore & McAlear, 1961) or paramural bodies (Marchant & Robards, 1968), contribute to cell-wall formation. Membranous structures appearing as primitive dictyosomes and beeing associated with fat-inclusions very likely enables blastospores and germinating conidia to store or mobilize reserve substances. The high number of dictyosomes and other organelles such as mitochondria, ribosomes and endoplasmic reticulum in blastospores certainly account for the short time they need for germination (2-3 h) in contrast to conidia (12-24 h).

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THE EFFECT OF DESTRUXINS ON THE STRUCTURE AND FUNCTION OF INSECT MALPIGHIAN TUBULES

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Summary
Destruxins are cyclic peptide lactone toxins, isolated from the entomopathogenic fungus *Metarhizium anisopliae*. Destruxin A inhibits fluid secretion *in vitro* by Malpighian tubules of the desert locust *Schistocerca gregaria*. A sublethal dose of destruxin A also causes a significant decrease in the ability of Malpighian tubules of adult blowflies, *Calliphora vomitoria*, to excrete amaranth dye *in vivo*. However, the ultrastructure of locust tubules incubated for 1h in 100μM destruxin A is not significantly different from that of controls. This suggests that destruxin A is not acting as a cytolytic agent, but has specific actions on the cell's regulatory machinery.

1. Introduction

The insect pathogenic fungus, *Metarhizium anisopliae*, produces a family of cyclic peptide lactone toxins , destruxins, which may play a part in pathogenesis. The acute toxicity of destruxins varies according to the species tested, Lepidoptera and Diptera being particularly suscepible (Roberts, 1980; Samuels *et al.*, 1988). Destruxins cause tetanic paralysis of lepidopteran muscle (Samuels *et al*, 1988), interfere with the immune system of the desert locust (Huxham *et al, 1989)* and inhibit secretion of ecdysteroids from the prothoracic gland of *Manduca sexta* (Sloman and Reynolds, 1993). We have found that destruxins A, A_2, B and E all inhibit fluid secretion *in vitro* by Malpighian tubules of the desert locust (James *et al, 1993)*. The aim of this study was to confirm the effects of destruxin on Malpighian tubule function *in vivo*, using the blowfly, *Calliphora vomitoria*, and then to investigate the effect of destruxin A on the ultrastructure of desert locust Malpighian tubules, in order to shed further light on the mode of action of destruxins.

2. Materials and Methods

Desert locusts were from our own breeding colony at the University of Bath, adult females 4-14d old were used. Blowflies, *C. vomitoria*, were obtained as pharate pupae from a supplier, 6d old males were used in the experiments. Destruxins were purified from cultures as described previously (Samuels *et al.*, 1988). The rate of production of primary urine by *Schistocerca gregaria* Malpighian tubules *in vitro* was measured using the method described by Anstee and Bell (1975). The effect of destruxin on the secretory rate of blowflies was followed by determining the rate of removal from the blood of an injected dose of amaranth. 4μl of 0.5% amaranth in Ephrussi and Beadle saline (with or without destruxin) was injected through the scutellum. Blood

Fig 1

Rate of secretion of a single Malpighian tubule exposed to (●) locust saline then (O) 100μM destruxin A.

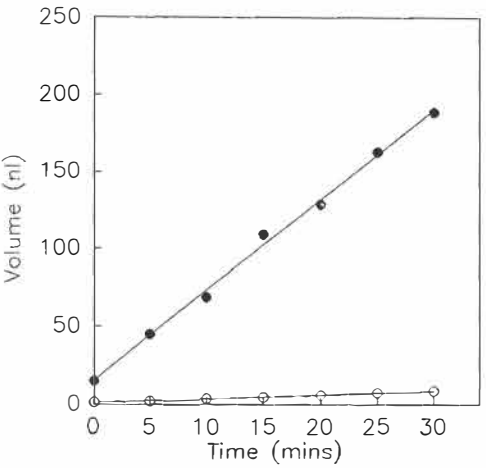
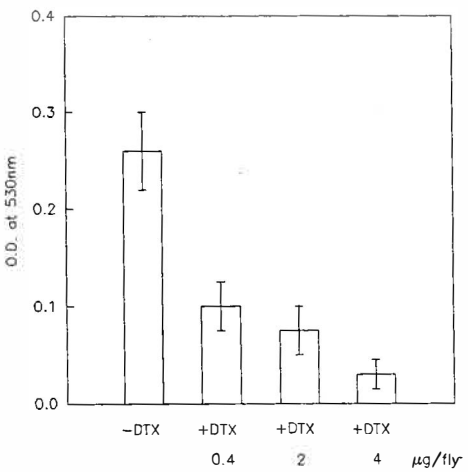


Fig 2

Fall in concentration of amaranth in the haemolymph of male *Calliphora vomitoria* between 30 and 90 min after injection with three doses of destruxin A (dtx).



samples, 1μl, were taken from the same site 30min and 90 min after injection, spotted onto a thin layer chromatography plate and scanned using a chromoscan at 530nm. The difference between the 30min and 90min peak heights indicated the drop in amaranth concentration in the haemocoel, due to excretion, during this time. The effect of destruxin A on the ultrastructure of locust Malpighian tubules was determined using the *in vitro* preparation. The tubules were exposed to 100μM destruxin A for 1h at 28°C, then processed for transmission electron microscopy essentially as described by Charnley (1982).

3. Results and Discussion

Destruxin inhibits secretion by *Schistocerca* Malpighian tubules *in vitro* (Fig 1.). This inhibition is dose dependent and partially reversible (James *et al*, 1993). Three doses of destruxin all reduced the ability of blowfly Malpighian tubules to secrete amaranth dye. The drop in amaranth concentration in the blood was significantly greater for control than experimental flies for all three doses used (Fig 2).

The ultrastructure of *Schistocerca* tubules incubated for 1h in 100μM destruxin was not significantly different from that of controls (electron micrographs not shown here). Thus a dose of destruxin which causes a 70% reduction in Malpighian tubule secretory rate does not have any obvious structural effects. This suggests that destruxin A is not acting as a cytolytic agent, but has specific actions on the cell's regulatory machinery. This work must be contrasted with that of Vey and Quiot (1989) who found adverse effects on the ultrastructure of the Malpighian tubules of waxmoth larvae, *Galleria mellonella*, after injection with destruxin.

Although there was no difference in ultrastructure between controls and destruxin treated tubules, after an hour's incubation, both treatments showed certain changes in ultrastructure from their appearance at time zero. This type of *in vitro* Malpighian tubule preparation is widely used in physiological experiments, but this is the first time that the ultrastructure of the tubules has been examined during the course of an experiment. The ultrastructural changes viz slight vacuolisation of the basal infolds and swelling of the mitochondria, are consistent with the tissue being slightly anoxic. A decrease in the oxygen supply to tissues would prove inhibitory to ion pumps and interfere with osmoregulation of the cells, resulting in the observed cell vacuolisation and swelling of organelles. It should be noted, however, that despite these changes in ultrastructure the control tubules continue to secrete normally.

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PROGRESS WITH THE DEVELOPMENT OF *METARHIZIUM FLAVOVIRIDE* FOR CONTROL OF LOCUSTS AND GRASSHOPPERS

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Abstract

The IIBC/IITA/DFPV Research Programme for the Biological Control of Locusts and Grasshoppers has demonstrated the feasibility of formulating conidia of Deuteromycete fungi in oil diluents suitable for use with existing controlled droplet application machinery at ultra-low volume (< 5 l/ha) rates. Formulations of *Metarhizium spp.* are highly infective at the standard assay conditions of 35% relative humidity 30°C. The chosen isolate of *Metarhizium flavoviride* from Niger is virulent to a wide range of acridoid pests including *Schistocerca gregaria, Locusta migratoria, Chortoicetes terminifera, Nomadacris succincta* and important members of the Sahelian grasshopper complex. Similar virulent isolates are available from 14 locust-affected countries in Africa, Asia and Australia.

In 1992, field trials were conducted in Niger, Mali and Benin. In large mesh cages in Niger, mortality of sprayed adult *S. gregaria* was ≈95% after 12 days. In 1 ha trials against *Hieroglyphus daganensis* in open grasslands in Niger, applications by hand held spinning disc sprayers resulted in 80% mortality by 20 days and a trial using the 'Ulvamast' vehicle-mounted ULV sprayer gave 70% mortality with grasshoppers confined in field cages. In dense rice and volunteer sorghum in northern Benin, best results against grasshoppers were 50% mortality after 7 days, rising slowly to 85% after 21 days, suggesting initial problems of droplet penetration into denser vegetation, with an important component of secondary uptake from foliage: this was also apparent in other field trials in the first seven days following spraying.

Introduction

In their synopsis of the desert locust plague of the mid 1980s Showler and Potter (1991) envisaged a control strategy involving pesticides that were compatible with: environmental concerns, human safety, efficacy, shelf-life in African conditions, available application equipment, cost and life stage of the target population. Biological control constitutes an obvious approach to satisfying the first two requirements and Prior and Greathead (1989) identified deuteromycete fungi as being amongst the most promising agents against acridids. The IIBC/IITA/DFPV Research Programme for the Biological Control of Locusts and Grasshoppers[§1] has demonstrated that lipophilic conidia of these fungi could easily be formulated in an oil, for application with Ultra-low Volume (ULV) spraying equipment that is normally used for locust control (Bateman, these proceedings). Oil-based *Metarhizium* formulations are highly infective at the standard assay relative humidity of 35% and temperatures of 30°C (Bateman *et al.*, 1993).

Preliminary screening has now taken place for 152 isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin, *M. flavoviride* Gams & Rozsypal and *Beauveria bassiana* (Balsamo) Vuillemin. A culture derived from a single conidium of *M. flavoviride* (IMI 330189ss) isolated from *Ornithacris cavroisi* in Niger is used as a "standard strain". This has been compared with other isolates for initial assays and has subsequently proved to be an effective standard for both laboratory and field experiments. Isolates were obtained from culture collections, mainly the USDA Agricultural Research Collection of Entomopathogenic Fungi (ARSEF), the American Type Culture Collection (ATCC), the CentraalBureau voor Schimmelcultures (CBS) and the International Mycological Institute (IMI). Many of the isolates found by the project have come from ecological studies in Benin, however specimens

§1: This is funded by: the Canadian International Development Agency (CIDA), the Directorate General for Development Cooperation of the Netherlands (DGIS), the Swiss Development Corporation (SDC) and the Overseas Development Administration of the UK (ODA).

have also been obtained from explorations in Pakistan and Oman and the use of a network of personnel in West Africa (Kooyman and Shah 1992).

Schistocerca gregaria: Large Cage Trials, Niamey 1992

In 1992 we were not able to gain access to any populations of *Schistocerca gregaria*. Two groups of large semi-permanent cages measuring 9 x 3 x 2 metres tall were constructed in Niamey in order to test formulations under "near field conditions" - especially outside the normal field season.

Spraying must take place under calm conditions using a Micron 'Ulvafan' air assisted sprayer to provide sufficient turbulence to achieve good coverage on insects and plants. Although the cage walls help to act as a wind break, displacement of droplets by small (often immeasurable) air currents still may occur during spraying and the sides of the cages were covered with matting during treatment. In an attempt to reduce background insect mortality, approximately 4 m^2 of the roof was covered by bamboo matting and 12 hills of millet were also planted in each cage, to reduce stress by providing shade.

Fig 1. shows the effects of treatments consisting of:

i. conidial formulation with normal, small (approximately 50μm VMD§2) droplets,
ii. condial formulation with enlarged (approximately 80 μm VMD) droplets,
iii. controls (blank oil: 50% groundnut oil, 50% kerosene) with $\approx$ 50μm VMD droplets.

"Small" and "enlarged" droplet sizes were produced by modifying the 'Ulvafan' so that the spinning disc was powered with 12 v and 6 v respectively. The fan was powered by an independent circuit and rotated at its normal speed in all treatments. The conidial formulation was a suspension containing 3.7 x 10^{12} conidia/l sprayed at a volume application rate equivalent to $\approx$ 2 l/ha.

The climatic conditions in these cages were very similar to the open air, with temperatures ranging from 22-45° C and RH from 20-80%. Sprays of *M. flavoviride* oil-based formulations at field rates killed >90% *S. gregaria* adults in 10 days, but there was <20% mortality in the control cages. There was no significant difference in efficacy between the two droplet sizes used.

1992 Field Trials At Malanville, Northern Benin

Malanville is an irrigated area of rice and other crops on the banks of the Niger river in North Benin. Grasshopper problems can be very serious and spraying is often carried out by the Service de Protection des Végétaux. A trial was done on small 0.15 hectare plots at a site called PRPIM (Projet Rehabilitation de Perimetre Irrige de Malanville). Rates of *M. flavoviride* application were 2 x 10^{12} and 2 x 10^{13} conidia per hectare. Samples were taken immediately (0 days) and 3 days after spraying (Shah *et al.*, 1992).

Best results against grasshoppers, mainly *Hieroglyphus daganensis*, were 50% mortality 7 days after application, rising slowly to 85% after 21 days. This suggests initial problems of droplet penetration into the denser vegetation, with an important component of secondary uptake from the foliage. The potential importance of secondary uptake continues to stimulate formulation research, including the addition of protectants against ultra-violet radiation (Moore *et al.*, 1993). At the height of the cropping season the vegetation can be dense, which poses application problems because ULV drift sprays do not penetrate into the lower layers. This was also apparent in other field trials, including the Niger Ulvamast trial, in the first seven days following spraying.

Ulvamast Trials In Niger

Hand-held equipment is useful for preliminary trial and small-scale pest control including treatment of hopper bands. Even with a 10m track spacing an operator must walk 1.1 km/ha so the maximum area that a single operator is able to treat rarely exceeds 10 ha/day.

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§2: Volume Median Diameter ($D_{V,0.5}$)

Migratory pest control often necessitates the rapid spraying of larger arid areas, so vehicle mounted ULV sprayers such as the Micron 'Ulvamast' are commonly used.

In 1 ha trials against *Hieroglyphus daganensis* and other species in open grasslands in Niger, a pilot trial in 1992 using the 'Ulvamast' applied 4×10^{12} conidia/ha at 2 l/ha. The sample taken 3 days after application showed 70% mortality after 14 days in laboratory cages. Control mortality was high, suggesting damage to the sampled insects by sweep-netting. In 1993 this trial is being repeated at Tam, near Maïne Soroa in the east of the country, using 9 ha plots.

1993 Trial On *Zonocerus variegatus* At Mono, Southern Benin

In 1992, the use an oil-based formulation of conidia of another strain of *M. flavoviride* (I91 609) proved effective against *Zonocerus variegatus* (ZVA) in S. Benin (Lomer et al., 1993). A further trial on ZVA inhabiting *Chromolaena odorata* undergrowth in Mono teak forest in southern Benin, involved ULV applications to 1 ha plots of 2×10^{12} conidia/ha in 2 l/ha, and caused a population reduction from 3.0 ZVA/m^2 to 0.3 ZVA/m^2 in 15 days.

Collaborative Tests

The Australian Plague Locust *Chortoicetes terminifera* forms gregarious bands in open pasture. The bands are detected by aerial survey and then sprayed from the ground, to prevent swarming. Chemical pesticides are increasingly restricted and there are very good prospects for integrating *Metarhizium* into control programmes. An Australian isolate of *Metarhizium* sp., ARSEF 324, is highly virulent and will be field tested in November 1993 by Dr Richard Milner of CSIRO in collaboration with the IIBC/IITA/DFPV programme and the Australian Plague Locust Commission. Laboratory and preliminary field tests have been carried out in collaboration with the Plant Protection Research Institutes of: Vietnam (on Bombay locusts: *Nomadacris succincta)*, South Africa (on Brown Locusts: *Locustana pardalina)* and with the Ministry of Food Production in Trinidad (on Moruga locusts: *Coscineuta virens)*; all shown that adult insects are susceptible to oil-based sprays containing the standard isolate.

Future Plans: Field Tests On Acridoids

The programme has now entered into a second phase, to run from 1993 to 1995. The major objective is to validate our experimental results on a range of major pest species in a series of large scale field trials, up to 100 ha in extent, thereby demonstrating that effective control can be achieved under operational conditions.

In Phase 2 of the programme, field tests are planned on all of the above pest species and also other grasshoppers (many spp. including *Oedaleus senegalensis*) of the Sahel and the Moroccan locust, *Dociostaurus maroccanus*.

The major excitement at present is the widespread outbreak of Desert Locust in Asia and Africa. A major objective for us is to do trials on Desert Locusts, first on hopper bands and later on adults. We were unable to do any in 1992 because of low populations. However, this year, with assistance from GTZ, SDC and FAO we are preparing for trials in Mauritania and Pakistan and possibly Sudan and Ethiopia.

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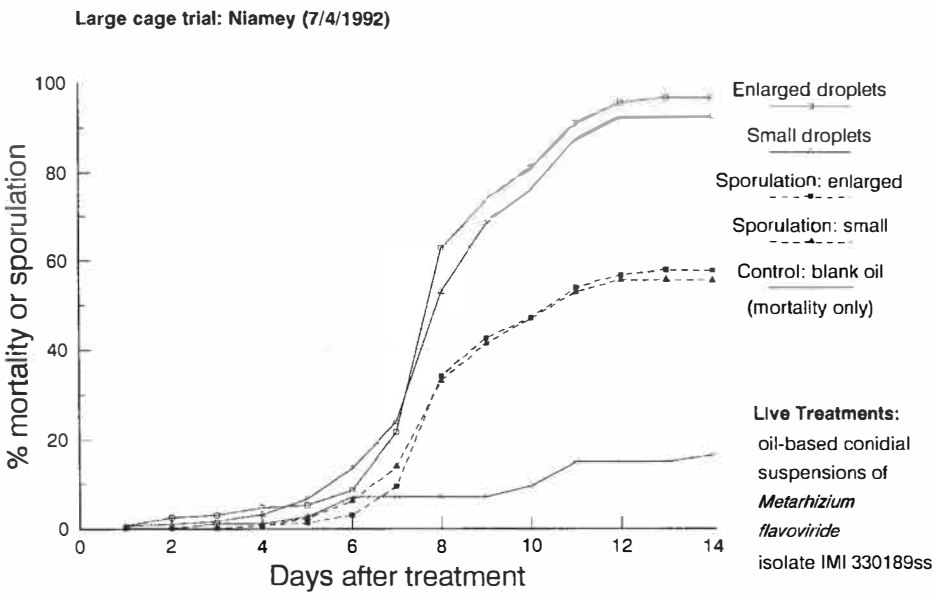
Fig. 1.



GROWTH AND SURVIABILITY OF THE ENTOMOPATHOGENIC FUNGUS VERTICILLIUM LECANII IN THE SOIL

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The entomopathogenic fungus **Verticillium lecanii** has a broad spectrum of hosts and its application is preferable for the biological control of sucking insects. In our investigation program for biological control of such pests in greenhouses we have collected, selected and breed several strains with different virulence to hosts e. g. to white fly, aphids and thrips species (Hirte et al., 1989). But it was observed, that many strains are variable in their quantitative and species-specific virulence (Sermann et al., 1991), indeed it exist a little number of natural isolates, which are constant in their typical and high virulence. With such a strain we studied the effectivness against the flower thrips **Frankliniella occidentalis** . In the other publication of this meeting (Sermann et al., 1993) we could demonstrate, that it is possible to infect and control the soil states of **F. occidentalis** with **V. lecanii** .

V. lecanii is not a typical soil-inhibitant. Therefore it is necessary to investigate its viability and surviability in the soil. The mean asks for clarifying under aspects of practical importance are: 1. how long are **V. lecanii** spores in the soil survival and evident and 2. have the spores a vegetative development in the soil.

In our first studies we investigate the behaviour of **V. lecanii** in model tests in the laboratory under definite conditions of temperature and soil moisture in a typical greenhouse soil. For detection of the viability of **V. lecanii** we use the plate method of Koch with suitable media to regognize and differ macroscopic and microscopic **V. lecanii** colonies from other soil fungi. But the colony producing units (cpu) origate from spores and mycelia, so that it is not possible to differentiate between vegetative or resting states (spores). Therefore we studied the promotion of **V. lecanii** in sterile soils without the concurrence of the soil microflora.

Fig. 1 shows that **V. lecanii** are capable to promote in a sterile soil and the curve character demonstrate a typical form of growth curves. That is not so under conditions with a rivaling soil microflora. Here the number of fungi decreases remarkably (Fig. 2). This reducing is more intensive in

the first period after inoculation (10 - 20 days) and can retard then to a level for a longer time.

The curves after blastospore inoculation decrease to a lower level (Fig. 2, 3), the conidiopsore-curves stop on a higher level, so that the tenacity of inoculated conidiospores is longer than of inoculated blastospores. In relation to the sporeform and also to the quantity of inoculated spores (Fig. 3) it is possible to keep a certain, desirable sporetiter in a distinct time (10- 30 days).

The decrease of V. lecanii is also influenced by soil conditions. This shows experiments with different incubation temperatures: In one variant the soil was incubated by the constant temperature of 22°C, in another variant the incubation temperature was oscillative changed as following: 12 h 20°C, 12 h increasing temperature till 32°C. In Fig. 4 it is demonstrated that by varying soil temperature the decrease of the inoculated fungi is faster.

For practical use we tested the infiltration of V. lecanii in the soil after spraying a spore suspension on the soil surface. Table 1 shows, that the spores are holding back to a great extend in the uppermost soil layer. Further it is important that there are no differences of tenacity in the tested soil layers (Table 2).

All experiments demonstrates that Verticillium lecanii have in the soil a typical behaviour of an allochthonic microorganism (Hirte, 1977): Under sterile soil conditions the inoculated spores of V. lecanii germinate and the fungus can develop in form of a typical growth curve. But in a natural soil in concurrence with the autochthonic microflora the number (cpu) of this entomopathogenic fungus decreases remarkably, at first faster than slower.

Under consideration of distinct conditions (e. g. applicated sporeform, quantity of inoculated spores, soil temperature and moisture) it is therefore possible to keep a necessary sporetiter in a certain time (10 - 30 days) sufficient effective enough for infection of pestinsects in soil, especially in sensible stages.

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soil layer	cpu / g soil
0 - 3 cm	$2,5 \times 10^7$
3 - 6,5 cm	$7,5 \times 10^3$
6,5 - 10 cm	$7,0 \times 10^3$

Tab. 1 Infiltration of V. lecanii into the soil after spraying the surface ($5,3 \times 10^7$ blastospores / cm^2 soil surface)

Tab. 2 Tenacity of V. lecanii in different soil depths

soil depths	cpu x 10^6 / g soil				
	1	4	11	21	40
	days after application				
0 - 3 cm	20,0	13,1	0,25	0,09	0,06
3 - 6,5 cm	20,0	10,4	0,24	0,09	0,05
6,5 - 10 cm	20,0	20,5	0,11	0,11	0,05

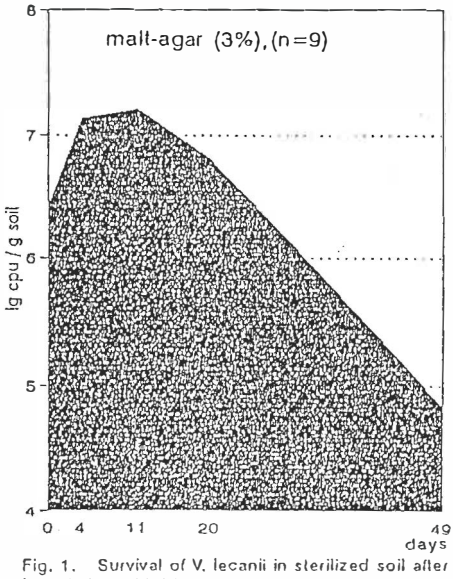

Fig. 1. Survival of V. lecanii in sterilized soil after inoculation with blastospores

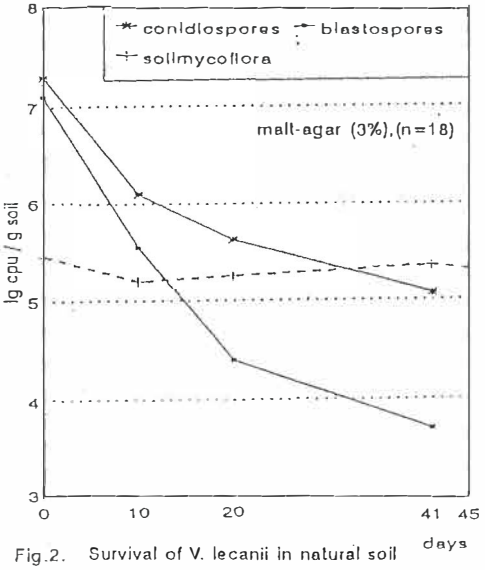

Fig.2. Survival of V. lecanii in natural soil

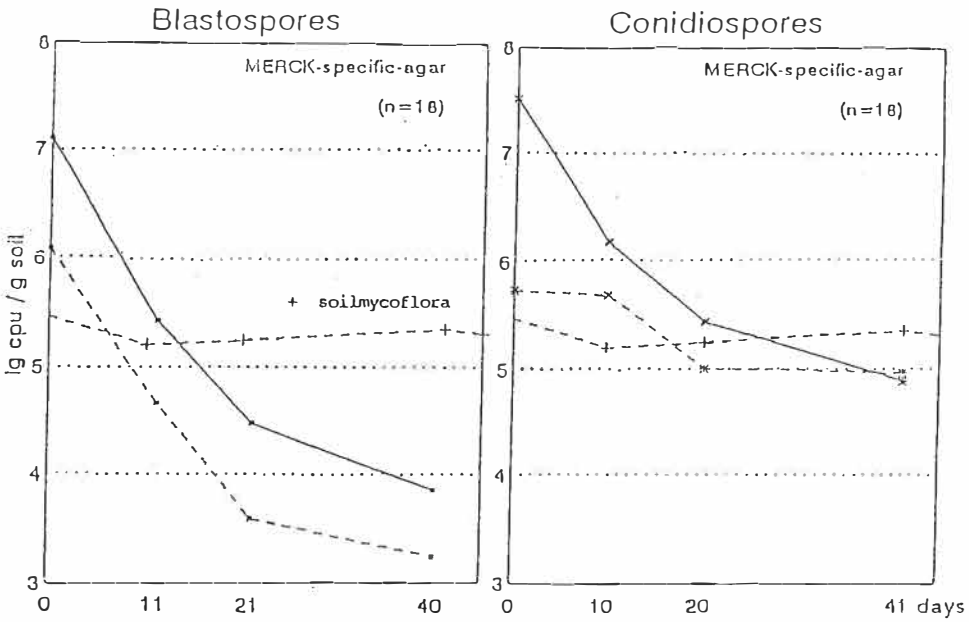

Fig. 3. Tenacity (Survival) of V. lecanii in natural soil in relation to different sporeforms and inoculation quantities

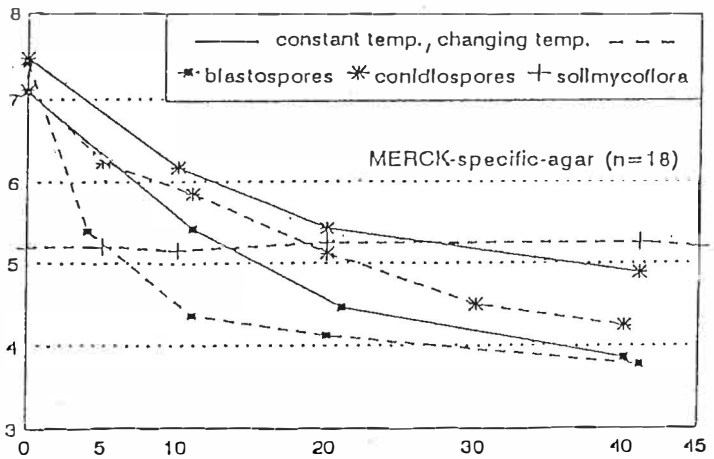

Fig. 4. Tenacity of V. lecanii in natural soil under different temperature regimes

EFFECTIVENESS OF A SOIL APPLICATION OF VERTICILLIUM LECANII ON SOILBORNE STAGES OF FRANKLINIELLA OCCIDENTALIS

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Introduction

The entomopathogenic fungus *Verticillium lecanii* can exists saprophytically on organic substances in soil. The californian flowerthrips *Frankliniella occidentalis* takes place a part of his development in soil, so contacts of pest and entomopathogen in the soil are possible. In directed laboratory tests it was proofed , if *V. lecanii* infect old larvae and nymphs of *F. occidentalis* in soil and what mortality can be obtained with a fungus soil application.

Materials and Methods

Laboratory tests allow a direct evidence of infections for soilborne stages of *F. occidentalis*.

For tests old larvae of *F. occidentalis* were bred on chrysanthemum plants in special cages under greenhouse conditions. Petri dishes (diameter: 10 cm) were used, in which glassrings (diameter: 2 cm; height: 1cm) were fixed in stearin. Each glassring was filled with 0,3 gr.of sterilized or natural humus soil (water content: 30-40% by weight). Five old larvae of *F.occidentalis* were put on the soil

- before (=direct application) or
- 2 days after (= indirect application)

treatment (handsprayer) with V. lecanii-spores suspension. Per variant 35-45 old larvae were included. After all applications the glassrings were coverd with a glasscover. Trials were carried out in a phytotron with 20°C.The air humidity in the glassrings was approximately 98 % R.H. In all tests isolate V 24 was provided from our *V. lecanii* isolat collection. Test designs are listed in table 1.

The larvae were checked 3, 5, 7, 9 and 11 days after introduction. The living and dead larvae and those with fungus mycelium were counted respectively. Hatched adults from surviving animals were put on chrysanthemum flowers and checked again after 7 days.

table 1: Design of tests on soil applications of *Verticillium lecanii* to soilborn stages of *Frankliniella occidentalis*

tests/ variants	soil substrate	type of application	treatment (5ml/dish)	type of spores	cfu/ml
1 Kd	humus	direct	water	-	-
1 Vd	soil	direct	V24-susp.	blastosp.	10^7
1 Ki	2x autoclaved	indirect	water	-	-
1 Vi	2x autoclaved	indirect	V24-susp.	blastosp.	10^7
2 Kd	humus	direct	water	-	-
2 Vd	soil	direct	V24-susp.	blastosp.	10^7
2 Ki	natural	indirect	water	-	-
2 Vi		indirect	V24-susp.	blastosp.	10^7
3 Kd	humus	direct	water	-	-
3 Vd	soil	direct	V24-susp.	blastosp.	10^6
3 Ki	natural	indirect	water	-	-
3 Vi		indirect	V24-susp.	blastosp.	10^6
4 Kd	humus	direct	water	-	-
4 Vd	soil	direct	V24-susp.	conidiosp.	10^6
4 Ki	natural	indirect	water	-	-
4 Vi		indirect	V24-susp.	conidiosp.	10^6

Results

Fungus infections of old larvae could be found statistically significant in all variants of the *V. lecanii* application. Important results were:

- The direct and indirect fungus application induced nearly the same mortality of larvae (FIG. 5).
- The applied concentration of 10^6 blastospores/ml differs insignificantly from the concentration 10^7. (FIG.5).
- The mortality of old larvae was higher in sterilized soil (FIG 8).
- Conodiospores of *V. lecanii* seem to induce a higher mortality than blastospores.-
  The larvae mortality increased to the 7^{th} observation day and seems to be dependent from counting rhythm(FIG. 6).
- On the most of dead larvae growing V. *lecanii* mycelium appeared.
- Surviving animals from the application tests died in adult phase (FIG. 7). The total mortality rate induced by *V. lecanii* enlarged by that phenomen (FIG 8).

Conclusion

Soil application of *V. lecanii* spores induce infections of soilborne stages of *F. occidentalis*. Remarkable rates of infection were found in laboratory tests.

Both the initial mortality effect on old larvae and a observed late mortality effect on adults demonstrate new possibilities to biocontrol *F. occidentalis* in soil by *V. lecanii,* particular for our isolate V 24.

FIG.5 Effect of direct (d) and indirect (i) soil application of Verticillium lecanii (isolat V 24) on old larvae of Frankliniella occidentalis in laboratory tests

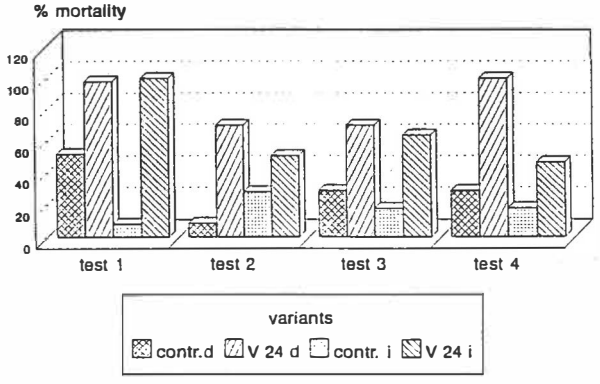


FIG.6 Development of mortality and mycel by old larvae of Frankliniella occidentalis after soil application of Verticillium lecanii in laboratory tests

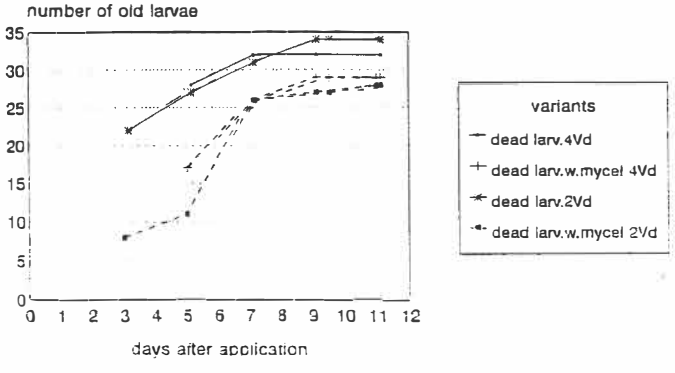


FIG.7 Late effect on adults after a soil application of Verticillium lecanii (isolat V 24) on old larvae of Frankliniella occidentalis in laboratory tests

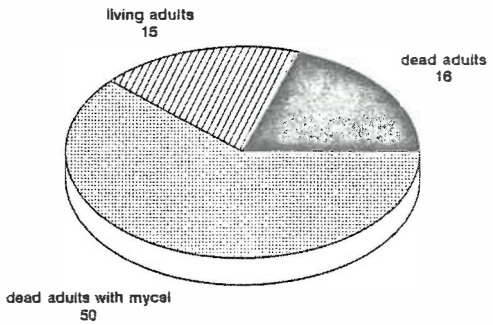
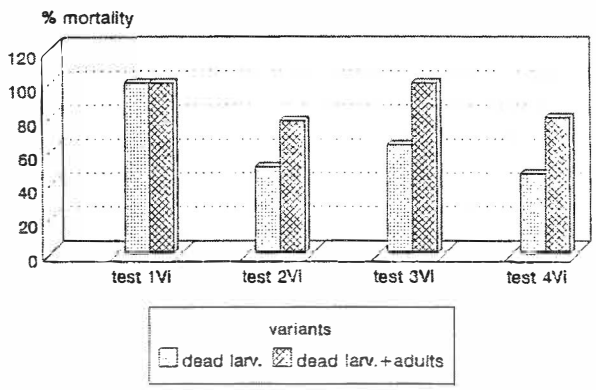


FIG.8 Mortality of old larvae and adults of Frankliniella occidentalis after soil application (i) with Verticillium lecanii (isolat V 24) in laboratory tests



Side-effects of pesticides on *Verticillium lecanii:* in vivo tests on whitefly and aphids

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1. Introduction

Two products based on two different strains of the entomopathogenic fungus *Verticillium lecanii* are now being used in IPM programmes. These are Mycotal for control of whitefly, mainly in cucumber and Vertalec for control of aphids, mainly in cucumber and chrysanthemum. Both products are formulated as wettable powders and registered in the U.K. and Switzerland, Mycotal is registered in The Netherlands and both products can be used in Denmark and Sweden. In IPM programmes they are used in conjunction with fungicides, insecticides and acaricides for disease and pest control. Compatibility studies of chemical and biological control agents are necessary to be able to give proper recommendations for their integrated use.

A number of compatibility studies have been carried out with *V. lecanii* (Wilding, 1972; Olmert & Kenneth, 1974; Tuset, 1975; Hall, 1981; Saito, 1988). An overview of side-effect studies is given by Schuler (1991).
In most of these studies the effects of pesticides were examined in vitro by investigating the inhibition of germination and mycelial growth on agar. These were performed with watery solutions of spores obtained from laboratory cultures of different isolates of the fungus. Only some authors (e.g. Hall, 1981) tested the inhibitory effects of chemicals in a bioassay using insects and plants.
In this study the effects of pesticides were investigated on the formulated products Mycotal and Vertalec. The studies were performed in a bioassay using leaf discs and insects at environmental conditions comparable to those in protected crops. Since the work of Hall (1981) a number of new chemical pesticides have become available which have not been tested yet. The emphasis in this study lies on those chemicals which are commonly used on cucumber and chrysanthemum. The direct effect of certain combinations as well as the short-term persistent effect of some fungicides were investigated. The effect of spore-type on compatibility was also investigated. The results are compared with previously documented results.

2. Material and methods

Chemical compounds and *V. lecanii* products were tested at label recommended rates for cucumber and/or chrysanthemum (table 1). Mycotal was sprayed at a concentration of 10^7 conidiospores/ml, Vertalec with 10^6 blastospores/ml.

2.1 The bioassay

To test efficacy of fungal preparations on insects a bioassay was developed using leaf discs instead of plants. The bioassay tray consisted of a round "petri dish-type" tray with a lid (ϕ 77 mm; height 31 mm; Bock, Art.Nr.41113).

In order to allow sufficient gaseous exchange four holes were made in the sides of the tray (ϕ 1 cm) and one in the lid (ϕ 6,5 cm) covered by nylon mesh. Leaf discs were supported on water agar (1%) of a minimum depth of 5 mm. In this way leaves remained in good condition for about 14 days. Care was taken to insure leaf discs covered the surface of the agar completely to prevent dehydration.

Treatments were applied with an air-assisted spray tower. Pesticides were sprayed till leaf discs were covered with a fine mist. For the *Verticillium* products the spraying apparatus was adjusted to give the recommended number of spores per mm², i.e. for Mycotal 300 spores/mm², for Vertalec 30 spores/mm². To obtain the desired relative humidity (RH) trays were incubated upside down above a saturated salt solution of KCl (=85% RH) and KNO_3 (=92% RH).

2.2 Studies with whitefly

Treatments were applied to first and second instars of the greenhouse whitefly *Trialeurodes vaporariorum* on tabacco. The direct effect of pesticides was investigated by spraying leaf discs with the chemical. After the spray solution had dried, Mycotal was applied. After drying, trays were incubated at a RH of 85% and 23 C° ± 1 C°. Residual effects were investigated by spraying Mycotal three days after the chemical treatment. Three replicates were used per treatment. Mortality was assessed ten days after the Mycotal treatment. A water treatment, a Mycotal treatment and a chemical treatment were carried out to determine the separate effect of each of these variables.

2.3 Studies with aphids

Treatments were applied to first and second instars of the cotton aphid *Aphis gossypii* on cucumber. Only direct effects were tested. The procedure used was the same as that for whitefly. Incubation took place at a RH of 92% and 23 C° ± 1 C°.

Five replicates were used per treatment. Mortality was assessed after 8 days. Newly born nymphs were not included in the assessment.

3. Results and discussion

The effect of a fungicide on the activity of *V. lecanii* was calculated as the percentage inhibition of activity of the fungal product, corrected for the mortality in the water treatment and the mortality in the pesticide treatment (Abbott's correction). These effects are given in table 1. Fifteen fungicides were tested on Mycotal and four commonly used insecticides/acaracides. Only a limited number of pesticides were tested on Vertalec, five fungicides and two insecticides. Pirimicarb, a commonly used aphicide, could be tested on *Aphis gossypii* since this species is tolerant to this chemical.

Table 1: Side-effects of pesticides on two *Verticillium lecanii* products: %inhibition in activity on whitefly and aphids.

			MYCOTAL		VERTALEC
Active ingredient and concentration		Product name	%inhibition	%inhibition after 3days	%inhibition
bitertanol	0.1%	Baycor	56	–	–
bupirimate	0.2%	Nimrod	0	–	4
captan	0.2%	Captan	100	100	–
carbendazim	0.05%	Carbendazim	4	4	–
chlorothalonil	0.3%	Daconil	100	100	–
fenarimol	0.02%	Rubigan	0	–	–
imazalil	0.025%	Fungaflor	43	34	75
iprodione	0.05%	Rovral	0	0	–
oxycarboxin	0.05%	Plantvax	42	66	72
procymidone	0.05%	Sumisclex	0	0	–
propiconazole	0.05%	Radar	54	7	82
thiophanate-methyl	0.05%	Topsin M	48	21	–
tofylfluanid	0.15%	Eupareen M	83	78	–
triforine	0.1%	Funginex	–	–	67
vinclozolin	0.1%	Ronilan	6	0	–
buprofezin	0.03%	Applaud	–	–	26
fenbutatin oxide	0.1%	Torque	12	0	–
hexythiazox	0.05%	Nissorun	0	0	–
pirimicarb	0.05%	Pirimor	0	0	21

Variation was large between replicates for all different treatments. Mortality of test insects caused by the pesticides themselves varied considerably. This made interpretation difficult. The results indicate that the fungicides captan, chlorothalonil and tolylfluanid are very harmful to Mycotal and they remained so after three days. This agrees with the results Hall (1981) obtained from studies on both agar and aphids.

Bitertanol, imazalil, oxycarboxin, propiconazole and thiophanate-methyl were moderately harmful. Bitertanol was not tested after three days because it was thought to be safe. The effects of propiconazole and thiophanate-methyl were reduced after three days. This is not the case with imazalil and oxycarboxin.

Bupirimate, carbendazim, fenarimol, iprodione, procymidone, vinclozolin, fenbutatin oxide, hexythiazox and pirimicarb were safe to Mycotal. The effects of both triforine and buprofezin could not be determined as they caused a very high mortality to whitefly.

Only a small number of pesticides were tested with Vertalec since we expected rather similar results to those obtained with Mycotal. The most frequently used fungicides for powdery mildew in cucumber and white rust in chrysanthemum were tested. Imazalil, oxycarboxin, propiconazole and triforine were harmful. Bupirimate, buprofezin and pirimicarb were safe.
Vertalec appears to be much more effected by pesticides than Mycotal. This might be explained by differences in strain or spore-type. A comparison between blastospores and conidiospores of both strains, formulated as wettable powders, was conducted with imazalil and bupirimate. No significant difference in the inhibitory activity of these fungicides on either type of spores of both strains were detected (table 2). The differences between Mycotal and Vertalec may be attributed to strain sensitivity although the host plant, the host insect and the number of spores (10x more in Mycotal) may influence these results. However differences between strains are generally found to occur (Olmert & Kenneth, 1974; Hall, 1981).

Table 2:
Inhibition (%) of two fungicides on blastospores and conidiospores of Mycotal and Vertalec.

active ingredient	conc.	product name	Mycotal		Vertalec	
			blastosp.	conidiosp.	blastosp.	conidiosp.
bupirimate	0.2%	Nimrod	-	-	30	24
imazalil	0.025%	Fungaflor	0	8	67	86

4. Conclusions

From this study we conclude that the fungicides bupirimate, carbendazim, fenarimol, iprodione, procymidone and vinclozolin can be used together with *V. lecanii* products in IPM programmes. The fungicides bitertanol, imazalil, oxycarboxin, propiconazole and thiophanate-methyl can only be used with great care and after a suitable interval between treatments.
A three day interval between the application of a harmful chemical and a *V. lecanii* product is not enough to overcome any negative effects.
The long-term persistent effect of fungicides on *V. lecanii* cannot be tested by this bioassay method because leaf discs deteriorate after about 10-14 days and the instars develop into late instars, pupae and adults each with different susceptability to the fungus.

A different method using whole plants needs to be developed to determine the long-term persistent effects.
Captan, chlorothalonil and tofylfluanid cannot be used in conjunction with Mycotal and Vertalec. The greater sensitivity of Vertalec to chemicals means extra care is needed when using this product.
If the results found in this study are compared with results on mycelial growth and germination reported by other authors, it becomes clear that tests on agar have little predictive value. Only if inhibition on agar is very low or absent can there be expected to be no reduction in ability to infect insects. In all other cases a test on insects should be conducted to determine the inhibitory activity.

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TOLYPOCLADIUM SP. (*FUNGI IMPERFECTII: HYPHOMYCETES*) IN LARVAE OF *AGROTIS SEGETUM* (LEP.: NOCTUIDAE)

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Larvae of the turnip moth *Agrotis segetum* (Schiff.) (cutworms) are polyphagous insects feeding on a range of crops in Europe, Africa and Asia. The fully fed last instar larva (L6) hibernates in the soil, and in late spring (April-May) moves to a more shallow position for pupation. During a study of the influence of biotic winter mortality factors on cutworm populations, larvae from one collection site (out of a total of 10 collection sites) was found to be infected to a large extent by a phialidic fungus with conidia in slimy heads (68% of cutworms collected in May were infected). The fungus has been observed in cutworms at this specific location for several consecutive years and has so far been identified as a *Tolypocladium* species.

Semifield experiments initiated in late October, where a spore suspension was applied to buckets containing soil and hibernating cutworms, showed that the fungus had infected 100% of the recollected cutworms by the end of May. A similar result was found for larvae kept in soil from the location where *Tolypocladium* sp. originally was found. However, already in early January (82 days after starting the experiment) 70% of the recollected cutworms proved to be infected by the fungus despite the relatively low soil temperatures at this time of year. The majority of these larvae were dead and sporulating in the soil at the time of sampling.

The fungus grows on SDA and MEA within the temperature range 2 °C - 30 °C Preliminary tests indicate that the host range may be restricted, as no infection has been obtained in larvae of *Tenebrio molitor* L., *Galleria mellonella* L. or *Trichoplusia ni* using different inoculation methods.

Based on these results *Tolypocladium* sp. seems an interesting species, especially as it is able to infect and kill its host at low soil temperatures.

ACTION OF *BEAUVERIA BRONGNIARTII* AGAINST *MELOLONTHA MELOLONTHA* AND ITS PERSISTENCY IN VALLE D' AOSTA*

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In Valle d' Aosta, Northwest Italy, a region characterized by very particular soil and climatic conditions, the deuteromycete *Beauveria brongniartii* (Sacc.) Petch, an efficient control agent of the European cockchafer *Melolontha melolontha* L., is found within the larval populations of the beetle at a very low frequencv (less than 0.5%). This phenomenon has prompted study of the performance of *B. brongniartii* under the environmental conditions peculiar to Valle d' Aosta.

To this end, the action of the fungus upon the larvae of *M. melolontha*, its distribution and its persistency in the soil have been studied.

Test plots in Signayes (Aosta), heavily infested by second stage European cockchafer larvae were inoculated with $1x10^8$ conidia/m^2 of soil (equal to 5x10 conidia/g dry weight) of a highly virulent strain of *B. brongniartii*. Test plots were treated once or twice at a distance of one year apart between treatments.

Samples made the following 2 years to evaluate the effect of the fungus on the second and third stage larval populations showed that the average mortality of the insects in the treated plots was significantly 21.9% higher compared to the control. No significant differences in larval mortality were found between plots treated once and plots treated twice.

The fungus, 10 months after inoculation, in the treated plots had reached levels between a minimum of $5x10^4$ and a maximum of $4x10^5$ conidia/g dry weight of soil. Three years after treatment, in the plots inoculated once, the fungal presence of the soil was between $1.2x10^3$ and $4.9x10^4$ conidia/g dry weight. Two years after the second treatment, in the plots inoculated twice, the levels were between $6.7x10^3$ and $1.2x10^5$ conidia/g dry weight.

The fungus was never isolated from the control plots.

The results obtained indicate that in the soils peculiar to Valle d'Aosta, inoculation with *B. brongniartii* may be an effective control measure for *M. melolontha*.

*This research was funded by a grant of the Regione Autonoma Valle d' Aosta.

7. Meeting of the subgroup entomopathogenic viruses and bacteria.

CHARACTERIZATION OF A PICORNA-LIKE VIRUS ISOLATED FROM THE MAIZE STEM BORER *SESAMIA CRETICA* LED. (NOCTUIDAE) IN EGYPT

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Summary

A small RNA virus was isolated from larvae of *Sesamia Cretica* (Lepidoptera Noctuidae), the most important corn borer in Egypt. Some properties of this virus (Maize Stem Borer Virus : MSBV) have been studied.

Electron microscopic observations of the purified suspension showed the presence of non-envelopped isometric viral particles, 30 nm in diameter. The virus capsid contained three major proteins (VP1, VP3, VP4) with molecular weights of 60,000, 54,000 and 28,000, as well as one minor (VP2) with molecular weight of 58,000 daltons. The viral genome was composed of one single strand RNA with molecular weight of 9,4 Kb.This viral RNA presents a terminal Poly Adenylate sequence as the members of the Picornaviridae family.

Reliable and sensitive viral diagnosis tools, based on immunoenzymatic test (ELISA), and non-radioactive nucleic probe, have been developed.

This virus is capable to cause important mortality (96 % in 8 days) by oral infection with high virus concentration, for last instard larvae. This bioassay shows that MSBV is highly pathogenic and thus presents interesting potentialities as a biocontrol agent.

Introduction

Among the major corn borer in Egypt, *Sesamia cretica* Lederer, 1857 (Lepidoptera : Noctuidae) is the most frequently observed. This maize stem borer is a polyphagous insect on graminaceous, especially *Zea mays*, *Saccharum officiarum* and *Sorghum vulgare*. Control of this pest was limited in the use of chemical insecticides, and microbiological control by insect viruses had to be considered.

Two viruses were recorded in *S. cretica*, i.e. Granulosis virus (ScGV)(Fédière et al., 1992 a) and small RNA virus (Fédière et al., 1991). The present investigation aims to characterize the small RNA virus designated Maize Stem Borer Virus (MSBV) in order to provide its classification.

Results

1 - Virus strain

Dead infected larvae were collected from maize fields at El Badrashin in june 1990. The virus was purified and propagated from this time in laboratory reared larvae infected *per os*.

2 - Electron microscopy

Examination of purified viral suspension by electron microscope revealed large number of non-envelopped isometric particles, 30 nm in diameter.

3 - Spectrophotometric measurements

U.V. absorption of viral suspension was examined through wawelenghts between 320 and 220 nm. The average ratio of extinction at 260 nm to that at 280 nm was 1.4.

4 - Electrophoresis of the viral proteins

Molecular weigth and number of proteins were assessed by comparing their electrophoretic mobilities, in 9% polyacrylamide gels, with those of standard marker proteins. Electrophoresis revealed three major bands with molecular weights of 60,000 (VP1), 54,000 (VP3) and 28,000 daltons (VP4), as well as one minor band with molecular weight of 58,000 daltons (VP2).

5 - Antisera and immunological tests

Antisera were prepared in rabbits. Immunoenzymatic test ELISA between MSBV and two other picorna-like viruses of *Latoia viridissima* (Fédière, 1983) and *Turnaca rufisquamata* (Fédière et al. 1992 b),revealed that these viruses were serologically different.

6 - Characterization of the viral RNA

Virus samples were tested for nucleic acid by RNAse and DNAse before electrophoresis. This test indicated the RNA nature of the genome.

The molecular weight and the number of fragments were calculated after migration in 1% agarose gel in denaturing conditions and comparison with RNAs genomes of Drosophila C virus (Jousset et al. 1977) and L. viridissima Picornavirus (Fédière et al., 1990, Zeddam et al., 1990). In these conditions the extracted RNA demonstrated to be 9.4 Kb and that only one band was present.

The retaining of the RNA by an oligo (dT) cellulose column indicated the presence of a poly(A)+ tract.

The viral RNA was used as template to generate a cDNA genomic bank. Following the cloning of cDNA, plasmids were obtained containing viral inserts of different sizes. One of them, designated PSc39, contained an insert of 1.6-2.0 Kbp. The physical map of this insert revealed a Hind III-Bam HI fragment of 250 bp which was used to prepare a cold nucleic probe.

Conclusions

By severals of its properties, including a single-stranded RNA genome approximatively 9.4 Kb containing a poly(A)3' sequence this virus presents the salient features of the members of the Picornaviridae family. Both the ELISA tests and the nucleic probe provide efficients tools for epidemiological studies in field conditions.

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RESTRICTION ENDONUCLEASE ANALYSIS OF THE GRANULOSIS VIRUS OF *ADOXOPHYES ORANA* F.V.R. (LEP., TORTRICIDAE)

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Summary

Two types of replication of the granulosis virus of *Adoxophyes orana* (AoGV) are known: a nuclear type with replication of the virions in the nucleus, and a cytoplasmic type with replication in the cytoplasm of cells with intact nuclei. Pure lines of the two AoGV replication types have been isolated. In order to find out whether or not the different phenotypes represent different genotypes, restriction endonuclease analysis was conducted on the viral genome of the AoGV. The eight restriction enzymes Sal I, Eco RI, Hind III, Bgl I, Kpn I, Bam HI, Xho I and Sma I cut the AoGV DNA into 19, 18, 16, 5, 5, 4, 2, and 1 fragment(s), respectively. The size of the AoGV genome was estimated to be 89 kbp. These viral DNA samples from the two types of AoGV were further analysed using the four restriction endonucleases, Sal I, Eco RI, Hind III and Bgl I. Both AoGV types have the same restriction endonuclease profiles. No so called "submolar" band representing genotypic heterogeneity was found in the examined samples. This suggests that the viral genome of the AoGV is homogeneous, even if two phenotypes are found in viral replication.

Introduction

A granulosis virus (AoGV) isolated from *Adoxophyes orana*, a serious pest of the apple and pear cultures in Switzerland, is being investigated in our laboratory since a few years and a viral insecticide to control the pest was developed. A speciality of *A. orana* granulosis is the slow death of the infected larvae. Instead of dying quickly they survive longer than normal larvae and die late as large dauerlarvae (Benz, 1979). Electron microscopical studies demonstrated that AoGV caused two types of infection, a nuclear type (N-type) in which the virus replicates in the nucleus and the nuclear membrane disrupts after development of virions, and a cytoplasmic type (C-type) in which the virus develops only in the cytoplasm and the nucleus remains intact (Schmid et al., 1983).

These two GV types were separated by *in vivo* methods (Drolet and Benz 1986, Drolet, 1989). Bioassay results showed some differences between the two GV types, but the primary biochemical analyses on viral proteins and DNA seemed insufficient for characterizing them. Recently, the structural proteins from both types of AoGV were analysed by SDS-PAGE, high resolution two-dimensional electrophoresis and immunoblot (Li, 1993). Here we report the results of restriction enzyme analyses on the AoGV genome. The DNAs from both GV types were compared to find out whether the two GV types are two different viruses or different phenotypes of the same virus.

Materials and Methods

The two GV types (N-type and C-type), separated from the original viral suspension of Schmid et al.(1983) by Drolet (1989) were used in this study. Third instar larvae of *A. orana* were infected with either of the two GV types. To achieve lower mortality, the purified capsule solutions from the N- and C-type were serially diluted for infecting the larvae. Viral DNA was extracted from individual infected larvae as described by Smith and Crook (1988). The viral DNA was digested with 8 restriction endonucleases: Sal I, Eco RI, Hind III, Bgl I, Kpn I, Bam HI, Sma I and Xho I under the conditions recommended by the supplier (Boehringer Mannheim, Biochemica). Double digestion was carried out with 4 enzymes (Sal I, Eco RI, Bgl I, Xho I) using the same incubation buffer (H buffer). The digested fragments were subjected to electrophoresis at 90 V for 3 h in 0.6% agarose gels using a 0.04 M Tris-acetate-0.002 M EDTA buffer system (Maniatis et al. 1982).

Results and Discussion

Of the eight enzymes used to digest the viral DNA of the AoGV Sal I, Eco RI and Hind III cut AoGV DNA into 19, 18 and 16 fragments, respectively, Bgl I and Kpn I each cut the AoGV DNA at 5 sites, Bam HI at 4 sites, Xho I at 2 sites, and Sma I at 1 site (Fig. 1). The size of the AoGV genome was estimated to be approximately 89 kbp (adding together the sizes of the fragments digested by a restriction endonuclease gives the genome size).

Restriction endonuclease analysis is the most useful tool in identifying virus species and variants. Each viral species has its unique restriction enzyme pattern. The AoGV restriction enzyme profiles are not similar to any of the published profiles of other GVs. This indicates that the AoGV is a very special isolate. But some common characteristics of a GV genome can be found. The enzyme Sal I, for example, always cuts GV genomes into numerous smaller fragments, while the enzyme Sma I, which has a base recognition sequence of 6 bp (a G/C triplet followed by a C/G triplet), has only few sites on the GV genomes (Crook 1992). Our results are consistent with these characteristics.

Since the two types of AoGV have different places of replication and cause distinct cytopathic effects in the infected cells, it could be expected that they have different genotypes. It is reported that in some GV isolates genotypic heterogeneity is reflected in their DNA restriction endonuclease profiles in the form of submolar bands.

To compare the genomes of the two GV types, individually collected DNA samples of both GV types were digested with four restriction enzymes, Eco RI, Sal I, Hind III and Bgl I, producing 19, 18, 16, 5 DNA fragments, respectively. Both GV types have similar profiles and no submolar bands could be found in these profiles.

The individual genotypes can be separated from a genetically heterogeneous mixture by an *in vivo* isolation method, namely low mortality dose infection (Smith and Crook 1988). This method is based on the independent action hypothesis of microbial pathogenicity. When low larval mortality (10%) is caused by a very low infection dose, most of the infected larvae (95%) should have been infected by only a single virus particle (Huber and Hughes 1984). Thus, such an infected larva contains a clone of the pathogen.

In an attempt to detect different genotypes of the N-type and the C-type, DNA samples of individual larvae infected with relatively low doses of N-type AoGV (65 and 30% mortalities in two experiments) and C-type AoGV (19% and 13% mortalities in two experiments), were analysed with the four restriction enzymes mentioned above. Although at least in the C-type infections the mortality rates were very close to the critical threshold of 10% mortality, the DNA samples extracted from these larvae had similar restriction enzyme profiles to those shown in Fig. 1, i.e. no individually viral genotypes could be detected (data not shown). However, the number of our individual profiles is not large enough to exclude the possibility that some minute genetic heterogeneity exists within and between both replication types. If more viral DNA samples were analysed, the chances of finding such differences would increase.

Double digestion of the AoGV DNA was also carried out with four restriction endonucleases (data not shown). These profiles will be useful for mapping the AoGV genome in the future.

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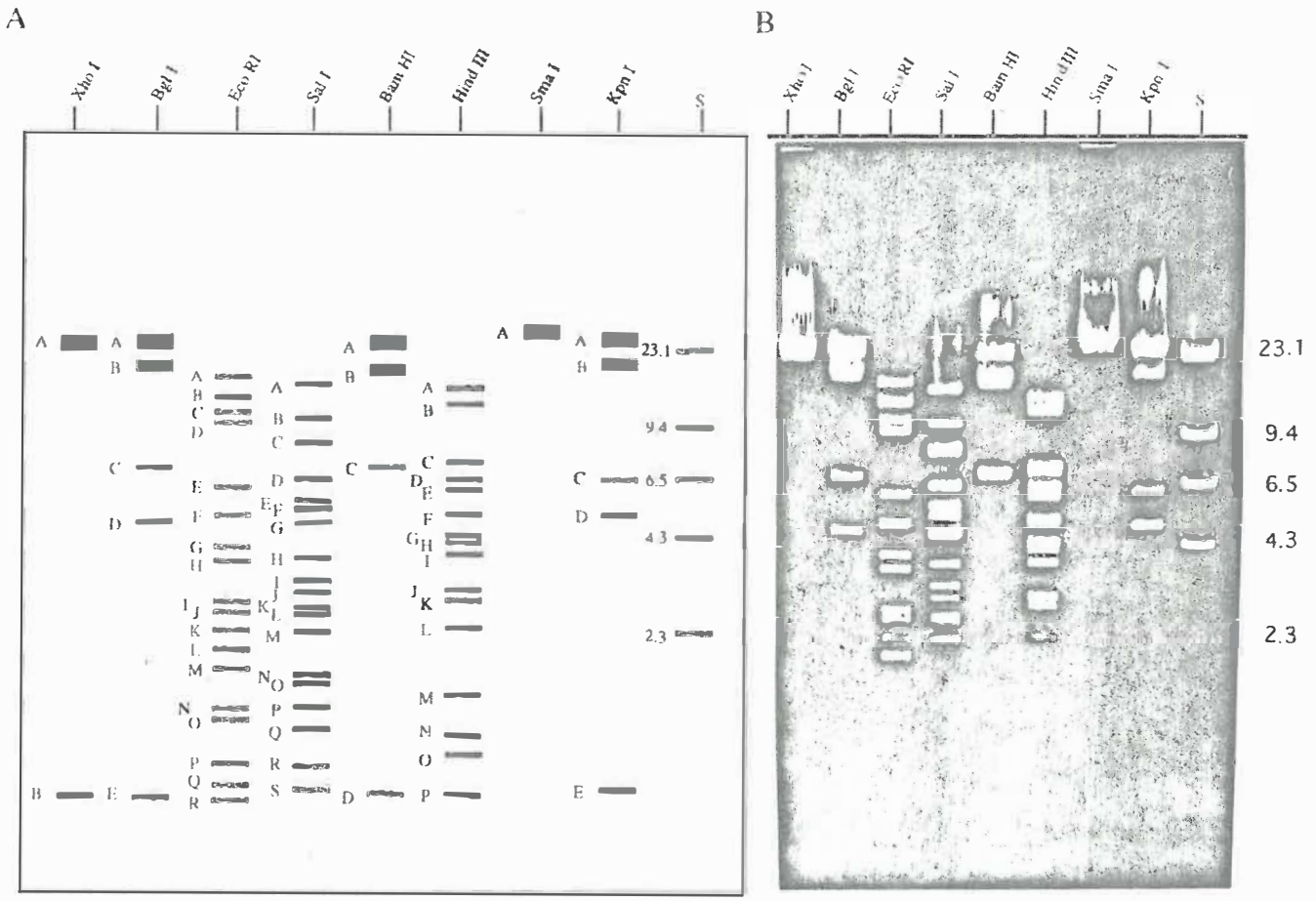

Fig. 1. Diagram of restriction endonuclease profiles and 0.6% agarose gel electrophoresis of AoGV DNA digested with Xho I, Bgl I, Eco RI, Sal I, Bam HI, Hind III, Sma I, Kpn I. S is Hind III-cleaved λ phage DNA standard (in kbp).

INTERACTIONS BETWEEN TWO NATURAL VIRUS PATHOGENS OF *Ocnogyna baetica* (LEP., ARCTIIDAE) LARVAE

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Summary

The interactions between a baculovirus (ObGV) and an entomopoxvirus (ObEPV) affecting larval populations of the winter webworm, *Ocnogyna baetica* (Rambur) in Southern Spain have been studied by laboratory bioassays. Third-instar *O. baetica* larvae were fed individually with known doses of both viruses either separately or combined simultaneously. Fat body cells were susceptible to infection of either viruses. There were fat body cells with simultaneous infections of both viruses but double infections were not observed in any single cell. ObGV inclusion bodies were detected also in hypodermis and Malpighian tubules cells. The simultaneous treatments involving the lowest dosage of one virus and the highest dosage of the other one caused lower mortalities than those expected according to an independent action. Significant antagonist effect was observed when the highest dosage of ObGV was in the combination.

1. Introduction

The arctiid *Ocnogyna baetica* (Rambur) is an endemic species of the Mediterranean region (Gómez de Aizpúrua, 1986), whose larvae are polyphagous and live gregariously in large colonies beneath a nest of white webbing on the ground or on weeds growing on wasteland (Benítez Morera, 1927; Cañizo 1928). After the third molt, these larvae scatter in all directions and even wander over considerable distances in search of food. Thus, the winter webworm could produce economical injuries, feeding on crop plants as alfalfa, beans, artichoke, lettuce, sugar beet, wheat and grapevines (Benítez Morera, 1927, 1942; Cañizo, 1928; Domínguez, 1976; Silvestri 1905).

Although the winter webworm has a great number of natural enemies in Southern Spain (Lipa et al., 1993c) pest control measures are needed. The use of entomopathogens (Barreiro and Santiago-Álvarez, 1985) against this pest have been proposed as an alternative for reducing the use of chemical insecticides.

A baculovirus of the granulosis type (ObGV) and an entomopoxvirus (ObEPV), have been recently described (Vargas-Osuna et al., 1991; Vargas-Osuna et al., 1993a; Lipa et al., 1993a,b) from *O. baetica* larvae. Both viruses have been found infecting larvae of the same population but individuals with a double infection were not recorded (Lipa et al. 1993c). The most

common and widespread is the ObGV (Lipa et al., 1993c) that has shown a good insecticidal activity in laboratory (Vargas-Osuna et al., 1993a) and field assays (Vargas-Osuna et al., 1993b).

There is the possibility that the two viruses, if used as biological insecticides, might interact antagonistically or synergistically. This investigation was undertaken to determine the effects of exposure of *O. baetica* larvae to both pathogens simultaneously.

2. Materials and methods

The *O. baetica* GV and EPV were obtained from field collected larvae in Córdoba, and were produced by infecting *O. baetica* larvae with the original suspension. They were then purified according to the method previously described (Lipa et al., 1993a; Vargas-Osuna et al., 1993a).

The larvae used in our tests were field collected as first- and second-instar and maintained at laboratory conditions (26±2°C, 60±5% RH and 16 h day length) on a semidefined medium (Santiago-Álvarez y Vargas-Osuna, 1986). Then newly molted third-instar larvae were chosen for the assays.

Both pathogens were bioassayed individually or simultaneously. Selected larvae, exposed to both virus individually, were placed in individual plastic cups (30 mm in diameter), one per cup, and were fed alfalfa leaf disks (5 mm diameter) treated by means of a microapplicator with 3 µl of ObGV or ObEPV suspension of a known concentration which give the following dosages: 34 IBs/larva and 4938 IBs/larva for GV; 1380 IBs/larva and 30200 IBs/larva for EPV. The larvae exposed to both viruses simultaneously fed alfalfa leaf disks that contained: 1) the two lowest dosages of both viruses; 2) and 3) the lowest dosage of one virus and the highest dosage of the other one.

These suspensions were made in sterile distilled water to which 0,1 % of a spreader (AGRAL) had been added. As a control, leaf disks were treated with 3 µl of sterile distilled water plus AGRAL. At least 70 larvae were used for each dose. Larvae that had not eaten the treated disks after 36 hours were discarded; the others were transferred to new plastic cups and supplied with untreated semidefined media. The assay was conducted at 26±2°C. The mortality of larvae was recorded every 24 hours until pupation and the cause of the death was determined by examination of their tissues with phase contrast microscope.

Five days post-treatment, every 48 hr, the same number of infected and control larvae were fixed in alcoholic Bouin, embedded on paraffin and 8µ sections were stained according to the method of Hamm (1966) and observed under the light microscope to record the development of the diseases.

Chi-square test was used for comparison of expected mortality with the observed mortality. Synergism or antagonism, respectively, are indicated when the observed percent mortality is significantly greater or less than expected.

3. Results and discussion

Table 1 shows the mortality of third-instar *O. baetica* larvae in test. Since the larvae used were field collected is not surprising to observe mortality caused by some of the natural factors limiting populations of the winter webworm in Southern Spain (Lipa et al., 1993c). Most of the larvae were killed by a solitary parasitoid, *Cotesia plutellae* (Hymenoptera: Braconidae), that emerged from 1 to 6 days post-treatment so that parasitized larvae could be excluded from the bioassay. This is the most abundant parasitoid of *O. baetica* larvae in Southern Spain that normally emerge from second or third-instar larvae (Lipa et al., 1993c).

Table 1. Mortality of *Ocnogyna baetica* larvae treated with ObGV and ObEPV.

		No. larvae killed by			
Treatment	N	Parasite	GV	EPV	GV+EPV
Control	101	54	1	2	0
g	75	25	8	1	0
G	122	65	29	3	0
e	78	44	0	3	0
E	105	70	2	9	0
g + e	85	39	3	7	4
g + E	72	38	0	9	2
G + e	74	43	9	1	2

N = Number of larvae in test.
g, e: the lowest dosages of GV and EPV, respectively.
G, E: the highest dosages of GV and EPV, respectively.

The mortality of third-instar *O. baetica* larvae caused by the viral suspensions is summarized in Table 2. Although a slight incidence of ObGV and ObEPV on *O. baetica* natural population larvae was observed, the mortality due to each virus disease increased with dosage. The ObGV dosages caused a mortality 2-fold higher than those concerning ObEPV.

In every one of the simultaneous treatments the total mortality, that was caused by both viruses, gave values of the same order (Table 2). The percentage of the larvae died by each virus, on the three combinations, was directly related with the dosage, nevertheless the mortality due to GV was lowered in respect to the single treatment (Fig. 1). On the other hand, some larvae died by double infection as has been observed on *Choristoneura fumiferana* and *Malacosoma disstria* treated with combinations of a Baculoviridae and a Reoviridae (Harper, 1986).

Table 2. Mortality percentages of *Ocnogyna baetica* larvae treated with ObGV and ObEPV.

Treatment	n	% Mortality due to				
		GV	EPV	GV+EPV	Total	Corrected
Control	47	2.13	4.26	0.00	6,38	-
g	50	16.00	2.00	0.00	18,00	12,41
G	57	50.88	5.26	0.00	56,14	53,15
e	34	0.00	8.82	0.00	8,82	2,61
E	35	5.71	25.71	0.00	31,43	26,76
g + e	46	6.52	15.22	8.70	30,44	25,70
g + E	34	0.00	26.47	5.88	32,35	27,74
G + e	31	29.03	3.23	6.45	38,71	34,53

n = Number of larvae in test, excluding parasitized larvae

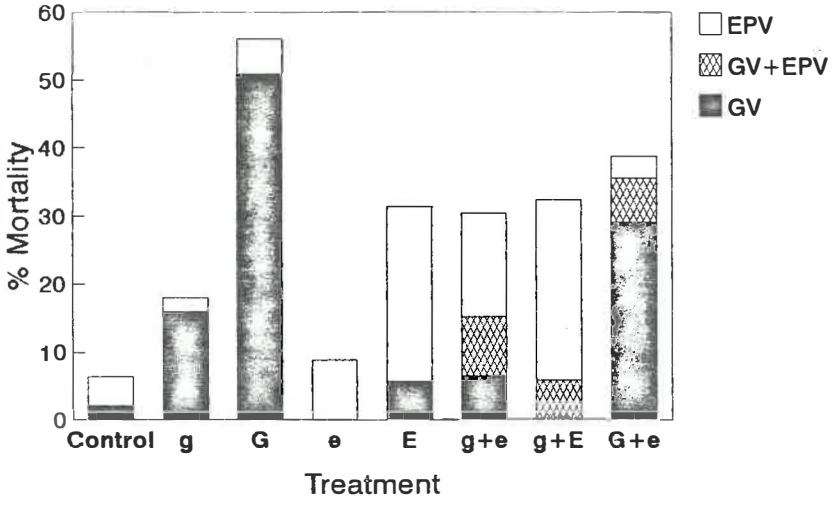

Figure 1.- Comparison of the mortality percentages of *Ocnogyna baetica* larvae treated with ObGV and ObEPV.

When the larvae were simultaneously treated with the lowest dosages of the two viruses, the total mortality was slightly higher than the sum of the mortalities caused by each virus alone. On the other hand, the simultaneous treatments involving the lowest dosage of one virus and the highest of the other one caused lower mortalities than those expected according to independent action. An antagonism effect (χ^2 = 5.681; 1 d.f.) occurred when the highest dosage of ObGV was in the combination. A lower mortality than either virus separately has been also reported when NPV and GV were fed simultaneously to *Heliothis armigera* (Wihtlock, 1977).

Histopathologically differences between viruses were observed. The ObGV inclusion bodies were detected in fat body, hypodermis, and Malpighian tubules cells, whereas the ObEPV spheroids were only present in the fat body as occur with the majority of lepidopteran entomopoxviruses (Granados, 1981).

The histopathology of larvae exposed simultaneously to both viruses showed four different outcomes: 1) larvae with tissues only infected by ObGV; 2) larvae with tissue only infected by ObEPV; 3) larvae with fat body infected by both viruses on adjacent cells; and 4) larvae with the fat body only infected by EPV, and the GV infection was restricted to the hypodermis. These results suggest a competition between GV and EPV to partitioning the fat body cells for replication (Harper, 1986). This interaction appear to be disadvantageous for GV that can not be able to utilize the fat body as efficiently as in single infections.

The observed interaction at tissue level could explain why the same dose of GV produce different mortalities when is given alone or in combination with de EPV.

These observations may have a significant implication for winter webworm pest management with viral insecticides.

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OIL FORMULATION OF INSECT VIRUSES

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Summary

Each of 6 vegetable oils, 12 mineral oils and an anti-evaporant were assessed for palatability on cotton leaves to *Spodoptera littoralis* larvae and for their effect on potency of unpurified *S. littoralis* nuclear polyhedrosis virus (NPV) stored in oils over 18 months at 4°C. Results show vegetable oils to be more palatable than mineral oils, but with a water control being most preferred. Rates of pupation in some treatment groups were significantly lower than in the control group. Potency ratios over the period of storage fell to a mean of 0.07 relative to a standard NPV. NPV stored in arachis oil gave the least reduction in potency and on that basis appeared to be the best candidate for use in oil-based formulations.

The solubilities and suspension stabilising properties of a range of additives were assessed in arachis oil. Two promising additives were further investigated through settling tests with oil-milled unpurified NPV and suspensions stable for several hours were achieved.

1. Introduction

Insect pathogenic baculoviruses offer a method of pest control that is safe, cost-effective and environmentally benign. They are particularly suited for use in developing countries where they can be produced locally. Research at the Natural Resources Institute has been directed at the development of baculoviruses for control of a number of major crop pests of developing countries, including *Spodoptera* spp. and *Helicoverpa armigera*. A wettable powder formulation of a NPV has been produced for control of *S. littoralis* on cotton (McKinley *et al.*, 1989). Research is now concentrated on the development of a range of formulations for application against pests under varying environmental conditions and in different crops. This paper reports initial results with the development of an oil-based formulation for ultra-low volume application. In this study *S. littoralis* NPV has been used as a model system.

2. Materials and methods.

Assessment of palatability

Each of 20 treatments (listed in table 1) including a distilled water control were applied to both sides of

cotton leaves excised from plants at the 6-10 leaf stage. Third instar larvae of *S. littoralis* were fed on treated leaves in Petri dishes for 16 hours and then individually transferred to $30cm^3$ pots with artificial diet (McKinley et al., 1984) where they were allowed to continue development until pupation. Leaf areas consumed were determined by image analysis. The experiment was replicated five times.

Assessment of potency after storage:

Spray-dried, unpurified NPV was added to samples of the 19 treatments to achieve suspensions of approximately $1x10^8$ polyhedral inclusion bodies (pib) ml^{-1}. Samples were thoroughly mixed and stored at 4°C in complete darkness.

Virus was recovered at intervals over an 18 month period from each oil by low speed centrifugation followed by acetone extraction and resuspended in distilled water. In addition to the 19 treatments, two control treatments were prepared. The first comprised the same spray-dried NPV washed in acetone then resuspended in distilled water, the second comprised spray-dried powder resuspended in water alone. From each treatment five doses were prepared in a five-fold dilution series with top dose of approximately $2.0x10^7$ pib ml^{-1}. Virus samples were bioassayed in *S. littoralis* neonate larvae using a modification of the standard droplet dosing technique (Hughes and Wood, 1981). A purified aqueous suspension *S. littoralis* NPV was also included in each bioassay as a standard treatment. Natural mortality was determined in each assay by a single water control treatment.

Seven day larval mortality data were subjected to probit analysis using the Maximum Likelihood Program (Rothamsted Experimental Station, Harpenden, UK) and LC_{50} values were obtained from slopes constrained to parallelism. Relative potencies were derived from standard suspension LC_{50}s divided by sample LC_{50}s.

Suspension of virus in oil

Suspensions of spray-dried, unpurified NPV were prepared in arachis oil (0.5g in 250ml) with and without a range of stabilising additives comprising alkylated vinylpyrrolidone polymers, sorbitan oleates, ethoxylated phosphate esters and polyethoxylated organics at 1, 3 and 5% (weight/volume). The latter were selected on the basis of their low hydrophillic/lipophillic balance values coupled with probable solubility in arachis oil. Times for 90% sedimentation were measured (19cm drop at 30°C in a vibration free bath). Unpurified NPV milled in oil to give a mean particle diameter of approximately 3μm was likewise suspended in oil with and without stabilising additives.

3. Results

Palatability

Mean leaf areas eaten ranged from 1113.8mm^3 for the control to 370.5mm^3 for light paraffinic mineral oil. Analysis of variance showed that significant differences ($p=0.0065$) exist between treatment means. None of the vegetable oils gave means significantly different from the control ($p=0.05$). All pure mineral oils with the exception of Norpar 12 and mineral seal oil gave means significantly lower than the control ($p=0.05$). The anti-evaporant and control means were not significantly different.

Significant differences in pupation rates ($p=0.0127$) also existed between some treatments. The highest rate of pupation (100%) occurred in the control group. None of the vegetable oils caused significantly lower pupation. Five treatments, all mineral oils, gave significantly lower pupation.

Storage

Final potency ratios relative to the standard treatment ranged from 0.6173 for NPV stored in arachis oil (14 months storage) to 0.0013 for refined and deodorized soya oil. There was no clear division between vegetable and mineral oils.

Suspension of virus in oil

Unpurified freeze-dried NPV sieved into fractions, with particle sizes ranging from 500μm to 80μm, and blended with pure arachis oil all showed >90% sedimentation in under 0.5hr even with the addition of china clays to the finest fraction to increase suspension viscosity. No improvement in suspension time was recorded with any of the additives. Virus milled in oil however recorded a stable suspension in excess of three hours which with the addition of either a sorbitan oleate or an ethoyxlated phosphate ester increased to in excess of six hours with the sediment readily resuspended after standing for several days.

4. Discussion

Arachis oil has been chosen as a carrier for NPV in low volume applications on the basis of these preliminary studies because it did not demonstrate anti-feedant properties (essential as NPV has to be ingested) and sustained higher NPV potency over 18 months storage than any of the other treatments. Further work on long-term

storage in arachis oil at 26°C is being carried out now to confirm these findings.

Clumping is a general feature of solids in oils and is due to insufficient charges on particles to keep them apart. The combination of oil milling and the addition of soluble surfactants produced stable suspensions. Future work will consider application techniques and the palatability of surfactants in suspension.

Table 1. List of treatments.

Sample	Source
Isopar M	Exxon
Isopar V	Exxon
Norpar 12	Exxon
Norpar 13	Exxon
Norpar 15	Exxon
Mineral seal oil	BP
Light paraffinic mineral oil	BP
50 spindle	BP
150 solv neutral	BP
Refined & deodourised rapeseed oil	Seatons
Rapeseed refined	Seatons
Refined & deodourised soya oil	Seatons
Refined & deodourised cottonseed oil	Seatons
Risella oil (L)	Shell
Risella oil (EL)	Shell
Actipron	BP Research
Anti-evaporant	Shell
Alkali refined linseed	Seatons
BP Arachis oil	Seatons
Distilled water	

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COMPARISON OF TWO *BACILLUS THURINGIENSIS* ISOLATES *IN VIVO* AND *IN VITRO*

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Introduction

As mentioned by BENZ and JOERESSEN (1993) the new *Bacillus thuringiensis*-isolate K24 belongs to the same H serotype 14 as the well known ssp. *israelensis* (ISR). However, although the plasmid spectra of the two isolates are similar, they are not identical, and although ISR and K24 are toxic for mosquito larvae, only K24 is pathogenic to sawfly larvae. This knowledge led us to more detailed comparative investigations in order to find out how far the two strains differ.

Materials and Methods

Pathogens and purification of δ-endotoxins

K24 is described by BENZ and JOERESSEN (1993). The ISR strain 4444 was obtained from Prof. P. LÜTHY of the Microbiology Institute of ETH Zurich. The strains were cultivated in Fernbach bottles on a medium containing 0.3% glucose (to produce larger parasporal bodies). The spores plus parasporal bodies (SP + PB) were harvested from the cultures after lysis of the sporangia. Purification and solubilization of parasporal bodies were carried out as described by HUBER *et al.* (1981) and CHEUNG and HAMMOCK (1985). The parasporal δ-endotoxins were dissolved in 50 mM Na_2CO_3, pH 11 and dialysed against PBS-buffer.

Biotests with insects

Biotests with mosquito larvae were made by adding defined concentrations of SP + PB to the water in the test beakers. Feeding-stop as a function of the time-dosis-effect relationship was determined by adding wettable soot to the test beakers. The soot particles are fed by healthy larvae and their gut becomes soon black, whereas the guts of intoxicated larvae remains lucid.

Several sawfly species (see BENZ and JOERESSEN, 1993) were tested by feeding 4th instar larvae *ad libitum* on foliage of their host plants treated with the SP + PB complex of either ISR or K24 or by microfeeding the larvae with droplets of known volume (2 or 5 µl) from micropipets containing 50% sucrose as phagostimulant and different SP + PB concentrations. Intralymphal injection with 2 or 4 µl of SP + PK suspensions or dissolved δ-endotoxin was carried out via an abdominal proleg of 4th instar larvae.

Haemolytic activity

Washed human erythrocytes were suspended in phosphate buffered solution contain-

ing 800 µg dissolved ISR- or K24-δ-endotoxin per ml and incubated at 15°C. The control was treated with KCN which caused 100% haemolysis. The free Fe^{2+} was oxidized to Fe^{3+}, coupled to cyanid, and measured at 546 nm.

Cytotoxic activity in mammalian V79 cells in vitro (s. JOERESSEN *et al.*, 1993).

Competitive binding to cell receptors

In order to investigate whether the two δ-endotoxins share a common receptor or receptors in close vicinity on the V79 cells, exposure to the ISR endotoxin (33 µg/200 µl medium) was combined with K24 endotoxin exposure levels of 0, 33, 66, and 99 µg / 200 µl, synchronously or subsequently. The endotoxin activity was measured by the neutral red uptake (s. JOERESSEN *et al.*, 1993).

Analysis of δ-endotoxin polypeptides

Continuous gradient SDS-polyacrylamide gel-electrophoresis (10-15% SDS-PAGE) was carried out. δ-endotoxin samples were dissolved by incubating for 10 min at 100°C in SDS-sample buffer (pH 6.75). Gels were stained with Coomassie brilliant blue.

Polyclonal antibodies

Polyclonal antibodies were produced in rabbits against tree antigens: (i) the 25 kDa peptide of the ISR δ-endotoxin, (ii) the mixture of the six 23 to 25.5 kDa peptides of the K24 δ-endotoxin, (iii) the three K24 specific low molecular weight (17.5-18.5 kDa) peptides of the K24 δ-endotoxin. ELISA was used for measuring cross reactions.

Results

Biotests with insects

The pathogenicity of the two tested strains to mosquito larvae was not different. However, Sandoz Inc. has an ISR strain that is three times more toxic for *Aedes aegypti* than K24 (D.BASSAND, oral comm.) and thus the ISR strain 4444. There was also no difference between ISR and K24 in the toxicity for the beneficial *Chironomus tentans*. However, a tenfold higher dose is needed than for *Aedes*.

In contrast to the above results, the parallel biotests with sawfly larvae of the families Pamphiliidae, Diprionidae and Tenthredinidae demonstrated a great difference between ISR and K24. Microfeeding with a dose of 7.2×10^4 K24 SP+PB per ml caused 100 % mortality in *Gilpinia hercyniae* larvae, whereas SP+PB of ISR had no effect. If the dissolved δ-endotoxins alone were microfed, none of the larvae died. Upon *ad libitum* feeding, too, only the ingestion of K24 SP+PB exerted dose dependent pathogenic effects in the larvae of *Acantholyda erythrocephala, Cephalcia abietis, Gilpinia hercyniae*, *Neodiprion sertifer*, *Pristiphora abietina*, *Nematus melanaspis*, and *Athalia rosae*. In contrast to the usually rapid effect of *B. thuringiensis* after it is fed by sensitive species the pathogenic effects of K24 develop slowly in sawfly larvae. They die after 5 to 7 days without a foregoing feeding-stop. Intralymphal injection of up to 4400 ng dissolved K24 or ISR endotoxin per larva led to rapid paralysis and death. No

strain-specific difference could be observed. The same is true after intralymphal injection with SP+PB which caused rapid death with both strains, beginning 5 hr after injection and reaching a maximum after 24 hr as a consequence of the growth of vegetative cells in the haemolymph.

In vitro toxicity

The ISR δ-endotoxin is strongly cytolytic to human erythrocytes (300 µg endotoxin per 400 µl suspension) and to V79 Chinese hamster lung fibroblasts (s. JOERESSEN *et al.* 1993). For K24 similar effects were only observed with the highest dose and longest incubation time.

Competitive binding to cell receptors

The binding studies on V79 cells indicate that the endotoxins of both, ISR and K24, bind to the same cell receptors but only the ISR-endotoxin is cytotoxic. Simultaneous application of ISR- and K24-toxin (100 µg K24- and 33 µg ISR-toxin) to V79 cells reduced the toxicity of the ISR-endotoxin by 55 %. Pre-incubation with non-cytotoxic K24-endotoxin for 30 minutes decreased the toxic effect of ISR-endotoxin by 90% whereas replacement of the cell medium after pre-incubation (i.e. removal of the non-bound K24-toxin molecules prior to the exposure of the cells to the ISR-endotoxin) leads to a reduction of 70% only.

Analysis of δ-endotoxin polypeptides

The δ-endotoxins of ISR and K24 differ in their peptid spectra (Fig. 1). In SDS-PAGE a cluster of six bands (23, 23.5, 24, 24.5, 25 and 25.5 kDa) is observed for K24 instead of the single haemolytic and cytotoxic 25 kDa peptide in ISR. The K24-endotoxin contains 3 specific peptides (16.5, 17.5 and 18.5 kDa) that are missing in the ISR-endotoxin (and in other *B.t.* subspecies).

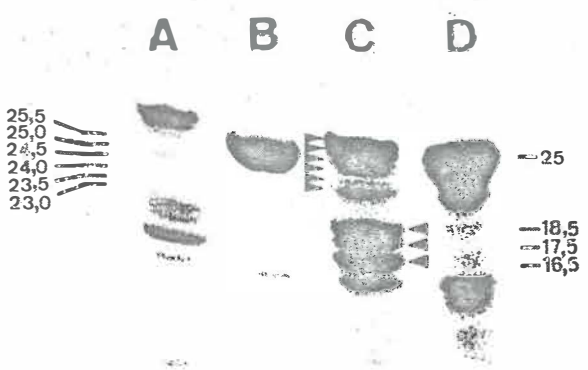

Fig. 1: Polypeptide spectra of the δ-endotoxins of the K24 (A, C) and ISR (B, D) strains of *Bacillus thuringiensis* on a continuous 5 to 20% SDS-PAGE gradient gel. A,B with 50 µl, C,D with 80 µl δ-endotoxin solution.

Immunological studies

The immunological studies show that antibodies against the cytotoxic 25 kDa peptide of the ISR-endotoxin (CHEUNG and HAMMOCK , 1985) react strongly with the full ISR-endotoxin but - in agreement with the SDS-PAGE results - cross-react only weakly with the full K24-endotoxin. The antiserum against the K24 splitted bands of 23 to 25.5 kDa cross reacts but weakly with the full ISR-endotoxin, and less than the antiserum against the three 16.5 to 18.5 kDa K24 peptides. Thus, although one of the six K24 peptides with 23 to 25.5 kDa has the MW of 25 kD, it is probably not identical with the 25 kDa peptide of the ISR-endotoxin. On the other hand, the three lower molecular peptides of K24 must be part of the ISR-endotoxin as it is weakly recognized by their antibodies.

Discussion

Biotests with insects: Although no difference in the mosquitocidal activity of the two strains was found, a clear difference exists in their sawfly pathogenicity. Only K24 infects sawfly larvae. The difference seems to depend on the power to overcome the gut wall. As soon as the two organisms (spores and/or δ-endotoxin) are in the blood, they are pathogenic. Since microfeeding with K24 δ-endotoxin does not harm sawfly larvae, the pathogenicity of K24 seems to depend mostly on the spores. This is also suggested by the observation of the slow development of the disease and that no early feeding-stop can be observed. This slow pathogenesis stands in contrast to the quick effects of the δ-endotoxins as it is also found in the mosquitocidal action of the K24 δ-endotoxin.

In vitro toxicity on mammalian cells: It is astonishing that, although the ISR- and K24-endotoxin bind to the same cell receptors of hamster fibroblasts, only the ISR-endotoxin is cytotoxic. The underlying mechanism is not known. The same mechanism is probably also responsible for the haemolytic activity of the ISR-endotoxin.

The polypeptide spectra: There can be little doubt that the differences between the two B.t. strains depend to a large degree an the different polypeptide spectra of the specific δ-endotoxins. The absence of haemolytic and cytotoxic activity of the K24-endotoxin could be due to the replacement of the 25 kDa peptide by a series of 6 peptides.

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Natural occurrence of *Bacillus thuringiensis* on cauliflower and grass foliage.

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SUMMARY:
The population dynamics of naturally occurring *Bacillus thuringiensis* strains on the phylloplane of cauliflower was studied. A technique using stomacher blending was shown to be the most effective for extraction of sporulated *Bacilli* spp. from phylloplane material. A fluctuation in the number of extracted sporulated Bt isolates was seen during the growth season. Based on diversity studies of a limited number of isolates (20), we were able to divide the strains into different groups using biochemical reactions, crystal morphology, bioassay, PCR and RAPD.

INTRODUCTION:

The bacterium *Bacillus thuringiensis* (Bt) has been found to occur naturally in many different habitats. The spores have been recovered from nearly every kind of soil in most parts of the world (Martin and Travers, 1989), from diseased insects (Ohba *et al.*, 1979), and more recently, from the phylloplane of several deciduous and conifer tree species (Smith and Couche, 1991).
Bt is known to multiply in susceptible insects that have ingested a sufficient amount of spores and crystals. Many experiments have been carried out to evaluate the ability of Bt spores to germinate and multiply in soil (Pruett *et al.*, 1980; West *et al.*, 1984). However, nearly nothing is known about Bt´s population dynamics on the phylloplane.
The objective of this study was to describe the dynamics of naturally occurring Bt strain(s) over time. We defined the time frame as one growth season and cauliflower as the phylloplane to be analyzed. In conjunction with the phylloplane sampling, insect species present on the phylloplane and soil beneath the plants were also sampled. Bt strains were isolated from these habitats and characterised by the use of various methods, to clarify the possible diversity of the isolates. In addition grass foliage was sampled as a part of the methodology test.

METHODS AND MATERIALS:

Sampling:

Cauliflower foliage was collected from an organic field (Slangerup, Denmark), with no previous history of Bt-application. Five leaves were collected regularly during the growth season, from mid-June to mid-October. The leaves were stored at -10^{0}C for up to three months before processing.
Grass foliage (*Lolium perenne*) was collected from a grazing pasture (Wageningen, The Netherlands).

Testing of method for extraction from phylloplane material:

Grass foliage was cut into pieces of approximately 1 cm; 1 g of foliage was used per extraction. From two cauliflower leaves, 13 discs were prepared (Ø=13 mm) and used for each extraction ($\approx$ 0.75 g wt/wt). The experiment was repeated twice.
The plant material was shaken with 20 ml sterile PBS or water at 200 RPM on a rotary shaker for 2 h, or blended in 20 ml PBS or water for 3 x 30 sec using a Stomacher®. The supernatant was collected, spun down (25 min, 4300 x *g*), and the pellet was resuspended in 2 ml PBS and heat treated for 30 min at 65^{0}C. Suspensions were plated in triplicate on T_3 sporulation agar (Travers *et al.*, 1987). Plates were incubated at 30^{0}C for 24 h upon which the number of sporulated *Bacilli* was recorded.

Isolation procedure for population dynamics:
From each day of sampling, 5 g of cauliflower foliage from two leaves were cut into small pieces. These were extracted in 20 ml PBS for 3 x 30 sec using a stomacher. The supernatant was collected and spun down (25 min, 4300 x *g*), and the resulting pellet was resuspended in 2 ml PBS and heat-treated for 30 min at 65°C. Suspensions were plated in triplicate on T_3 sporulation agar (Travers *et al.*, 1987). Plates were incubated at 30°C and colonies counted after 24 h. Colonies having a rugose and "ice-crystal" appearance and a diameter > 2 mm were selected and subcultured on sporulation agar. Isolates were examined using a phase-contrast microscope and cells containing a crystal inclusion in the sporangium were determined to be Bt, whereas the rest were classified as *Bacillus cereus*. Crystal morphology was noted.

Characterization:
10 putative Bt isolates from each of two cauliflower leaves sampled in mid June were selected for further characterization.

Biochemical tests: A ID32 STAPH identification kit (bioMérieux, France), containing 26 enzyme and fermentation reactions was used to characterize the isolated bacteria. *Staphylococcus haemolyticus* (ATCC 15796), *Staphylococcus sciuri* (ATCC 49574) and *Staphylococcus lentus* (ATCC 49575) were used as positive and negative controls.

Bioassay: Bt isolates were inoculated in 20 ml Nutrient Broth (Difco) and incubated for 5 days at 30°C on a rotary shaker, after which microscopic examination was used to verify the presence of spores and crystals. These were recovered by centrifugation (30 min, 4300 x *g*) and resuspended in 20 ml sterile water + 500 µl Triton X-100. 15 µl of this suspension were spotted onto 24 15 mm cabbage-leaf discs and air-dried. One *Pieris brassicae* larvae (L_2-L_3) was placed on each disc, and incubated at 20°C. Mortality was recorded after 72 h.

Polymerase Chain Reaction: 0.1 µg DNA was used in a PCR reaction containing 0.1 U Taq-polymerase, 0.2 mM dNTP, 2 mM MgCl. Specific primers: 2 sets of primers specific for cry-IA were used in these reactions (Lep1 and Lep2) (Carozzi *et al.*, 1991). DNA was amplified on a Thermal Cycler (Perkin Elmer) using the sequence: 5 sec at 94°C, 45 sec at 45°C and 2 min at 72°C. Random Amplified Polymorfic DNA (RAPD): PCR was performed using a 10-mer primer (5'AGTCACCCAC3'). The DNA was amplified using the following programme: 5 sec at 94°C, 1 min at 32°C and 2 min at 72°C.

RESULTS AND DISCUSSION:

EXTRACTION PROCEDURE:
Data from the extraction of cauliflower leaf-discs using shaking and stomacher blending is shown in Table 1. Stomacher blending extracts the highest count of spore-forming *Bacillus* spp. and there seems to be no difference between the various extractants. This pattern was also observed using grass-foliage. Donegan and co-workers have also reported the use of stomacher blending to be the most efficient method for extraction of the gram-negative *Enterobacter* spp. from bean leaves (Donegan *et al.*, 1991).

POPULATION DYNAMICS:
The data from sampling of isolates in the cauliflower field indicate that the mean Bt bacterial count fluctuates only to a small degree (Fig. 1). Bt constituted about 10 % of the total Bt/Bc pool present on the leaves.

The number of sporulated Bt was in the range of $8x10^1$- $1.7x10^3$ CFU/cm^2 cauliflower leaf (weight/area of cauliflower leaves was 0.04 g/cm^2). The range is in the same order of magnitude previously found on the phylloplane of several deciduous and conifer tree species (Smith and Couche, 1991).

Table 1. Efficiencies of techniques used to extract sporulated *Bacilli* from cauliflower foliage.

Mean (log CFU/g)	Treatment
2,62 A[a]	2 h shaking (water)
2,67 A	2 h shaking (PBS)
3,00 B	Stomacher blending (Water)
3,02 B	Stomacher blending (PBS)

[a] Proc ANOVA, SAS: means followed by the same letter are not significantly different at the 5% level.

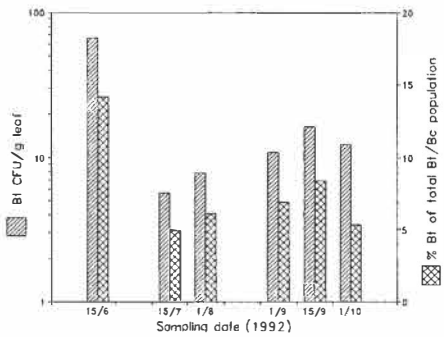

Fig. 1. Mean number of sporulated naturally occurring *Bacillus thuringiensis* (CFU/g leaf) and percentage sporulated Bt of the total Bt/*Bacillus cereus* population (%), on cauliflower leaves.

CHARACTERIZATION OF STRAINS:

26 biochemical tests was used to separate the Bt isolates into three major groups. Nine isolates (DBt 9, 12, 16, 18, 36, 37, 39, 41, 44) have 100 % homology in all the tests. The next group of seven isolates (DBt 11, 14, 38, 40, 42, 43, 45) have differences in six reactions compared to the first group. When looking at the biochemical reaction pattern, it is obvious that these two groups are very similar based on their phenotype. The last four isolates comprise a diverse group. They do not react positively in the arginine dihydrolase (ADH) or urease (URE) reactions, and further seven reactions gives results different from the first group.

These findings differ only slightly from data of Sneath (1986), who found all Bt and Bc strains to have positive glucose (GLU) and maltose (MAL) reactions, which we do not find. The opposite is the case for ADH and alkaline phosphatase reactions.

There is a major discrepancy between our results and those of a study using biochemical tests to analyze diversity of 854 Bt isolates from soil (Martin *et al.*, 1985). They find only 43 % of the isolates to be positive in the Voges-Proskauer reaction, 42 % positive in the esculin reaction and 6 % positive in the URE reaction. In contrast, we find 80-100 % of our isolates giving positive reactions in all three of these tests.

Nevertheless some phenotype diversity between Bt strains is expected, as indicated by de Barjac and Frachon (1990). They use diversity in reactions which are considered to account for all Bt strains, to distinguish different Bt serotypes.

In the experiments set up to describe diversity among the 20 selected isolates, it can be seen that the use of crystal morphology, bioassay and PCR gave similar results (Table 2). Strains having crystal inclusions with a bipyramidal shape, used as visual evidence of cry-gene activity, showed insecticidal activity against *Pieris brassicae*, and gave PCR signals of the size expected for cryIA-genes. It is surprising, however, that all of the bipyramidal-crystal-forming isolates showed activity against *Pieris brassicae*. In a similar experiment using isolates from an animal feed mill, only 50 % of the bipyramidal-crystal-producing isolates showed activity against *Pieris brassicae* (Meadows *et al.*, 1992). Bipyramidal crystals are also produced by Bt subsp. *israelensis* containing cryIVA and cryIVB genes active against Dipterans (Beegle and Yamamoto, 1992).

In terms of the insecticidal specificity of the strains, we find 16 of 20 isolates to be active against Lepidopterans. This toxicity group tends to be predominant in soil (Martin and Travers, 1989), in dust (Meadows *et al.*, 1992) and on living phylloplane (Smith and Couche, 1991), although predominance of Coleopteran-specific strains on dried tobacco leaves has been reported (Kaelin and Gadani, 1993).

The use of PCR enabled us to determine that the majority of the isolates contain cryIA genes, the exceptions were DBt 10, 13, 15 and 17. CryIB and CryIC are also active against *Pieris brassicae* (Höfte and Whiteley, 1989; Visser et al., 1988), but the 4 isolates also do not appear to produce these toxins, since no activity

The use of PCR enables us to determine that the majority of the isolates contain cryIA genes, the exceptions are DBt 10, 13, 15 and 17. Nor do these four isolates appear to contain cryIB or cryIC, since no insecticidal effect against *Pieris brassicae* was detected (Höfte and Whiteley, 1989; Visser *et al.*, 1988).
Using RAPD, four distinct groups (A-C) of band patterns were found. Within group A, a subdivision into two groups was made. Group A2 lacks one band present in group A1. Group A includes 16 of the 20 isolates and is similar to the group having bipyramidal crystals and Lepidopteran activity. The remaining four isolates were grouped according to three different patterns, thus subdividing the isolates with spherical crystals. The differentiation between strains DBt 13 and 15 is also seen by the biochemical tests. These tests also separate DBt 10 from DBt 17, neither of which are detected by the RAPD pattern with the primer used.

One of the strains isolated from grass leaves obtained from The Netherlands had a remarkable large crystal in comparision with other subsp. Transmission Electron Microscopy (TEM) revealed the crystal to be located within the exosporium and we believe this to be the reason for the association of spore and crystal after sporulation. The isolate was serotyped to subsp. *japonensis*, previously only described in Japan (Ohba and Aizawa, 1986). Bioassays using *Pieris brassicae* (Lep.) and *Tipula oleracea* (Dip.) showed no activity of our strain. Bioassays are in progress to test for activity against Coleopteran spp.

In conclusion, we found the use of stomacker blending for extraction of sporulated *Bacilli* to be the most effective technique. The number of extracted sporulated Bt isolates fluctuates during the growth season and the number of Bt isolates found on foliage is within the range previously reported for naturally occurring Bt on the phylloplane. From the diversity studies of a limited number of isolates (20), we were able to separate these strains into different groups using biochemical reactions, crystal morphology, bioassay, PCR and RAPD.

	Bioassay (mortality)	Crystal morphology	PCR LEP 1	PCR LEP 2	RAPD pattern
DBt 9	+ + +	BP	490	986	A1
DBt 11	+ + +	BP	490	986	A1
DBt 37	+ + +	BP	490	986	A1
DBt 40	+ + +	BP	490	986	A1
DBt 41	+ + +	BP	490	986	A1
DBt 44	+ + +	BP	490	986	A1
DBt 12	+ + +	BP	490	986, 908	A1
DBt 14	+ + +	BP	490	986, 908	A1
DBt 16	+ + +	BP	490	986, 908	A1
DBt 18	+ + +	BP	490	986, 908	A1
DBt 38	+ + +	BP	490	986, 908	A1
DBt 42	+ + +	BP	490	986, 908	A1
DBt 45	+ + +	BP	490	986, 908	A1
DBt 39	+ + +	BP	490	986	A2
DBt 36	+ + +	BP	490	986, 908	A2
DBt 43	+ + +	BP	490	986, 908	A2
DBt 10	-	S	NS	NS	B
DBt 17	-	S	NS	NS	B
DBt 13	-	Few S	NS	NS	C
DBt 15	+	Few S	NS	NS	D

Table 2. Characterization of 20 *Bacillus thuringiensis* isolates. Bioassay using *Pieris brassicae* larvae, + + +: 100% corrected mortality, +: < 10% corrected mortality, - : no mortality. Crystal morphology: BP = bipyramidal, S = spherical. PCR product profile using two sets of specific cryIA primers, LEP1 = 490bp. and LEP2 = 986, 906bp. Random Amplified Polymorfic DNA grouping pattern (A-D).

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PATHOGENICITY AND PRACTICAL USE OF TWO PROTOZOAN PATHOGENS ON *PROSTEPHANUS TRUNCATUS* (HORN) (COL., BOSTRICHIDAE) IN TOGO

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Summary
Two protozoans, a *Mattesia* species and a new species of the genus *Nosema*, were identified on *P. truncatus*, a dangerous storage pest in Togo.
In a jar trial the development of the pest population was observed after a treatment with protozoan spores. The mortality of the treated adults of *P. truncatus* was significantly higher than the mortality of the untreated adults with a natural protozoan infection. According to the high infection rate of 60 % of the dead beetles in the treated population it was considered that the spore-treatment caused the higher mortality of *P. truncatus*.
In two trials with a spore-application on stored maize cobs these results were not repeatable because of the low infection rate of the pest (between 2,5 and 32,5 %). Therefore the use of *Nosema* sp. and *Mattesia* sp. in practice seems to be not effective against the storage pest. Further research is needed to look for better application techniques, which lead to an increased infection on the Larger grain borer.

1. Introduction

The following investigations are part of a PhD-thesis, which were made in Togo at the "Service de la Protection des Végétaux" (Henning, 1993). The subject of the investigations was the storage pest *Prostephanus truncatus*, well-known as the Larger Grain Borer (LGB). This pest originates from Centralamerica and was accidentally introduced into Africa in the 70ies. On this continent LGB was found in the early 80ies in Tanzania (Dunstan & Magazini, 1981) and shortly afterwards in Togo (Krall, 1984). In both countries LGB causes high losses in traditional maize stores.
In order to control this dangerous pest in its new distribution areas in Africa, research on the existence and practical use of biological natural antagonists was initiated. For this reason a survey in several regions of Togo was made to look for natural insect pathogens on *P. truncatus*. Two protozoans, a *Mattesia* species, which was already known (Leliveldt et al., 1988), and a new species of the genus *Nosema* (Henning, 1993), were identified. A trial in jars and two trials under semi-practical conditions were made to examine the effectivity of the protozoans against LGB.

2. Methods

Jar trial

The aim of this trial was to find out, if the treatment with protozoan spores causes an increase of natural infection in the pest population, and furthermore what are the effects of the protozoans against LGB. Six jars were filled with an identical quantity of maize grains. Three jars were treated with spore powder containing 3 x 10^6 spores of *Nosema* sp. and 8 x 10^3 spores of *Mattesia* sp.; the other three remained untreated. Sixty adults of *P. truncatus* were placed into each jar. These beetles were taken out of a maize store and already naturally infected with protozoans.

Trials under semi-practical conditions

Two trials on nearly equal terms were made at different places. For the first trial little maize-stores, like those used in southern Togo, were built up in Cacaveli on the testing ground. The second trial were placed about 15 km to the north of Cacaveli.

The table shows the different treatments of the stores with protozoan-spore-powder and with the pyrethroid "Deltamethrin" (the commercial name of the insecticide is "K-Othrine"), which is recommended against LGB in this region.

Table: Treatment of maize stores with K-Othrine and with protozoan spores
(storage of 30 kg maize cobs per store)

variants	quantity of applicated preparation per store	dosage of active ingredient and spores per kg maize	
		trial I	trial II
control	untreated		
spore-treatment	75 g spore powder	$3x10^9$ *No** + $2,5x10^8$ *Ma**	$2,5x10^9$ *No* + $3x10^7$ *Ma*
insecticide-treatment	15 g K-Othrine dust	1 mg Deltamethrin	
insecticide-treatment	7,5 g K-Othrine dust	0,5 mg Deltamethrin	
combination of insecticide and spore-treatment	7,5 g K-Othrine dust + 75 g spore powder	0,5 mg Deltamethrin + $3x10^9$ *No* + $2,5x10^8$ *Ma*	0,5 mg Deltamethrin + $2,5x10^9$ *No* + $3x10^7$ *Ma*

* = spores of Nosema sp. and Mattesia sp. respectively

After the maize cobs were stored 40 healthy adults of *P. truncatus* were added into each store. Every 4 weeks a sample of 50 maize cobs per store was taken. The beetles of LGB were counted and examined for protozoan infection. The trials ended after 20 weeks of storage.

3. Results

Jar trial

The figure below shows the mortality of the adult *P. truncatus* in 4 following generations. The rates of mortality in the filialgenerations 1, 2 and 3 were significantly higher in the treated population than in the untreated. For the statistics the chi-square-test was used (at $\alpha = 0.05$). In all filialgenerations about 60 % of the dead and treated adults were diseased, whereas only 50 % and less of the untreated individuals were diseased. Therefore it was concluded that the spore-treatment caused the high mortality of LGB.

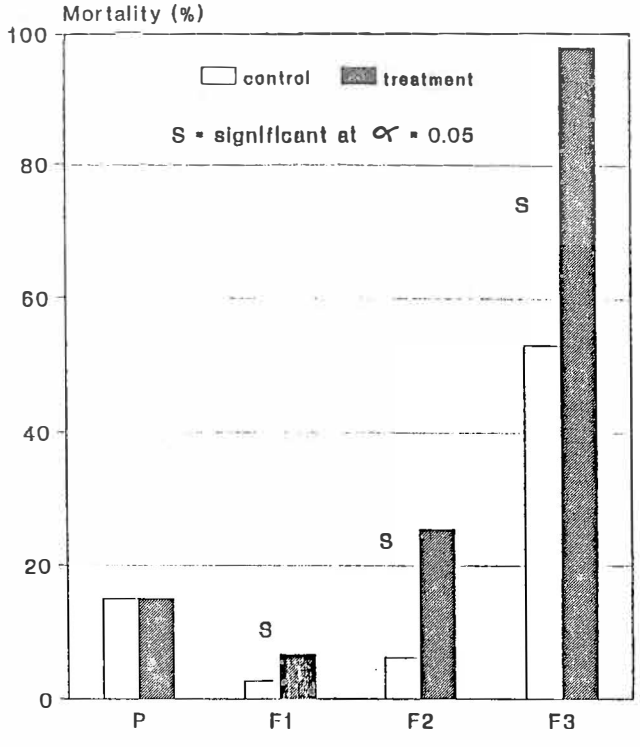

Fig.: Mortality of the adults in the untreated (control) and in the spore-treated populations of *Prostephanus truncatus* (treatment with $3x10^5$ *Nosema*- and $8x10^3$ *Mattesia*-spores per g substrate)
P = parental generation, F1-3 = filialgeneration 1,2 and 3)

Trials under semi-practical conditions

In both trials with the treatment of maize stores a development of LGB was observed only in the control and in the spore-treated stores. Obviously the application of the active ingredient "Deltamethrin" was very efficient against *P. truncatus*.
The treatment of stores with protozoan spores caused only limited infection on LGB. The infection rates ranged from 2,5 to 32,5 %.

4. Discussion

From the presented investigations it can be concluded that the proved effects of *Nosema* sp. and *Mattesia* sp. in the jar trial were not repeatable under semi-practical conditions. This based on the low infection rates of *P. truncatus* in the maize stores. Some further research is needed to think about better application techniques in practice, which increase the infection on LGB.

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NON-OCCLUDED BACULOVIRUS (NUDIBACULOVIRINAE) OF THE RED BUG, *PYRRHOCORIS APTERUS* (HETEROPTERA, PYRRHOCORIDAE)

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Introduction

In spring 1990 most *Pyrrhocoris apterus* larvae of our cultures died. The bugs of one of these cultures originated from Verscio (Ticino, Switzerland). In autumn 1990 and 1991 infected larvae were found at the same locality. They are easily recognized by their strongly swollen bodies.

Materials and Methods

Electron microscopy

The tissues were fixed in 3% glutaraldehyd, rinsed in 0.1 molar cacodylate buffer at pH 7.0 and fixed again in 1% OsO_4. After dehydration the tissues were embedded in Epon and cut. The tissue sections were mounted on copper grids and contrasted with 2% uranyl acetate and lead citrate. They were examined with a Philips CM 301 electron microscope.

Bioassays

Fifty 1st or 2nd instar larvae were used in each bioassay. The control group was fed with meal-worms as a protein source. Group A was fed on infected 4th instar *P. apterus* that had been immediately frozen at -10°C after killing. Group B was fed on infected 1st and 2nd instar *P. apterus* that had been stored at 2°C for three months.

Results

Electron microscopy

Electron microscopic investigations showed in the nuclei of the fat body cells particles looking like non-occluded baculoviruses (NOB) measuring 225 x 90 nm. The infected nuclei resemble "fried eggs" showing an electron dense centre surrounded by a less dense area.

The nucleocapsids are rod-shaped (160 x 60 nm) and are singly enveloped by a unit membrane, whose surface seems to be covered by a thin layer which possibly consists of paracristallin arranged lipoprotein molecules (Figure 1). Occlusion bodies were not found.

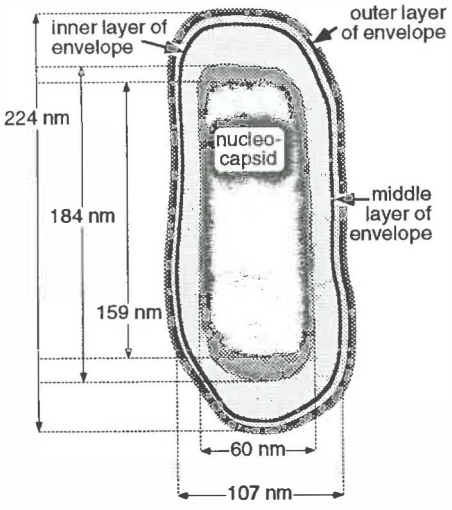

Fig. 1: Structure of NOB-like particle from fat body of *P. apterus*

Bioassays

High mortality rates of up to 94% were achieved by artificial contamination (Tab. 1). Larval stages are highly susceptible to the virus. Uptake is peroral either by sucking on infected specimens (living or dead) or through the uptake of water contaminated by cadavers of infected insects. The virus may also be spread by infected bugs piercing freshly molted instars and sucking hemolymph.

Table 1:

Assay	N(0)	N	N	Mortality
	April 2,1990	April 23,1990	May 3,1990	
Control	50	45	43	14 %
A	50	29	3	94 %
B	50	47	47	6 %

N(0) = Number of 1st or 2nd instar larvae at test beginn
N = Number of surviving bugs
A, B = Groups fed with infected larvae

Discussion

The virus of *Pyrrhocoris apterus* has the same ultrastructural features as other known non-occluded baculoviruses, such as the virus of the palm rhinoceros beetle, *Oryctes rhinoceros* (HUGER 1966), the so-called Hz-1 virus persistently established in the lepi-

dopteran cell line IMC-Hz-1 (GRANADOS et al. 1978) and the NOBs in the crickets *Gryllus campestris* (HUGER 1985) and *G. rubens* (BOUCIAS et al. 1989).

The virus of *Pyrrhocoris apterus* is the first known baculovirus-like particle in a heteropteran insect. Neither FRANCKI et al. (1991) nor HUGER & KRIEG (1991) or TANADA & KAYA (1993) mention such particles. Only MARTIGNONI & IWAI (1986) list two Reduviidae as possibly affected by a virosis without giving references as to the author(s) or type of virus.

Since the nucleic acid of the NOB-like particle of *P. apterus* has not yet been determined as DNA, our classification is based on ultrastructural characters alone. Future biochemical and biophysical studies may clarify the final systematic position.

Acknowledgements

The cultures used in these investigations were from Johannes Jenny (ETH-Zurich). He also helped with the bioassays.

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IMPROVING THE EFFICIENCY OF THE GRANULOSIS VIRUS OF *Adoxophyes orana* F.v.R. FOR MICROBIOLOGICAL CONTROL

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In Switzerland a granulosis virus (GV) preparation against the summer fruit tortrix, Adoxophyes orana F.v.R. (AoGV) is commercially available since a few years. Three major problems in the effective utilization of this GV in the field are: the cost of treatments, the lack of persistence due principally to inactivation by ultraviolet rays (UV), and the fact that infected larvae only die in the last instar as "overmature" dauerlarvae. The purpose of this study is to increase the resistance of the AoGV to UV and to increase its virulence so as to shorten the period between infection and death and/or to reduce the application dose for effective control. Two methods of research were used: selection for more effective virus strains and addition of different adjuvants to the virus spray.

Selection was carried out on the virus strains already existing without inducing mutations. UV resistance was selected for by exposing virus particles to a source of simulated sunlight. Increased virulence was selected for by feeding first instar larvae with a normally sublethal dose of the virus and subsequent isolation of the virus from the few individuals dying of granulosis. Selection was performed separately for both criteria. No significant enhancement of efficiency could be detected after more than 10 selection runs, neither for UV resistance nor for virulence increasing. These results indicate a great genetic homogeneity of our virus strains. It might be feasible to isolate a new genetically less homogeneous "wild" strain from larvae infected by high doses of a mixture of our Swiss AoGV strains with Japanese isolates, before selection with low doses is done.

In further attempts to increase the efficiency of the AoGV fife adjuvants were tested in order to increase the adhesion of the virus to the plant (a specific formulation of Pinolene®, Nu-Film 17), increase the stability of the virus by slowing down degradation by sunlight (the optical brighteners Leucophor BSB and Tinopal, the bio leaf fertilzer Humin-Vital 80), and increase the dose fed by the larvae before the virus is inactivated by increasing the appetite of the larvae (with skim-milk powder).

The laboratory experiments revealed that addition of Leucophor, Tinopal or Humin-Vital to the virus suspension before exposure to UV protects the virus and, therefore, leads to higher larval mortality by granulosis. However, further experiments - especially in the field - are needed.

DIFFERENTIAL CYTOTOXIC ACTIVITY OF TWO δ-ENDOTOXINS OF *Bacillus thuringiensis* IN MAMMALIAN CELLS

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Summary

The *Bacillus thuringiensis* ssp. *israelensis* (ISR) is toxic for mosquito larvae and has haemolytic activity in mammalian blood as well as cytotoxic activity in mammalian cells. Since the new sawfly pathogenic isolate K24 of *B. thuringiensis* (s. BENZ & JOERESSEN, in this issue) belongs to the same H serotype as ISR, has a similar plasmid spectrum, and is also toxic for mosquito larvae, we wanted to compare the mammalian cytotoxicity of the two strains.

Their δ-endotoxins were tested with V79 Chinese hamster fibroblast cells cultured in Eagle's minimal essential medium at 37°C. The endotoxins were dissolved in 50 mM Na_2CO_3, pH 11 and dialysed against PBS-buffer to a protein concentration of 1 µg/µl. The cells were exposed for 15 min, 1, 4, and 8 hours to logarithmic doses between 1 and 100 µg/200 µl medium. Cell toxicity was characterized by the lysosomal neutral red assay (NR test, basing on the uptake of neutral red by an active transsport system and the accumulation of NR in the lysosomes of viable cells), the mitochondrial succinat dehydrogenase activity (MTT test, measuring the blue formazan product formed from yellow MTT terazolium bromide by mitochondrial succinat dehydrogenase at 560 nm), the release of cellular lactat dehydrogenase into the medium (LDH test), and the measurement of reduced intracellular protein contents, using Bio-Rad reagents and bovine serum albumin as a standard.

The ISR δ-endotoxin shows clear mamammalian cytotoxic activity in all tests already at the lowest (1 µg/200 µl) or second lowest dose (3.3 µg/200 µl) after the shortest (15 min) or second shortest (1 hr) incubation period. The dose and time responses suggest a primary interaction of the ISR endotoxin with the cell membrane.

In contrast to the above results, the K24 δ-endotoxin showed none or only slight (nonsignificant) toxic activity and only at the highest dose (100 µg/200 µl) and longest exposure time (8 hr). The low toxicity of the K24 δ-endotoxin for mammalian cells distinguishes K24 clearly from the ssp. *israelensis*.

INSECTS ON POPLARS: REGIONAL DISTRIBUTION IN GERMANY AND THEIR SUSCEPTIBILITY TO *BACILLUS THURINGIENSIS* ISOLATES

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Summary

The overall objectives of the work are to assess the impact on representative elements of the poplar fauna of toxins derived from *Bacillus thuringiensis*. In two seasons the insect complex will be defined qualitatively on hybrid poplars, concentrated on Coleopterans and Lepidopterans. Three model insects *Melasoma populi, Phyllodecta vitellinae* and *Plagiodera versicolor* are in culture for bioassay screening. *Bacillus thuringiensis* subspec. *tenebrionis* and other isolates which are isolated from soil will be testet by bioassays.

INSECTS

To find the representative elements of the insect complex on hybrid poplars we chose three different regions in Germany for these investigations. The regions contain plants of different ages and different types of black poplar and balsam poplar hybrids. The first investigations were carried out with a beating tray. The beating tray of one square metre was put under a branch. The branch was beaten and the fallen insects could be collected. Some of the insects do not fall, when the branches are beaten. It is more effective to look directly at the branches.
"Region south" is near to Karlsruhe, it is about 100 km in the south of Darmstadt. "Region middle" is in and around Darmstadt. At least the "Region north" is about 300 km in the north of Darmstadt. It is in the "Lower Rhine Region" near to the Dutch border.
There were found about 50 different species in all regions. Some of the species were detected by only one individual in one region. For example: There was found only one individual of *Eupsilia satellitia* (Noctuidae) in the region middle only. *Melasoma populi* (Chrysomelidae) was found two times in the region north.
Insects like *Eupsilia satellitia* (Noctuidae), *Dasychira podibunda* (Lymantriidae) and *Phyllobius calcaratus* (Cur-culionidae) do not live only on poplars. They are polyphagous.

The insects which are listed in table 1 are very typical on poplars.
Table 1: Appearance of some insects in the different regions in the seasons 92 and 93

Insects	Region south		Region middle		Region north	
	black polar hybrids	balsam poplar hybrids	black poplar hybrids	balsam poplar hybrids	black poplar hybrids	balsam polar hybrids
Gypsonoma aceriana (Tortricidae)	93	93	92/93	92/93	92/93	92/93
Phyllocnistis suffusella (Phyllocnistidae)	92/93		92/93	92/93	92/93	92/93
Stigmella trimaculella (Stigmellidae)	92/93		92/93		92/93	92/93
Lithocolletis populifoliella (Lithocolletidae)	92/93	93	92/93	92/93	92	
Melasoma populi (Chrysomelidae)					93	
Phyllodecta vitellinae (Chrysomelidae)	92/93	92/93	92/93	92/93	92/93	92/93
Plagiodera versicolor (Chrysomelidae)				93	92	93

With the exception of *Plagiodera versicolor* (Chrysomelidae) and *Melasoma populi* (Chrysomelidae) all the listed insects were found in all regions. *Melasoma populi* seems to be quite seldom. It was found only in the region north where old trees were cut in 92. Not all insects were detected in every season in the different regions. For example *Gypsonoma aceriana* (Tortricidae) was not found in the season 92 in the region south.The difference between the seasons seems to be more important than between the regions

Another fact is the comparison between the presence of the insects on the different poplar hybrids. For example: *Stigmella trimaculella* (Stigmellidae) prefers black poplar hybrids in the regions south and middle. *Phyllocnistis suffusella* (Phyllocnistidae) prefers the black poplar hybrid in the region south and *Lithocolletis populifoliella* (Lithocolletidae) prefers it in the north.

BIOASSAYS

Melasoma populi (Chrysomelidae), *Phyllodecta vitellinae* (Chrysomelidae) and *Plagiodera versicolor* (Chrysomelidae) are typical Chrysomelidaes on poplars. They all are sensitive to *Bacillus thuringiensis* subspec. *tenebrionis*. The three species are in culture to make further bioassays with new isolates. These isolates were obtained from soil by the modified method of Aizawa (VRIESEN & KELLER 1991). All isolates for the bioassay produce flat plate shaped crystals like *B.t.* subspec. *tenebrionis*.
We started the bioassays with *Melasoma populi*. A suspension of the different *B.t.*isolates was produced. Two µl of the washed suspension were pipetted on poplar leaf discs with a diameter of 4 mm. Each larvae got one of these prepared leaf discs. The testet *B.t.* isolates are listed in the following table.

Table 2: Efficacy of some *B.t.* isolates from soil against *Melasoma populi*

*B.t.*isolate	Efficacy +/-	*B.t.*isolate	Efficacy +/-
L1/3	+	4a3/4	+
L1T3/3	+	5a2/11	+
L2/5	+	7b3/2	+
1a2/12	-	8b2/1	-
2a1/2	-	9b2/10	-
2b1/3	+	13a2/20	+
4a2/1	+	13ba/24	-

Nine of the listed isolates were positive now it will be interesting to investigate, if these isolates are identical to *Bacillus thuringiensis* subspec. *tenebrionis*. The next step will be the comparison of the efficiency of the "positive" isolates with the efficiency of the *B.t.t.*-standard. Then it will be interesting to characterize these isolates by other methods like SDS-PAGE, plasmid analysis and serological tests.

Ref.: Vriesen,S. & Keller, B., 1991. Investigations on the occurrence of *Bacillus thuringiensis* in different soils. IOBC/WPRS XIV/7, 147

IMPACT OF ENTOMOPATHOGENIC AGENTS ON THE OLIVE FRUIT FLY

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The dipteran olive fruit fly [*Bactrocera* (*Dacus*) *oleae*, Tephritidae] is the key pest of olive cultures in Mediterranean countries. The use of microbial preparations for the control of the insect is possible only for adults, because larvae develop inside the olive fruit.

In the present study an estimation of the effectiveness of some entomopathogenic agents, which has been provided to adults of the insect, has been carried out.

Artificial inoculations by injection using suspensions of 9 pathogenic viruses showed mortality of the adults in the following cases : For the Reovirus of the olive fruit fly, mortality of 97% occurred within 16 days. The CrPV (Picornavirus) killed the flies within 3 days, the CIV (Iridovirus) within 7 days, while the Densovirus of *Junonia coenia* and Reovirus I of *Ceratitis capitata* induced a lower mortality than to the previous ones. The NPV *Autographa californica*, NPV *Tipula paludosa*, Picornavirus C *Drosophila melanogaster* and non classified virus *Ceratitis capitata* had no effect on the insects. The effectiveness of this pathogenic viruses are listed in TABLE 1.

Percentage of the cummulative mortality in young adults of the olive fruit fly, inoculated with Reovirus *Dacus oleae*, CrPV and CIV presented in FIGURE 1 and FIGURE 2.

TABLE 1. Effectiveness of pathogenic viruses inoculated in adults of the olive fruit fly.

	Virus	Effectiveness
1	Reovirus *Dacus oleae*	++
2	NPV *Autographa californica*	-
3	NPV *Tipula paludosa*	-
4	CIV *Chilo suppresalis*	++
5	Densovirus *Junonia coenia*	+
6	CrPV *Teleogryllus oceanicus*	++
7	Picornavirus C, *Drosophila melanogaster*	-
8	Reovirus I *Ceratitis capitata*	+
9	Non-classified virus *Ceratitis capitata*	-

++= mortality >97%, += mortality >50%, -= no effect

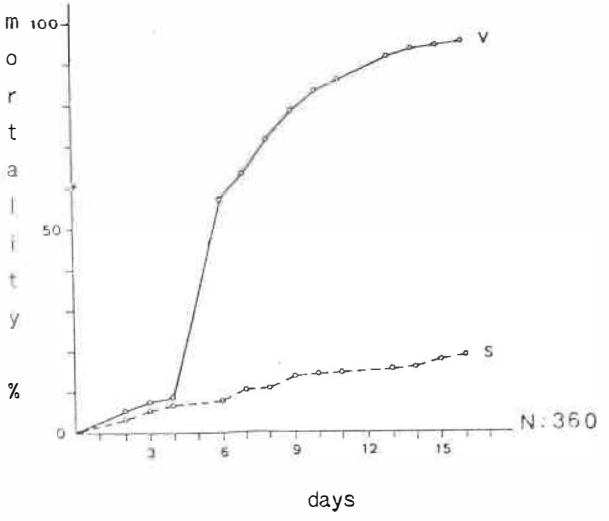

FIGURE 1. Percentage mortality of olive fruit fly inoculated with Reovirus of *Dacus oleae* (V) compared to the control (S) inoculated with Ringer solution. Insect numbers in every case N=360.

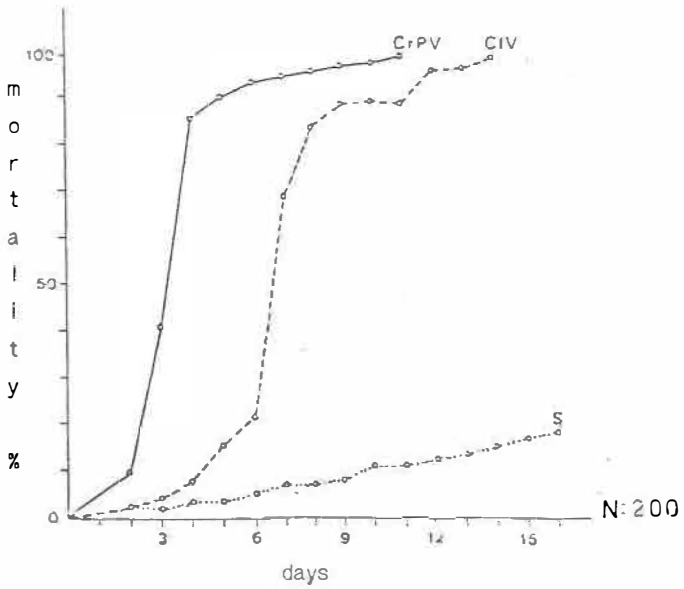

FIGURE 2. Percentage mortality of young adults of the olive fruit fly after experimental infections with CrPV, CIV and S (Control inoculated with Ringer solution). Insect number in every case N=200.

Laboratory infections using sugar suspensions of commercial microbial preparations, *per os*, proved that the olive fruit fly is sensitive to some of them. The results are presented in TABLE 2.

From the above Laboratory tests an interesting result has been obtained which indicates that the provision *per os* of sugar suspensions of *Beauveria bassiana* caused high mortality within 3 days in young adults of the olive fruit fly.

More research and development needs to be done with persistent formulations of microbial pesticides to improve their effectiveness which is often a major criteria for the control of the olive fruit fly.

TABLE 2. Sensitivity of adults of the olive fruit fly in some microbial preparations.

	Preparations	Sensitivity
1	*Bacillus thuringiensis* subsp. *israelensis* "VECTOBAC" Abbott Laboratories, USA	-
2	Endotoxin *B. thuringiensis* subsp. *kurstaki* "MCP" Bioincecticide-Mycogen Corporation "SHELL Company"	-
3	*Beauveria bassiana* "Naturalis-d" Fermone Corporation INC.	++
4	*Steinernema carpocapsae* "Exhibit" Biosys - "CIBA-GEIGY"	-

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STRUCTURAL PROTEIN ANALYSIS OF THE *ADOXOPHYES ORANA* GRANULOSIS VIRUS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS AND IMMUNOBLOT

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The structural proteins of purified *Adoxophyes orana* granulosis virus were analysed by two-dimensional (2-D) gel electrophoresis and by immunoblot. Isoelectric focusing (IEF) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) allowed resolution of 42 structural proteins in the enveloped nucleocapsid (ENC) of the virus ranging in molecular weight (MW) from 16,000 to 110,000 Da. Most viral proteins have their isoelectric points (pI) ranging from pH 4.5-6.6, but V34 has its pI at pH 8.5. Nonequilibrium pH gel electrophoresis (NEpHGE) followed by SDS-PAGE allowed resolution of 28 structural proteins in the ENC of the virus. No other alkaline protein than V34 was found in the ENC. Immunoblot was carried out. The ENC proteins, which were separated in the IEF/SDS-PAGE and NEpHGE/SDS-PAGE two dimensional systems, were transferred to a nitrocellulose membrane and treated with three antibodies of different viral components. The positive reactions demonstrated the viral nature of the proteins. The capsules contain an alkaline proteinase, which can digest the granulin into smaller peptides. Granulin isolated from the capsules, in which the proteinase has been inactivated, shows a single protein spot. Its MW is 28,000 Da and its pI lies at pH 5.1.

POSTER SESSION

SYNTHESIS OF VIRAL PROTEINS IN GRANULOSIS VIRUS-INFECTED LARVAL TISSUES

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Replication of the *Adoxophyes orana* granulosis virus (AoGV) was investigated *in vivo*. Three larval tissues, the fat body, the haemolymph and the midgut, were chosen for detecting viral protein synthesis. In the fat body, SDS-PAGE revealed changes in the host proteins after infection. One host protein band (L26) disappeared gradually, while two other bands (V28, V34) increased in size. Immunoblot with three specific rabbit antisera against different viral structures demonstrated that the two increasing bands are different viral structural proteins: V28 is granulin that forms the viral occlusion bodies (capsules), whereas V34 is one of the major bands of the enveloped nucleocapsid (ENC). Immunoblot analysis also showed that another viral band of low molecular weight (V16), which is thought to be the DNA-binding band of the ENC, greatly increases in size during viral replication, while in the Coomassie blue stained profile this tendency can not be seen.

The viral proteins were detected early on day 4 after the infection in the fat body and on day 7 in the haemolymph, but not in the midgut. This suggests that the fat body is the main tissue for AoGV replication. The AoGV may carry out its secondary infection in the haemolymph, but it does not replicate in the midgut. Another noticeable point is that one of our virus-specific antisera recognizes a larval protein. Whether this host protein is passively included into virus structures during viral replication or whether some viral component has a similar antigenic structure is unknown.

POSTER SESSION

USE OF NON-RADIOACTIVE NUCLEIC PROBES FOR EPIDEMIOLOGICAL SURVEY OF POTATO TUBER MOTH GRANULOSIS VIRUS

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Phthorimaea operculella (Lepidoptera: Gelechiidae), the Potato Tuber Moth (PTM), is the most important insect pest for potato crops under warm climates. Because of its worldwide extension, caterpillars of this lepidopterous insect are directly accountable for losses of millions tons of tubers each year, mainly in developing countries. Furthermore, the use of chemical insecticides, beside its toxic impact on the environment and arising resistance among treated insects, induces costly expenses for these counties.

By the end of the 60's, a granulosis virus (Baculovirus type B) was isolated from PTM larvae. Several investigations have pointed out the major interest for the use of this entomopathogenic agent alone or included in integrated pest management (IPM) programs. Significant trials have been carried out both in open helds and in storage. In such trials, the crucial point was to assign unequivocally the variations in the host population levels to the effect of the viral entomopathogen.

Actually, as the use of this low-cost producible virus is constantly spreading, the need for reliable and acute diagnosis tools is obviously growing.

The use of non-radioactive nucleic probes allows us to monitor the effectiveness of different viral applications, the persistence of the virus in treated plots and to evaluate the natural occurrence of the pathogen among the host-populations. So, we detected, for the first time, the presence of granulosis virus (PoGV) in PTM larvae originated from Turkey and Egypt. In this latter country, a three-year epidemiological survey concerning several hundreds of larvae collected from different locations have shown a relatively low prevalence of PoGV compared to neighbouring countries (i.e. Yemen), it could be related to severe local ecological factors (U.V. dryness...). On the other hand, after dispersal of PoGV in Egyptian cultivated potato plots, the high rates of mortality among PTM larval instars were directly correlated to the presence of the applied pathogen.

IMMUNO-ENZYMATIC DETECTION AND NUMERATION OF *PLASMODIUM YOELII YOELII* IN *ANOPHELES STEPHENSI* - OPTIMIZATION FOR FIELD USE

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A sandwich enzyme linked immunosorbent essay (ELISA) was developed for the detection of *Plasmodium yoelii yoelii* sporozoites in their laboratory vector *Anopheles stephensi* using polyclonal antibodies which are more accessible to moderately equipped field laboratories than monoclonal ones.

Preliminar calibration of the reaction was achieved in a previous study (P. Caubère) with homogeneous samples of female anopheles which are either infected or fed on normal mice for control.

Anti-sporozoite antibodies were prepared against mature sporozoite in two different mammal species. The reaction scheme was as follows: rabbit antibody-1/80 of a whole mosquito grinded-mouse antibody-peroxydase conjugate-orthotolidin as a substrate. Rough optic density results were referred by polynomial function transformation to the standard curve established for each plate with a purified suspension of mature sporozoites.

Serial samplings in the mosquito population led to the infestation curve of the vector between the 14th and 23rd days postinfection with a peak at J 21.

The ELISA calibrated values obtained with 118 infected and 111 control mosquitoes give 0,9% false positive. The 6,7% of false negatives are consistent with the informations obtained by dissection (1,6% of females without parasite and 12,9% of non infectious females).

The method proposed permits a good discrimination between infected and negative anopheles and an evaluation of the sporozoite charge thus avoiding the lasting and costly preparation of monoclonal antibodies.

9. Workshop on Entomophthorales.

IOBC/WPRS Working Group "Insect pathogens and insect parasitic nematodes"

Working with arthropod-pathogenic Entomophthorales

Workshop held in Zurich
9 - 10 September 1993

S. Keller
Federal Research Station for Agronomy
Reckenholz, CH-8046 Zürich

1. Fungal structures and their characters to be considered in taxonomy

Structure	Character(s)
Protoplasts	shape
	tpye of multiplication (occur in living hosts)
Hyphal bodies	shape
	number and size of nuclei (occur in hosts shortly before or after death)
Cystidia	presence/absence
	shape
	diameter
Rhizoids	presence/absence
	monohyphal/pseudorhizomorph
	endings
	diameter
	location on host
Conidiophores	simple/branched
	nature of branching
	number, size and distribution of nuclei
	diameter
Primary conidia	mode of discharge
	single/double walled
	shape
	dimensions
	content (vacuoles)
	number and size of nuclei
Sencondary conidia	mode of formation (type)
	shape
	dimensions
	mode of discharge
	mophology and dimensions of secondary conidiophores

Resting spores	shape
	dimensions
	colour
	mode of formation (zygo-/azygospores)
	ornamentation
	number of nuclei
	wall (structure, dimension)
Nuclei	structure
	size
	number per fungal structure
	distribution

Note: In the genera *Conidiobolus*, *Entomophaga*, *Entomophthora*, *Eryniopsis* (with an exception) and *Neozygites* the number of nuclei per conidium is the same as the number of nuclei per conidiophore or per fully developped hyphal body. Small differences are known to exist in *Entomophthora* spp. where a small percentage of the nuclei may remain in the conidiophore. But note also, that some hyphal bodies may develop to other structurs than conidia and eventually contain different numbers of nuclei.

The host (name, taxonomic position) and the symptoms of the disease are further important criteria for the identification.

2. Preparation

2.1. Collecting material for identification

The more material you collect the easier the identification is. Collect not only dead infected arthropods with all stages of development of the fungus, but also living arthropods suspected to be infected. The surviving living ones may be used to identify the host species. Living infected hosts contain the vegetative stages of the fungus (e.g. protoplasts). Fix a part of the living hosts and also a part of the sporulating fungal material immediately after collection. Transport the remaining material in a cool box to the laboratory.

2.2. How to get conidia (Fig. 1)

Primary conidia

Place cadavers individually or in groups in small Petri dishes on wet surface (cotten, filter-paper, water). Put slide over the cadaver in a distance of 1-2 mm. Primary conidia are projected on the slide within a few hours (Fig. 1.1.)

Secondary conidia

a. Take the slide with the primary conidia, place it in a Petri dish on wet filter paper. Place a second slide above the primary conidia held in a distance of about 1 mm by glass tubes or rods. Projected secondary conidia (type I conidia) are collected on the upper slide, capillary secondary conidia (type II conidia) remain on the lower slide (Fig. 1.2.).

b. When the fungus on the cadaver has sporulated on the water surface remove it after 3-4 hours. The primary conidia fallen onto the water surface start to produce secondary conidia. Collect the projected secondary conidia with a slide. Eventually you have to add some water for the correct projection distance (Fig. 1.3.). Remove slide. Fill water carefully in the Petri dish with the conidia until the water surface is convex. Touch with a slide carefully the water surface; upon contact with the water the conidia on the water surface adhere to the slide (Fig. 1.4.).

Best results usually are obtained with a combination given in figs. 1.1, 1.2. and 1.4. Some species may produce certain types of secondary conidia only under particular conditions.

2.3. Preparation of the cadavers

Cadavers usually are stored in 70% ethanol. Prepare the whole cadaver of small arthropods (e.g. mites, thrips, small aphids), but prepare only a portion of larger arthropods. Dissect the material in the staining or mounting fluid with tweezers or needles. The finer the dissection the better the fungal structures become visible. When wings are present do not distrub them. They are often good collectors of projected conidia especially from dipterans.

2.4. Mounting the material

The fungal material is usually stained and preserved in liquids based on lactophenol. The cover glass may be sealed with nail enamel. Problems often arise from untight seals especially when the stain countains volatile compounds like acetic acid.

2.5. Stains (see annex 1).

Number, size and structure of the nuclei are important taxonomic criteria. The stains usually used to stain the nuclei are the Feulgen reaction stain (FRS) or orcein. The FRS can be used to stain the nuclei in the conidia of all genera. Since it removes the plasmatic content of the conidia the method is particularly suitable for species (genera) with large conidia containing many nuclei like *Conidiobolus*, *Entomophaga* and *Entomophthora*. The disadvantage of the FRS is the rather complicated procedure which needs some experience.

Orcein stains the nuclei in all genera except in *Conidiobolus* where nuclei are only occasionally stained. Nuclei in conidia are less intensively stained than in other fungal structures (conidiophores, hyphal bodies). The nuclei in protoplasts are usually not or only weakly stained. Orcein can be added in different concentrations directly to lactophenol or solved in acetic acid. Therefore it is very suitable as routine nuclear stain.

2.6. Field book and slide labels

Notice all relevant data of the collection: date, locality, host, environment of the host, symptoms of the disease etc. Labels on the slides may contain the same data in an abbreviated form or refer to the field book. Sometimes indications to the used stains are helpful.

2.7. Herbarium material

Deposit Entomophthorales in a herbarium

- prepared and mounted on labeled slides
- on airdried cadavers (exsiccata) in labeled paper envelops, eventually with a piece of the supporting plant.

3. Isolation

3.1. Collecting material for isolation

Collect cadavers, preferably fresh ones. Place them individually in small boxes. If the cadavers are assumed to be infected with the same species you may place them together in larger boxes. Aphids: You may collect plant material with infected colonies. To prevent or to slow down sporulation transport the material in a cool box to the laboratory. If you can't start to isolate the fungi immediately after the collection you may store them air-dried under cool and dry conditions.

3.2. Methods of isolation (Fig. 2)

a. "Whole cadaver method"

This method is mainly used for small arthropods like aphids. Use fresh, preverably unsporulated cadavers or even living individuals from infected colonies, surface sterilize and wash them in sterile water and place them in media (Fig. 2.1.).

b. Conidia projection method

Place the cadavers on wet substrate (water, cotton, filter paper), place a sterile slide or cover glass 1-3 mm above the cadaver. Wipe off the projected conidia with a piece of medium and transfer it in culture tubes (Fig. 2.2.). The cadavers may be surface - sterilized or not and the device may be placed in a sterile chamber (e.g. Petri dish). Surface - sterilized cadavers may be placed above liquid media so that the projected conidia fall onto it.

c. Other methods

Dissect surface-sterilized infected, living or dead hosts under aseptic conditions. Transfer part of the body content to the medium.

3.3 Media (see annex 2)

4. Identification

The identification bases on the material from the host arthropod. Cultural aspects can be helpful and provide additional data.

A complete key for the identification of the genera of the arthropod-pathogenic Entomophthorales is provided in annex 3.

A (theoretical) procedure for the identification is given in Fig. 3.1. In reality the identification follows a more practical pattern:

You start with a cadaver. If it is a fly with a hole on the ventral side of the abdomen, you got a species of *Strongwellsea*. In the other cases you normally start with the projected conidia an/or the dissected cadaver. The shape of the primary and secondary conidia and the mode of formation of secondary conidia (Figs. 3.2 and 3.3.) allow to identify unequivocally the following genera:

Entomophthora: bell-shaped, bi- to multinucleate primary conidia

Erynia: mononucleate, bitunicate primary conidia, capilliconidia absent, two types of projected secondary conidia.

Eryniopsis: oligo - to multinucleate, unitunicate, pyriform to elongate primary conidia, two types of secondary conidia

Neozygites: rounded, 3- to 8-nucleate primary conidia, capilliconidia present

Zoophthora: mononucleate, bitunicate, elongate (subcylindrical) primary conidia; capilliconidia present.

The genera *Conidiobolus* and *Entomophaga* have the same conidial morphology and cannot be separated by this character. Use orcein and/or FRS as nuclear stain:

Conidiobolus: Nuclei in all fungal structures not or only weakly stained with orcein, diameter of nuclei less than 3 µm. FRS-stained nuclei measure less than 2 µm.

Entomophaga: Nuclei usually deeply stained with orcein, especially in conidophores, diameter more than 3 µm. FRS-stained nuclei measure less than 2 µm.

Further important structures used for the identification:

- Conidiophores (Fig. 3.4.)
  Simple (unbranched) in *Conidiobolus*, *Entomophaga*, *Entomophthora*, *Eryniopsis* (exceptions possible), *Neozygites*, *Strongwellsea*.
  Branched in *Erynia* and *Zoophthora*
- Rhizoids (Fig. 3.5.):
  normally absent in *Eryniopsis*, *Neozygites* and always absent in *Strongwellsea*,
  normally present in *Erynia* (monohyphal) and Zoophthora (pseudorhizomorph).
  Present or absent in the other genera (species characteristics).

Remarks

Two genera of arthropod-pathogenic Entomophthorales have not yet been considered here: *Massospora*, which attacks cicades and is not yet reported from Europe, and *Tarichium*, which includes species known only by their resting spores. If your collection consists of resting spores only it may be a species of *Tarichium* but it could also be the resting spore stage of a species of any other genera.

Fig. 1: How to get primary and secondary conidia

Fig. 1.1.

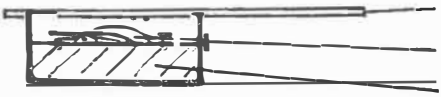


Fig. 1.2.

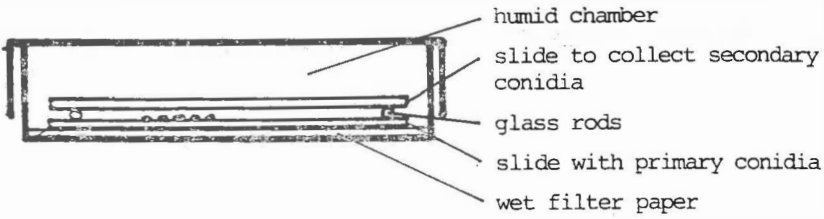


Fig. 1.1.

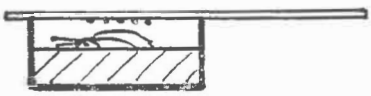


Fig. 1.3.

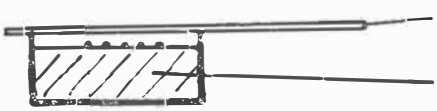


Fig. 1.4.

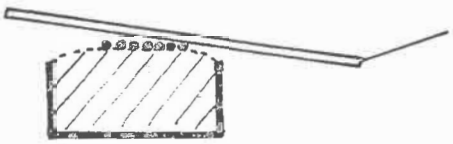


Fig. 2: Isolation of arthropod-pathogenic Entomophthorales

Fig. 2.1. Whole cadaver method

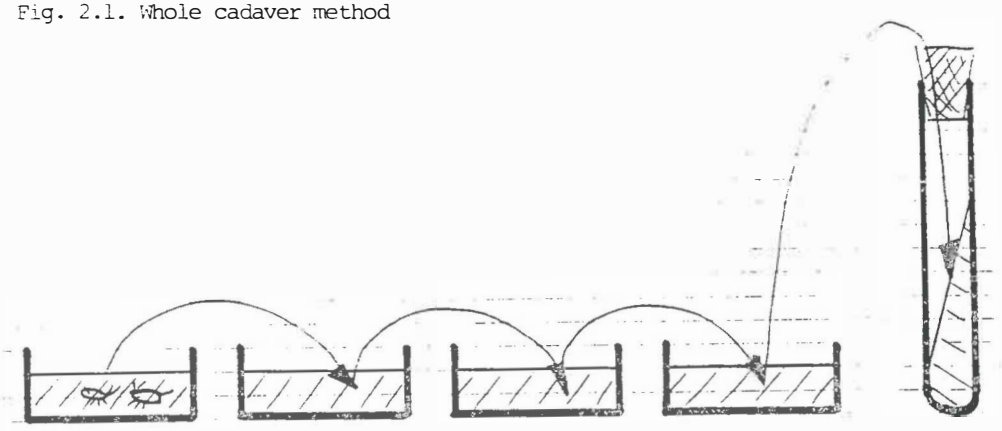
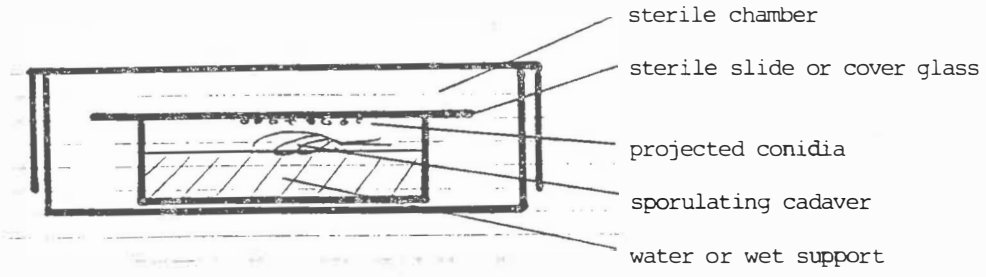


Fig. 2.2. Conidia projection method



The conidia projected on the slide or cover glass are removed with a piece of medium and inoculated into the cultural tube.

Key for the identification of arthropod-pathogenic Entomophthorales

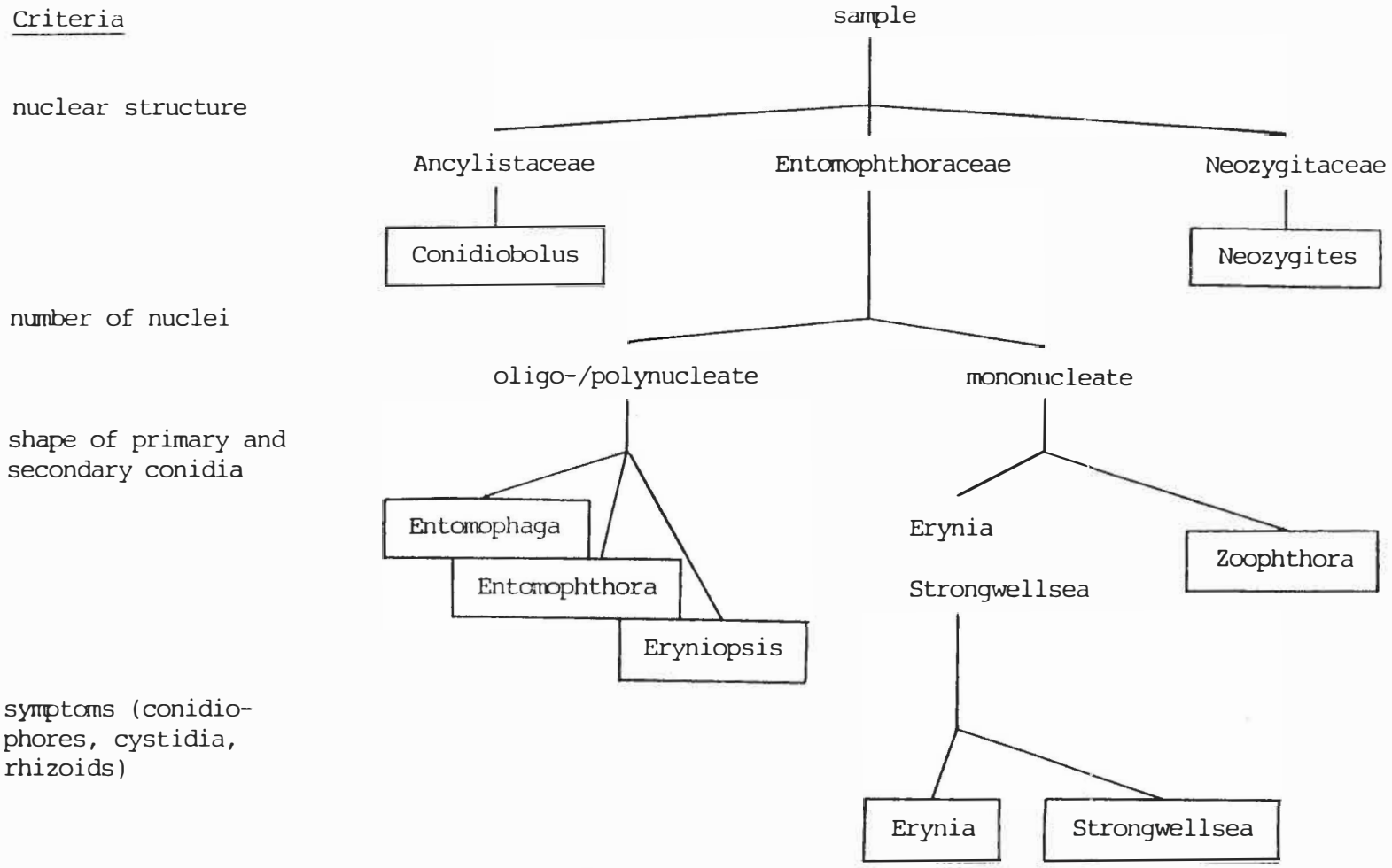


Fig. 3.1.

Fig. 3.2.: Primary conidia

A: Conidiobolus
B: Entomophaga
C: Entomophthora
D: Erynia
E: Eryniopsis
F: Neozygites
G: Zoophthora

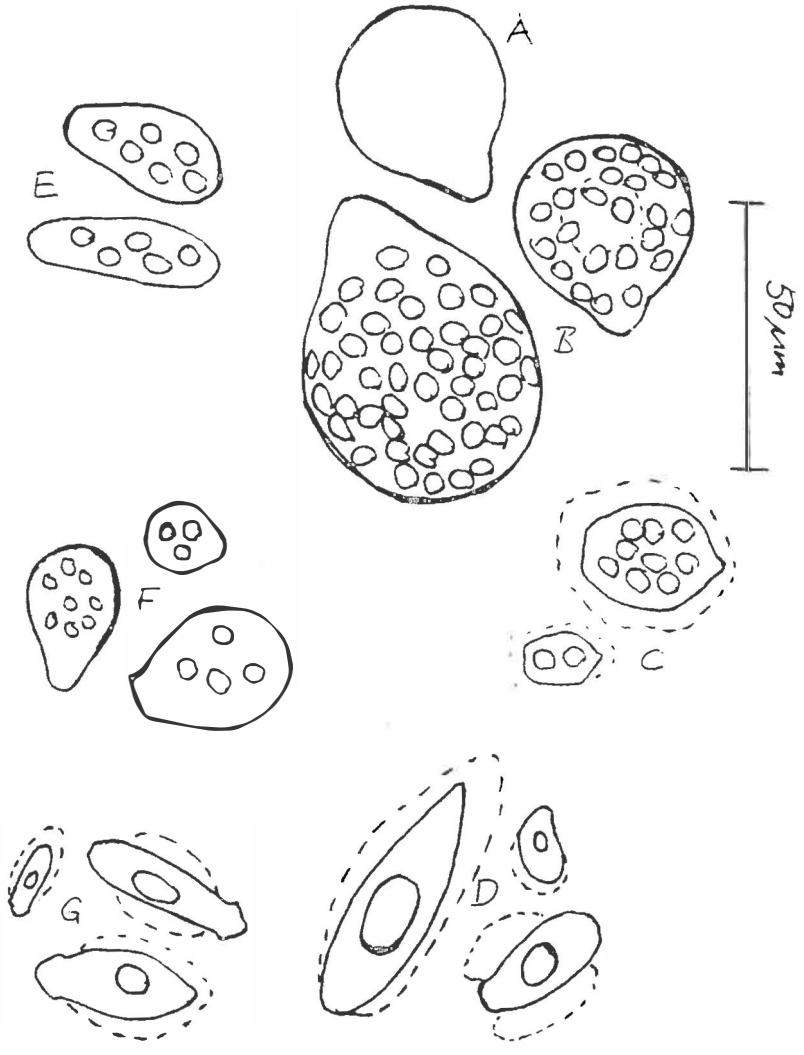


Fig. 3.3.: Secondary conidia

A: Conidiobolus
B: Entomophaga
C: Entomophthora
D: Erynia
E: Eryniopsis
F: Neozygites
G: Zoophthora

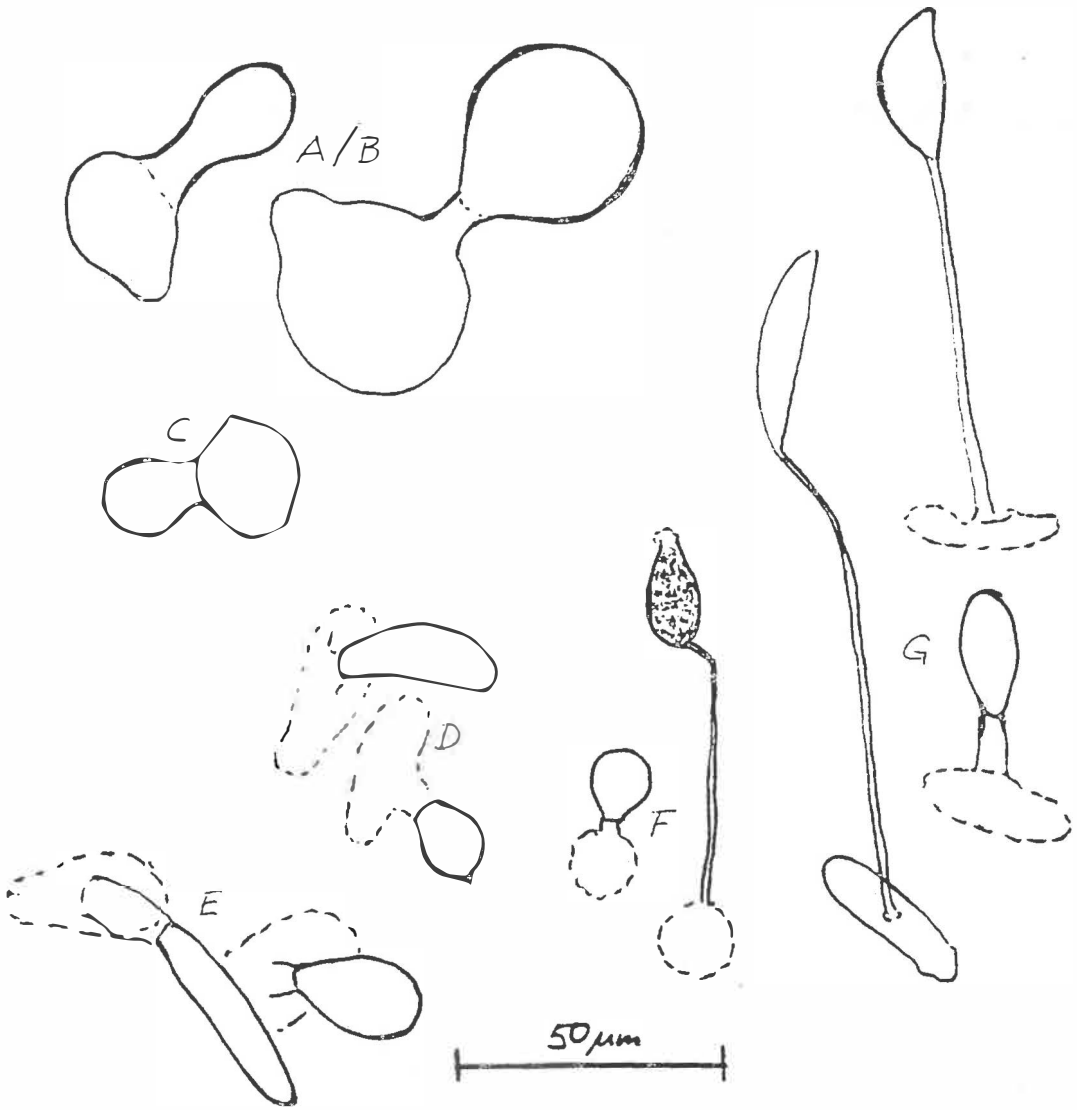


Fig. 3.4.: Conidiophores

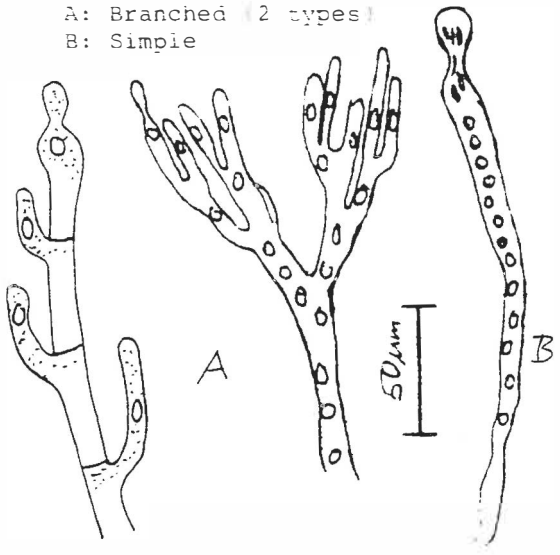
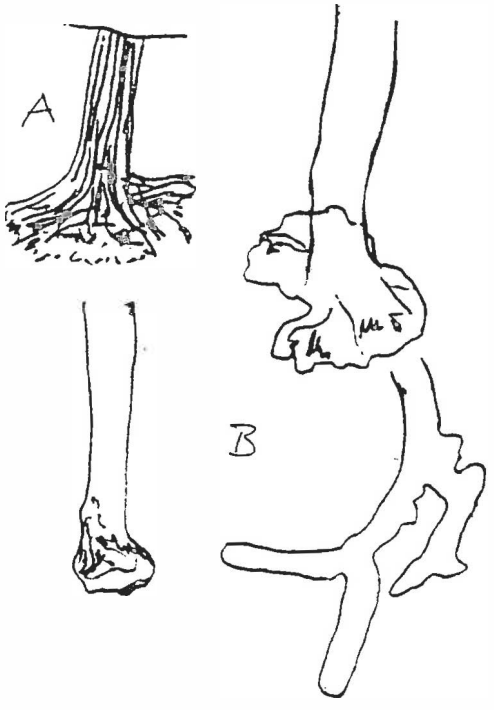


Fig. 3.5. Rhizoids: A: Compound (Pseudorhizomorph)
B: Monohyphal with 3 different endings



ANNEX 1

Stains

Stains (Keller, 1987)

Conidia and cadavers were mounted in lactophenol-cottonblue (LPCB) (0.1% cotton blue) or in lactophenol-aceto-orcein (LPAO) composed of the two following solutions: solution 1: lactophenol (LP) (Romeis, 1968): 20 g crist. phenol, 20 ml lactic acid, 10 ml glycerol, 20 ml dist. water. Solution 2: aceto-orcein (AO): 1 g orcein powder solved in 100 ml 50% acetic acid (v/v). These two solutions were used in the following compositions: 1 part LP + 1 part AO (suitable for nuclear staining in the genera *Conidiobolus*, *Entomophaga*, *Neozygites*, *Eryniopsis*), 2–3 parts LP + 1 part AO (suitable for nuclear staining in the genera *Entomophaga*, *Entomophthora*, *Eryniopsis*, *Erynia*, *Zoophthora*).

The Feulgen reaction stain (FRS) proved to be a suitable stain for nuclei in conidia of practically all considered species (Romeis, l. c.). In some species of Entomophthora good nuclear staining results were obtained with the method of Giemsa (Romeis, l. c.). This method was applied using whole cadavers instead of projected conidia. For the FRS and Giemsa methods fixation is needed prior to the staining process. This results in a shrinking of both the conidia and the nuclei. Both methods especially the FRS eliminate the cytoplasmic content of the conidia. the remaining stained nuclei appear on a clear background which facilitates the counting of nuclei. For this purpose the FRS is recommended for species of the genera *Conidiobolus*, *Entomophaga* and *Entomophthora*.

For histological sections the cadavers stored in ethanol were fixed in Bouin's fixative, embedded in plastic (JB4), sectioned in 4 µm sections and stained with haematoxilin (Romeis, l. c.).

A good nuclear stain is also achieved by adding 0,2-0,5% orcein powder to lactophenol or by placing pieces of infected insect for about 1-3 min in a concentrated aceto-orcein solution (e.g. 5% orcein in 50% acetic acid) and subsequent preparation in lactophenol.

Histochemischer Nachweis der Desoxyribonukleinsäure (DNS) nach F E U L G E N
FEULGEN - Reaction - Stain (FRS)

Herstellung der Lösungen:

n-Salzsäure: 100 ml konz. Salzsäure (spez. Gew. 1,19) werden mit dest. H_2O auf 1000 ml aufgefüllt.

Fuchsinschweflige Säure: (Schiffsches Reagens) 0.5 g Pararosanilin acridinfrei werden in 15 ml n-HCl ohne Erwärmen unter Schütteln vollständig gelöst. Anschliessend Zusatz einer Lösung von 0.5 g Kaliumpyrosulfit ($K_2S_2O_5$) in 85 ml dest. H_2O. Die klare, kräftig rote Lösung entfärbt sich allmählich und wird dabei blass gelblich.
24 Stunden nach dem Ansetzen wird sie mit 300 mg Aktivkohle (pulv.) 2 Min. geschüttelt und filtriert. Das farblose Filtrat ist im Kühlschrank in brauner Flasche mehrere Monate haltbar.

10 % Kalimpyrosulfit: 10 g $K_2S_2O_5$ in 100 ml dest. H_2O lösen. Gut verschlossen, haltbar

SO_2-haltige Spülflüssigkeit: 200 ml Leitungswasser werden mit 10 ml 10% $K_2S_2O_5$ und 10 ml n-HCl vermischt. Das Gemisch jeweils frisch herstellen. Es muss stark nach SO_2 riechen (Schwefeldioxydwasser).

Ausführung der REAKTION:

1) Hydrolyse in n-HCl bei 60°C im Wasserbad oder Wärmeschrank. 5-10 Min.
2) Unterbruch der Hydrolyse, Eintauchen des Objektträgers in Wasser und kurzes Auswaschen der Salzsäure.
3) Einstellen der Präparate in fuchsinschweflige Säure 1 -1 1/2 Stunden je nach Art des Präparates.
4) Auswaschen in SO_2-haltigem Wasser: 3 mal 2-5 Min.
5) Auswaschen in Leitungswasser 10 - 15 Minuten, längeres Auswaschen gibt sattere Färbung.

Achtung! Bei Präparaten die nicht dehydriert wurden ist das Plasmal der Zellen zu entfernen. Einstellen der Objektträger in 95% Alkohol für 24 Stunden. Auswaschen des Alkohols und anschliessend Hydrolyse.

Beim Auswaschen kann Material verloren gehen (z.B. Konidien). Behandeln der Objektträger mit Eiweissglycerin kann Verluste reduzieren.

rb 11.79.

Giemsa-Färbung:

Lösungen: Fixierlösung : 75 ml Alkohol 92 o/o
25 ml Eisessig
Farblösung : Giemsa (Azur-Eosin-Methylenblaulösung) MERCK Art.9204
Hydrolysierlsg: 1 N HCl , 100 ml konz. HCl und 900 ml H_2O dest.
Pufferlösung : pH 7 , oder Leitungswasser pH 7 .

Durchführung:

Zu untersuchende Objekte je nach Grösse , eine Stunde in der Fixierlösung nachfixieren , anschliessend gutes Auswaschen in Pufferlösung oder Leitungswasser. Hydrolysierung in 1 N HCl bei 60^{o} C während 6 Minuten. Auswaschen der HCl in Leitungswasser 10 Minuten, dann Färbung in Giemsa-Leitungswasser (6 Tropfen, 2.5 ml) während zwei Stunden je nach Material kürzer oder länger. Kurzes einlegen in Leitungswasser, dann zerzupfen des Objekts auf Objektträger , trocknen und in Malinol einschliessen. Hyphen hellblau, Kerne dunkel rotblau bis rotviolett.

References

KELLER, S. 1987. Arthropod-pathogenic Entomophthorales of Switzerland. I. Conidiobolus, Entomophaga and Entomophthora. - Sydowia 40: 122-167.

ROMEIS, B. 1968. Mikroskopische Technik. Oldenburg, München, 757 pp.

ANNEX 2

Media

Coagulated egg yolk (EY)

Egg yolk coagulated at 80°C for 40-5o min. (Müller-Kögler, 1959).

Coagulated egg yolk with milk (EYM)

1 part egg yolk diluted with 1 part sterile milk (commercially available UHT milk) coagulated at 80°C for about 70 min (Keller, 1987).

Sabouraud-dextrose-agar with egg yolk (SDAEG)

Autoclaved Sabouraud-dextrose- agar (SDA) enriched with egg at about 80°C (1 egg yolk per 200 ml SDA) (Keller, 1987).

Entomophthora-Complete-Medium (EMC) (BEN-ZE'EV, unpubl.)

Following is the recipe for 1L of solid (2% agar) ECM.

Trace elements solution

1. Dist. H_2O	95 ml
2. Citric acid·H_2O	5 gm
3. $ZnSO_4 \cdot 7H_2O$	5 gm
4. $[Fe(NH_4)_2](SO_4)_2 \cdot 6H_2O$	1 gm
5. $CuSo_4 \cdot 5H_2O$	250 mg
6. $MnSO_4 \cdot H_2O$	50 mg
7. H_3BO_3 annydr.	50 mg
8. $Na_2MoO_4 \cdot 2H_2O$	~~1 ml~~ 50 mg
9. Chloroform	1 ml

Dissolve the ingredients at room temp. while stirring. Sterilize by filtration. Keep in a sterilized bottle at room temp. Add the chloroform afte filtration. After Vogel, H.J. 1956. Microbiol. Gent. Bull. 13: 42-43.

Vitamins Solution

1. Sterile distilled H_2O	1000 ml
2. Thiamin	100 mg
3. Riboflavin	50 mg
4. Pyridoxin	50 mg
5. Ca·Pantothenate	200 mg
6. Para-amino-benzoic-acid	50 mg
7. Nicotinic acid	200 mg
8. Choline chloride	200 mg
9. Inositol	400 mg
10. Folic acid	50 mg

Sterilize by filtration and keep in quantities of 10 ml, in sterilized test tubes in deep freeze. The ingredients after Bradle and Tatum, 1945. Am. J. Bot. 32: 678-686.

Antibiotics Suspension

Sterily mix 6 gm Streptomycin sulphate, 10^6 I.U. Penicillin, and 100 ml sterile, distilled water, at room temp. Part of the antibiotics will not dissovle. Keep in deep freeze and use 10 ml suspension for each 1L. medium. After I. Ben-Ze`ev, 1980, Ph.D. Thesis.

Salts Solution

1. Distilled H_2O	750 ml
2. $Na_3Citrate \cdot 2H_2O$	123 gm
3. KH_2PO_4 annydrous	250 gm
4. $(NH_4)HPO_4$ (or so called Tri-ammonium ortophosphate	165 gm 118.4 gm)
5. $MgSO_4 \cdot 2H_2O$	10 gm
6. $CaCl_2 \cdot 2H_2O$	5 gm
7. Trace elements solution (see above)	5 ml
8. Chloroform	2 ml

Dissolve ingredients in this order, stirring continuously, at room temp. Heating will cause precipitation. Dissovle $CaCl_2$ slowly, in small amounts. Filter-sterilize and then add the chloroform. Keep at room temp. in pre-sterilized 100 ml bottles. Use 20 ml per 1L medium. Open bottles only in sterile conditions. Modified (Ben-Ze'ev, 1980) after Vogel (1956).

ECM 1 L.

Solution A

1.	Deionized H_2O	600 ml
2.	Salts solution	20 ml
3.	Tryptophane	100 mg
4.	Yeast extract (with no more than 2% NaCl)	10 gm
5.	Casein Hydrolysate (with no more than 2% NaCl)	10 gm
6.	Agar-agar	20 gm
1.	Deionized H_2O to complete to: a total of	700 ml

Solution B

7.	Carbon source (soluble starch or glucose, or maltose, etc.)	20 gm
1.	Deionized H_2O to make a total of	300 ml

Both solutions are prepared while heating and continuously stirring. When using soluble starch make sure that it is completely dissolved before autoclaving. The solutions are autoclaved separately and mixed afterwards. Plates or slants are poured when the medium has cooled to about 60°C. Vitamins solution (10 ml/L)* are added and mixed into the medium at 60°C, immediately before pouring plates or slants.

* and antibiotics suspension (10 ml/L)

To make liquid ECM omit the agar.

Solid ECM should be quite transparent but becomes opaque when stored in the refrigerator. It is best stored in Petri-dishes sealed in plastic bags, at 10° - 15°C.

Advantages of ECM

Most Entomophthorales grow on ECM as well as, or better, than on egg-yolk media. Being transparent, ECM allows microscopic observations, which are impaired by the opaqueness of egg-yolk. Some Entomophthorales will grow on Sabouraud's maltose + yeast extract medium (SMY) as well as on ECM and will allow direct isolation from the insect as well as on egg-yolk and on ECM, but some fungi seem to lose the ability to sporulate after prolonged subculturing on SMY. Some others, that have been isolated directly on ECM (like E. muscae), could not be isolated on SMY.

COMMON LIQUID MEDIA FOR LABORATORY PRODUCTION OF ENTOMOPHTHORALEANS

DY (Dextrose - Yeast Extract)

10.0 g/l	Dextrose (Clintose)
30.0 g/l	Yeast Extract (Amberex 1003)

CYP (Corn Syrup - Yeast Extract - Peptone)

40.0 ml	Corn Syrup (Karo, dark)
10.0 g	Yeast Extract (Amberex 1003)
10.0 g	Soytone (BBL)
960.0 ml	distilled water

SDY (Sabouraud Dextrose Broth - Yeast Extract)

40.0 g/l	Dextrose (Clintose)
10.0 g/l	Neopeptone (Difco)
10.0 g/l	Yeast Extract (Amberex 1003)

Liqid medium of BEAUVAIS & LATGE

2000 mg dextrose

3840 mg NaCl

3250 mg lactalbumin-hydrolysate

2500 mg yeast extract

976 mg MES buffer

500 ml Water

adjust to pH 7 with ln NaOH

autoclave

References

KELLER, S. 1987. Arthropod-pathogenic Entomophthorales of Switzerland. I. Conidiobolus, Entomophaga and Entomophthora. Sydowia 4o: 122-167.

MUELLER-KOEGLER, E. 1959. Zur Isolierung und Kultur insektenpathogener Entomophthoraceen. Entomophaga 4 (3): 261-274.

ANNEX 3

Entomophthorales: A key for the identification of the arthropod-pathogenic genera and their characterisation

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