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GROUPE DE TRAVAIL "LES ENTOMOPATHOGENES ET NEMATODES PARASITES D'INSECTES"

THIRD EUROPEAN MEETING "MICROBIAL CONTROL OF PESTS"

WAGENINGEN, 24 - 27 FEBRUARY 1991

EDITED BY
EDITE PAR *P.H. SMITS*

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INTERNATIONAL ORGANIZATION FOR BIOLOGICAL AND INTEGRATED CONTROL OF NOXIOUS ANIMALS AND PLANTS

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Introduction

This bulletin contains the proceedings of the 3rd meeting of the IOBC/WPRS Working Group on Insect Pathogens and Insect Parasitic Nematodes. The meeting took place at Wageningen, the Netherlands, from 24-27 February 1991. Its major themes were:

a) Diagnosis of insect diseases, with particular emphasis on neglected groups of micro-organisms.

b) "Behaviour" of insect pathogens in the environment, i.e. their persistence, ecology and epizootiology.

Furthermore, in two parallel sessions, the insect mycologists and their colleques working on insect parasitic nematodes discussed their specific problems and organised themselves in two formal subgroups of the Working Group. As leaders of the subgroups have been elected Dr. B. Papierok and Dr. R.-U. Ehlers, respectively. The meeting was attended by 110 scientists from 17 different countries, active on research on microbial pest control.

I would like to thank Dr. Peter Smits and Dr. Albert Minks and their staff for the excellent manner in which they organised the meeting in the convenient facilities of the International Agricultural Centre at Wageningen. After a brief period of convenership, I will step back from my position as convener, due to other commitments with the IOBC/WPRS. I wish my successor Dr. Peter Smits every success as the convener of our Working Group.

Jürg Huber
Convener

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EXPERIENCES IN DIAGNOSIS OF ARTHROPOD DISEASES WITH REFERENCE TO SOME UNPRECEDENTED PATHOLOGIES

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SUMMARY

The present status of diagnosis of arthropod diseases, especially in view of the increasing research on, and use of, pathogens and entomophagous arthropods for biological pest control, is outlined. As institutional diagnostic services are not properly established, routine procedures for rendering preliminary diagnoses are recommended and discussed. Once a tentative diagnosis is made and the causative agent is identified as to one of the higher taxonomic categories, the material should be forwarded to a specialist of this group who renders a definitive diagnosis, advises on practical aspects and how to overcome the disease condition in the arthropod rearing.

The importance of histo- and cytopathological investigations is stressed. Studies of this kind reveal a wealth of diagnostic information, such as pathways of infections, histo- and cytotropism of pathogens, pathogenesis, host defence reactions, and chronic, double or secondary infections.

Attention is drawn to unprecedented pathologies as exemplified by (1) baculovirus infections of adults of *Oryctes rhinoceros* which create an efficient 'factory' of virus reproduction in the midgut tube; (2) larval infections of *Costelytra zealandica* by *Serratia entomophila*, inducing fatal 'amber disease' by colonizing hosts only at the cardiac valve and at the intima of the crop; (3) the son-killer trait of the parasitoid wasp *Nasonia vitripennis* caused by a contagiously transmitted chronic bacterial infection.

As demonstrated by case studies, interactions of pathogens between hosts and beneficial arthropods deserve special attention in order to avoid setbacks in mass rearings and use of beneficials in biological pest control.

The need to establish institutional diagnostic services is emphasized.

IMPORTANCE D'UNE METHODOLOGIE APPROPRIEE POUR L'ETUDE DES DIFFERENTES MYCOSES D'INSECTES (RECOLTE, PREPARATION, ISOLEMENT)

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Summary

Entomopathogenic species are found in various fungal groups, almost everyone of them requiring appropriate methods for collecting and preparing the specimens and isolating the etiologic agents. Being mostly specialized in one or a few related groups, entomomycologists are rarely aware of the methodology for the other groups, which could originate a loss of informations in the course of investigations in the field. Considering methodology for entomopathogenic Deuteromycetes in aerial habitats is generally well known, it seemed useful to gather in this note references of papers where are described appropriate methodologies for others groups of fungi, i.e. Oomycetes and Deuteromycetes on aquatic dipteran larvae, Entomophthorales and **Cordyceps** spp. on aerial insects.

Des mycoses d'insectes se rencontrent dans le milieu aquatique aussi bien que dans le milieu aérien. Les espèces responsables appartiennent à des groupes très divers, de morphologie variable et de cycle biologique plus ou moins complexe. A chaque groupe correspondent donc des méthodes de récolte et de préparation des cadavres ainsi que des méthodes d'isolement appropriées, qui garantissent l'obtention du matériel nécessaire à l'identification du pathogène. Il n'est pas rare, dans ces conditions, que les mycologistes d'insectes, le plus souvent assez spécialisés, connaissent mal la méthodologie d'étude pour les groupes avec lesquels ils sont peu familiarisés, d'où une perte potentielle d'informations lorsqu'un chercheur donné prospecte sur le terrain. L'exemple des Entomophthorales et de leur présence sous les Tropiques est à ce titre révélateur. On sait que ces champignons s'y comportent comme des ennemis naturels particulièrement actifs d'insectes de divers ordres. Il faut avoir présent à l'esprit, cependant, que dans la majorité des cas, les données correspondantes sont récentes et qu'en fait, les Entomophthorales avaient été quelque peu négligées dans ces régions jusqu'à ces dernières années, comparativement à d'autres champignons entomopathogènes comme les Ascomycètes du genre **Cordyceps** et les Deutéromycètes (Papierok, 1987).

Aussi nous proposons-nous de rappeler ici des références d'articles où est décrite la marche à suivre pour tirer au mieux parti de différentes mycoses d'insectes que l'on peut trouver dans les conditions naturelles (récolte et préparation des cadavres, isolement de l'agent responsable). Trois grandes catégories de mycoses seront prises en considération; la première est due à des Oomycètes ou à des Deutéromycètes dans le milieu aquatique (sur larves de moustiques), la deuxième et la troisième respectivement à des Entomophthorales et à des Ascomycètes du genre **Cordyceps** dans le milieu aérien. Nous ne retiendrons pas le cas des mycoses à Deutéromycètes rencontrées dans ce même milieu ou dans le sol. La méthodologie est en effet bien connue et de plus, l'expérience montre que ce sont les mycoses d'insectes dont l'étude sur le terrain soulève le moins de difficultés.

Oomycètes et Deutéromycètes infectant les larves(aquatiques) de moustiques

La méthodologie proposée par Brey et Remaudière (1985) et par Brey et Papierok (1987) dans le cas des Oomycètes et des Deutéromycètes s'attaquant aux larves de moustiques (Diptères, Culicidae), a pour but de n'isoler que les seules espèces effectivement entomopathogènes. Pour cela, on fait évoluer des individus sains au contact des individus trouvés infectés et on contrôle qu'il y a transmission de la maladie. L'isolement proprement dit se fait directement à partir des cadavres, après stérilisation superficielle.

Entomophthorales

La méthodologie à appliquer avec les insectes tués par Entomophthorale est fondée sur la particularité qu'ont les conidies de ces Zygomycètes d'être projetées à partir des conidiophores. Nous avons récemment publié un descriptif détaillé et illustré de la marche à suivre (Papierok, 1989). L'attention du lecteur y est attirée notamment sur la nécessité de préparer rapidement les insectes présentant les symptômes d'une infection par Entomophthorale, compte tenu de la fragilité de certaines structures de ces champignons. L'importance qu'il y a à recueillir des conidies secondaires, obtenues par germination des conidies primaires, est également soulignée.

Ascomycètes du genre Cordyceps

La méthodologie d'isolement du champignon à partir de cadavres d'insectes portant des fructifications de **Cordyceps** sp. a été notamment précisée par Evans et Samson (1982). Les observations faites de notre côté, en Côte-d'Ivoire (Papierok et Charpentié, 1982) ou en Indonésie (Papierok et Desmier de Chenon, en préparation) par exemple, ont montré que le fait de maintenir les cadavres dans des conditions d'humidité saturante permet d'obtenir l'anamorphe d'une manière quasi-systématique.

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A microsporidium (Microspora: Pleistophoridae) in mass-rearings of the predatory mites *Amblyseius cucumeris* and *A. barkeri* (Acarina: Phytoseiidae): analysis of a problem

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Summary

The commercial production of the predatory mites *Amblyseius cucumeris* and *A. barkeri*, used for biological control of thrips in vegetables in greenhouses, is at stake, because of a reduced production and quality of these mites in mass-rearings. This problem is caused by a microsporidian disease (Microspora: Pleistophoridae), which is found in the predatory mites as well as in their prey in the rearings: *Acarus siro* or *Tyrophagus putrescentiae*. Aspects of the pathology and transmission are discussed.

Introduction

The predatory mites *Amblyseius cucumeris* (Oudemans) and *Amblyseius barkeri* (Hughes) are used for biological control of the thrips species *Frankliniella occidentalis* Pergande and *Thrips tabaci* Lindeman, both serious pests in greenhouse vegetables. The predators are part of the integrated pest management programmes of several vegetables in greenhouses in the Netherlands. For the commercial production of the predators, a mass-rearing method is used, originally developed by Ramakers and van Lieburg (1982).

The predators are reared in large aerated containers, which are filled with hundreds of liters of wheat bran. A temperature of approximately 22 °C and a high relative humidity (~90%) allows the growth of fungi on the bran. The fungi serve as food for stored product mites (*Acarus siro* L. or *Tyrophagus putrescentiae* (Schrank)), which in turn are fed upon by the predatory mites.

Recently, these mass-reared *Amblyseius* species became affected by a microsporidian disease (Ramakers *et al.*, 1989; M. Dissevelt & W. Ravensberg (Koppert B.V.), pers. comm.). Ramakers *et al.* (1989) suspected that the pathogen is a member of the genus *Nosema*, but more detailed investigations showed that we are probably dealing with a new species belonging to the family Pleistophoridae (A.M. Huger (Institut für biologische Schädlingsbekämpfung, Darmstadt, B.R.D.), pers. comm.).

The productivity of the mass-rearing decreased drastically as a consequence of the disease, and also quality of the predators produced may have been reduced. For these reasons, the disease is a threat for the biological control of thrips. If pesticides (such as pyrethroids) have to be used instead, the full program of integrated pest control measures is at stake, since the pesticides may interfere with the effectivity of other natural enemies.

Pathology

Microsporidiosis in the predatory mites *A. cucumeris* and *A. barkeri* is only recognizable in heavily infected mites when the disease is in an advanced stage and when many spores have been

produced. Such diseased mites have a swollen and whitish appearance and are sluggish in their movements. The symptoms are even more striking when the mites are observed under the light microscope: several organs (e.g., gut) are easily recognized in disease-free individuals, while internal structures in infected mites are hardly visible. Squash preparations of mites in an advanced stage of the disease show numerous spores which have been formed in the cells of the mites, leaving hardly any healthy tissue fragments behind.

In the mass-rearing, also infected prey mites have been observed. The symptoms of microsporidiosis in these mites are comparable to those in the predatory mites, although less conspicuous. Disease-free flour mites (and the other stored product mites) already have a whitish appearance and are slower in their movements than predatory mites.

Questions

In order to prevent future infestations in mass-rearings of predatory mites with microsporidia (or to cure already infected ones), it is important to know how the microsporidium persists in the culture. In other words: (i) Which conditions make it possible for the spores to survive outside the host? and (ii) How do mites become infected (or: how is the microsporidium transmitted)? The importance of the first question with respect to persistence of the disease in culture, depends on the number of free spores and on their contribution to transmission of the disease.

Persistence of the spores

Spores excreted (with faeces or other excretory products) by diseased mites, together with spores released after death of their hosts, form the "free-spore pool". On one hand, the pool increases in size by accumulation of spores; on the other hand it decreases since spores become inactivated by several factors. For many microsporidia ultraviolet light is one of the more destructive environmental factors (Ignoffo *et al.*, 1977; Kaya, 1975). In mass-rearings, spores are not directly exposed to sunlight, which probably means that UV-light does not play a prominent rôle in the inactivation of the spores. Temperature and relative humidity, on the other hand, may be important factors for survival of the free spores in mass-rearings. With increasing temperature and relative humidity, reduced spore viability has been observed (Gardner *et al.*, 1977), but tests on the persistence of spores under stored conditions, often vary with species of microsporidium: some microsporidia cannot even survive short periods of desiccation, while others require dry storage to prevent germination (Canning, 1982).

It is not known how the mass-rearing conditions (relative humidity ~90%; temperature ~22°C) affect the virulence of the spores. Also other conditions of the mass-rearings (e.g., CO_2 concentration) may be of importance for the longevity of the spores. In addition, conditions that are negative for spore persistence, may well be positive for the development of microsporidia in mites, and vice versa. For example, a high temperature might reduce the virulence of spores, and at the same time it might accelerate the development of the microsporidiosis in the host.

Transmission of the microsporidium

Since the microsporidium occurs at two trophic levels (the predator and the prey), the possible ways by which the microsporidium may be transmitted are more extended than in case of a restriction to one host species. In our opinion, five possible ways of transmission of microsporidia may be distinguished (see figure 1). In addition to vertical transmission (from parent(s) to offspring; v), four ways of horizontal transmission (h) may occur: by predation (h1), by contact with the free-spore pool (h2), by contact with the other species or with conspecifics (other than feeding or mating; h3), and by mating (h4).

Since in the rearings not only infected predators but also infected flour mites (or other stored product mites) can be found, a possible way of transmission is ingestion of the spores when predatory mites are feeding on infected flour mites (h1a). Predatory mites are known to be cannibalistic, which means that the infection may be established by consuming diseased

conspecifics as well (h1b). It seems less likely that flour mites become infected by diseased predatory mites; however, prey sometimes manages to escape from a predatory mite that started feeding on it (pers. obs. E.B.), and this may be just sufficient to transmit the microsporidium to the flour mite (h1a).

Contact with the free-spore pool may be an important way of transmission (h2), since the wheat bran and the fungi are contaminated by spores. By eating, or even tasting, all kinds of substances (like fungi), flour mites, and also predatory mites, may ingest spores. Even by cleaning their legs or mouthparts, infection might occur.

A male may transfer spores along with sperm during mating, or might obtain spores from a diseased female (h4). Many microsporidia are transmitted to the offspring of the host by means of vegetative phases (v; Canning, 1971), and this may well be the case with the microsporidium in the mass-rearing. The host may be either or both of the parents.

Physical contact with other mites (other than mating or feeding; h3) may be considered as a fifth possibility, but one should realize that spores probably have to be ingested to become infective, and that it is not likely that diseased mites carry any other spores on the cuticula than those obtained from the free-spore pool (= transmission by contact with free-spore pool).

Transmission probably depends on the incubation time and the infective dose. Once a host has been infected by a microsporidium, it takes time before spores are formed, before the infection has spread to the reproductive organs. Only then the microsporidium will be available for (horizontal or vertical) transmission. The rate at which the disease develops in a host, is probably set by the infective dose, *i.e.*, the number of spores that caused the infection.

In theory, it is possible the mites become immune to the microsporidium, especially when the infective dose is low. Once immune, they may become susceptible again. However, a memory component to the defence system, is thought to depend on life expectancy of animals. For short-lived organisms like mites, memory would be of limited value to their fitness (Anderson, 1986).

In case of transmission by predation (or cannibalism) the feeding behaviour of the mites has to be taken into account. Young predatory mites were found to eat only juvenile stages of flour

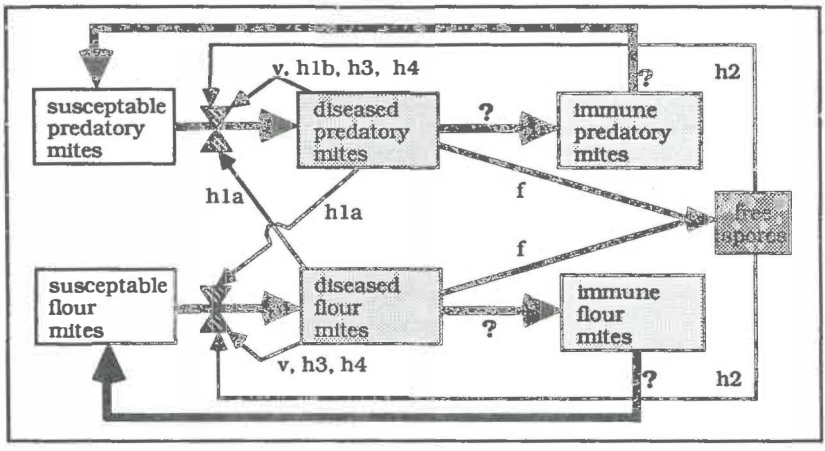

Figure 1: Possible ways of transmission of a microsporidium in the mass-rearing of the predatory mites Amblyseius cucumeris *and* A. barkeri *on the flour mite* Acarus siro *(or* Tyrophagus putrescentiae*).*

h1a: horizontal transmission by predation
h1b: horizontal transmission by cannibalism
h2: horizontal transmission by contact with free-spore pool
h3: horizontal transmission by contact with conspecifics or other species (not feeding or mating)
h4: horizontal transmission by mating
v: vertical transmission
f: contribution to free-spore pool by excretion by and death of diseased mites

mites or eggs, while the older predatory mites also preyed on the older ones (pers. obs. E.B.). These observations combined with the incubation time and infective dose dependency of the transmission, may very well restrict or even inhibit the transmission by predation.

Current and future research

Currently we focus on sorting out which of the transmission mechanisms hold true. To do so, an efficient biological assay method is indispensable. Recently, Beerling (unpublished) developed such a method for predatory mites and flour mites. By means of a bioassay, several aspects of the microsporidiosis can be studied; isolated mites are fed known dosages of spores, and the effect can be followed in time. Changes in behaviour and appearance can be observed, and the development of the disease can be studied histologically by making sections at different moments after infection. The virulence of the microsporidium (LD_{50}, LT_{50}) can be tested for different mite stages and for different mite species.

This bioassay method may also be used to test spores which have been exposed to viability-reducing treatments (*e.g.*, high and low temperatures and relative humidities; chemicals), or to study the effect of different environmental conditions on the development of microsporidiosis in the mites.

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SPIROPLASMAS - A NEGLECTED GROUP OF INSECT PATHOGENS

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Summary
Spiroplasmas are interesting but not yet well known group of insect pathogens. In many cases they are evidently commensals or symbiotes but some of them are definitely pathogenic to their hosts. For microbial pest control the greatest interest from the last group present Spiroplasma sp. from the Colorado potato beetle (Leptinotarsa decemlineata) at first discovered in the Northern America but during 1987-1989 recorded in populations in Poland, Germany and the Soviet Union. A recently discovered in Poland a new Spiroplasma sp. have some peotential in control of satin moth (Stilpnotia salicis).

1. Introduction

Spiroplasmas represent a good example of neglected group of insect pathogens and their potential in microbial control awaits thorough studies and practical exploitation.

It may be mentioned that soon after the first spiroplasmas infecting plants were found in 1972 (DAVIS et al., 1972) a strictly entomopathogenic spiroplasma was discovered by MARQUES and deMALGHAES (1973) in Drosophila sp. CLARK (1982) reviewed spiroplasmas known from insects but since that time there was a big progress in our knowledge of that peculiar and interesting group of prokaryotes (LIPA et al., 1988).

Spiroplasmas belong to the order Spiroplasmatales which together with Mycoplasmatales and Acholeplasmatales, belong to Mollicutes (Procaryotae) (WHITCOMB, 1981; BOVE, 1984; TULLY et al. 1987).

The genera Acholeplasma, Mycoplasma and Spiroplasma are associated with plants and various animals including insects and ticks.The members of the first genus occur also in sewage, compost and soil.

Cells of the genus Spiroplasma are pleomorphic, varying from helical and branched uni-helical filaments to spherical and ovoid. They are motile and have intracellular fibrills, though they are without flagella. They divide by binary fission and require cholesterol for growth. They have been found in vertebrates (mice), in the haemolymph and gut of insects and ticks, in the phloem of plants and on plant surfaces.

2. Spiroplasmas of plants

Spiroplasma citri and S. kunkelii are the best known plant spiroplasmas since they are causative agents of serious plant diseases (BOVE 1984, SMITH et al., 1988).

S. citri is a causative agent of citrus stubborn disease. It infects mainly plants belonging to family Rutaceae, but was transmitted to plant species from 15 families of mono- and

dicotyledones. It is transmitted by leafhoppers (Jassidae) e.g. in Europe by Neoliturus haematoceps.

S. kunkelii is causing corn stunt disease of Zea mays and Z. mays mexicana but can be also transmitted to many dicotyledonous plants.

S. floricola isolated from flowers and leaves of many plants, when injected to hemocoel of larvae of Galleria mellonella, caused striking cytopathological changes which indicate that it can develop in plants as well as in insects (ESKAFI et al. 1987).

4. Spiroplasmas of beneficial insects

First known entomopathogenic spiroplasma was recorded in Drosophila spp. (MARQUES and deMALGHAES, 1872; WILLIAMSON and POULSON, 1979). This spiroplasma destroys sex chromosome of males, and causing complete elimi-nation of males, may be responsible for the total collapse of rearing colonies used for genetic or toxicological research. For the reasons explained above this spiroplasma is named "sex regulating organism".

Special interest in spiroplasmas of insects arised when it was discovered that the so called "May-disease" of honey bee (Apis mellifera) is caused by Spiroplasma apis (CLARK, 1977; MOUCHES et al., 1983). This disease known for a very long time was thought to be caused by pollen-intoxication and is responsible for high mortality in bee colonies (CLARK, 1978).

Some years later a second species Spiroplasma melliferum (CLARK et al., 1985) was described from the honeybee (A. mellifera) being pathogenic to various insects.

5. Spiroplasmas of noxious insects

Since spiroplasmas can cause high mortality or sex disturbances in their insect hosts, there is an interest in their potential as microbial control agents.

Of special interest is Spiroplasma sp. discovered in the Colorado potato beetle (Leptinotarsa decemlineata) (CLARK, 1982; HACKETT and LYNN, 1985). This spiroplasma is host-specific and able to achieve titers of 10^{10} per mililiter of gut fluid. It is easily transmissible to both larvae and adults via regurgited gut fluid and feces and persists on potato leaves and in hibernating adults. The infection level of natural populations of L. decemlineata by spiroplasma varies from 10 to 100% in the USA.

I have initiated studies on Spiroplasma sp. from L. decemlineata in 1987 and found it in populations of this insect in 1987 in Poland and Germany (former the GDR area), and in 1988 and 1989 in the USSR. The infection level among adults collected in the field varied in various populations from 0 to 40%. The pathogenicity and the role of Spiroplasma sp. as the mortality factor in populations of its host has to be assessed.

Recently, we discovered (LIPA et al., 1988) Spiroplasma sp. infecting the satin moth Stilpnotia salicis which presents the first record of spiroplasmosis among Lepidoptera. This spiroplasma was present in 8 surveyed populations of this insect in Poland and the infection level varied from 8 to 20%.

It may be assummed that hundreds of spiroplasmas await to be discovered and this group should be an object of intensive studies of insect pathologists. Quite recently spiroplasmas were discovered in mosquito Aedes sollicitans (HUNG et al.,1987), Elateridae (HACKET et al., 1988), Diabrotica undecimpunctata (TULLY

et al., 1987), Tabanus spp. (TULLY et al., 1987) and others.

6. Methods of studies of spiroplasmas

Because of their helical structure, characteristic movement and length (5-10um) surveying of insects for spiroplasmas does not require using an electron microscope but especially equipped compound light microscope is sufficient. Although the length of spiroplasmas is relatively large, they are so thin (slender) that in the oridinary light microscope they are not seen due to their transparency. Therefore, dark field illumination and the magnification 600-1000x is needed. In case of large spiroplasmas phase contrast or similar optic principles may be useful.

When regurgitated gut fluid or drop of haemolymph is observed in the dark microscopic field, spiroplasmas are seen as bright, very thin, moving spirals. On fixed and stained with Giemsa's smeared preparations spiroplasmas are seen as filamentous structures (LIPA et al., 1988). In the scanning electron microscope the spiral structure of spiroplasmas is well seen.

7. Potential of spiroplasmas in biological control

Although so far no attempts were made as to the use of spiroplasmas for microbial control of insects this seems to be quite possible.

HACKETT et al. (1988) assume that spiroplasmas could be used in three ways: 1 - direct use of pathogenic strains; 2 - use of strains engineered for enhanced pathogenicity with exogenous genes e.g. toxin-producing; 3 - spiroplasma genes might be exploited by introducing them into other organisms. It may be expected that genes of Bacillus thuringiensis will be inserted to some spiroplasma. The other atractive possibility is to use the gene of Spiroplasma controlling male sterility in Drosophila and to insert it to other spiroplasmas or to insects. First attempts in this direction were already made (FITZ-EARLE et al., 1987).

8. Concluding remarques

Spiroplasmas are interesting but not yet well known pathogens of insects. In some casesthey are evidently commensals or symbiotes but some of them are definitly pathogenic to their hosts.

Intensive survey may result in finding several new records or strains of spiroplasmas useful for biological control of noxious insects.

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CLASSIFICATION AND ECOLOGY OF MOSQUITO SPIROPLASMAS

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Spiroplasmas (Mollicutes) are wall-free prokaryotes with a genome size of 10^9 Daltons, have a G+C content of 25% - 31%, require cholesterol for growth and exhibit helical morphology during the logarithmic growth phase. They are found in association with plants, flowers, insects and ticks. Some are highly pathogenic for plants (crop, citrus) or insects (domestic bees, beetles, fruit flies). They are classified into groups (from I to XXIV) and eventually subgroups and serovars according to their serological and molecular properties.

The first mosquito spiroplasma (msp) was isolated in August 1981 from a pool of female Aedes sollicitans caught in New Jersey (Slaff and Chen, 1982) and later described by Hung et al (1987) as Spiroplasma culicicola. Other msp were subsequently isolated from France (Chastel et al, 1985 ; Le Goff et al, 1990), the Far East (Rosen et al, 1986) and Alabama (Shaik et al, 1987).

At present, there are only three fully described msp : S. sabaudiense (group XIII) from Aedes sticticus/vexans collected in France (Abalain-Colloc et al, 1987), S. culicicola (group X) from Aedes sollicitans collected in New Jersey (Hung et al, 1987) and S. taiwanense (group XXII) from Culex tritaeniorhynchus collected in Taiwan (Abalain-

Colloc et al, 1988).

A number of other spiroplasmas has been isolated from mosquitoes originating from Taiwan, Japan and the U.S.A., but they remain insufficiently characterized. These are antigenically related to bee - (group I-2) or wasp - (group VII) spiroplasmas.

Of particular interest are the group XVI spiroplasmas presently known as the "Cantharis spiroplasma" because representative strains of this group have been isolated for a large variety of insects (two beetles, a wasp, a fire fly and many species of mosquitoes) in U.S.A. and France, and also from a flower, the thistle (Circium sp.) in France. Using serological methods, Tully et al (1987) have previously recognized at least three serovars among three of these strains. The classification of these spiroplasmas using a combination of serological and biochemical methods, which included protein SDS-PAGE, DNA/DNA hybridization and restriction enzyme analysis, has recently been conducted in our laboratory ; 17 strains from different hosts and geographical origins were classified into three proposed subgroups : XVI-1, XVI-2 and XVI-3 (Abalain-Colloc, Thesis, Brest, 1991).

Biological properties of msp are quite identical to those of other spiroplasmas infecting insects, ticks and plants.

Ecological studies have been carried out only in France, and in two different study areas : 1) Savoia, on flooded banks of mountain rivers, and 2) "Loire Atlantique" district, on salt marsh biotopes and banks of the Loire river (Chastel et al, 1987 ; Le Goff et al, 1990).

Only two msp species circulate among wild mosquito populations during spring-summer in France, namely S. sabaudiense and

the "Cantharis spiroplasma".

The following were shown to contribute to the ecology of these spiroplasmas : mosquito sex and species, the month and the method of collection of mosquitoes, the altitude of biotopes and the associated vertebrates. Isolations of msp succeeded only from pools of female mosquitoes of certain species (Aedes, Coquillettidia richiardii), from human baits and only during June and July, from biotopes of relatively low altitude (290-310m). Antibodies to the Cantharis spiroplasma were present in cows living in the same biotopes (Chastel et al, 1987).

Further studies in Savoia confirmed these previous observations, but antibodies in a bank vole (Clethrionomys glareolus) were found, while isolation attemps from individual mosquitoes demonstrated two important findings : Aedes sticticus is the main host of both S. sabaudiense and the "Cantharis spiroplasma" and msp are located within the mosquito body and not on to the integument. The "Cantharis spiroplasma" was isolated twice from a flower, the thistle (Circium sp.) taken from among 50 flower specimens from the same biotopes. Accordingly, a provisional "cycle" for the "Cantharis spiroplasma" has been proposed integrating data from both the U.S.A. and France (Chastel et al, 1990).

Finally, population dynamics of msp were studied in Savoia in 1987-1988 : msp were absent from mosquitoes in March, appeared progressively in June, exploded in July and August, and then disappeared suddenly in September. It was thus postulated that mosquitoes probably acquired their msp infection from other insects during nectar feeding.

Ecological studies recently conducted in "Loire

Atlantique" district showed that two salt-marsh mosquito species, Aedes detritus and Ae. caspius, are also hosts of msp. Studies of population dynamics in this area gave results very similar to those obtained in Savoia : after their explosive development during the summer months msp disappeared completly in September (Le Goff et al, 1990). However, attemps to isolate msp from 240 specimens of plants and flowers collected in the same Atlantic biotopes failed (unp. results, 1990).

In conclusion, only two species of msp actively circulate in France among mosquitoes, S. sabaudiense and the "Cantharis spiroplasma". S. sabaudiense appears as a strictly mosquito-adapted species but nothing is know concerning its life-history. On the contrary, the "Cantharis spiroplasma" seems more catholic in the choice of its hosts and is possibly acquired by mosquitoes in spring-summer months during nectar feeding from flowers infected by other insects.

For a more complete bibliography see : C. Chastel and I. Humphery-Smith : Mosquito spiroplasmas. Advances in Disease Vector Research, 1991, vol. VII, pp. 149-206 (Springer-Verlag Edit., New York).

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Xenorhabdus spp., bacteria associated with entomopathogenic nematodes

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Summary

Xenorhabdus spp. (**Enterobacteriaceae**) are symbiotically associated with **Steinernematidae** and **Heterorhabditidae**. During parasitization in most of the insect species there is no recognition of living bacteria and of nematodes by hemocytes and the defence reactions are avoided. In addition both partners of the bacterial-helminthic complex act together to kill insect preys by production of toxins and septicaemia. Nematodes reproduce in the host cadaver. **Steinernematidae** and **Heterorhabditidae** are also able to develop axenically on rich complex media or in symbiosis with bacteria on poor complex media. A little level of reproduction can be obtain with several Gram negative bacteria, but nowhere near the level to which they reproduce when cultured with their natural symbionts. *Xenorhabdus* spp. occur in two forms described as the result of a phase variation. Colonies of phase one adsorb dyes on agar plates, produce antibiotic compounds, and according to strains and species, are pigmented, produce bioluminescence, lecithinase and protease. The greatest yields of nematode production on cheap artificial diet are obtained with the symbionts phases one, specially with *Heterorhabditis-X.luminescens* associations. Conditions of phase variation induction are not still defined to day. Anaerobiosis seems to allow *in vitro* phase shifts which always appear during the stationary period of bacterial growth.

1°/ Introduction

The goal of this communication is to give the headlines of the present knowledge on *Xenorhabdus* spp. (Enterobacteriaceae). These bacteria are not pathogenic for insects when ingested (AKHURST,1982;BOEMARE,1983), but they are very infectious after inoculation (the LD_{50} is usually being less than 50 cells according to the test insects). They need to be inoculated into the insect hemolymph by their host nematode which act as a living seringue on the target insects. After the release of *Xenorhabdus* cells into hemolymph from the digestive tract of nematode 3rd instar larvae (L3), the beginning of the bacterial growth produce antibiotic compounds, efficient against a wide range of microorganisms, to inhibit a possible contamination (AKHURST, 1980; 1982). The death of the insect prey is mainly due to the septicaemia. Nematodes reproduce in the cadaver and this situation is quite equivalent as a monoxenic microcosm where nematodes find the suitable nutritional requirements for their development in a medium specially prepared by their symbionts.

Among the 128 insect species tested in laboratory, only 28 (mainly belonging to Diptera) were proved resistant to *Steinernema* strains (LAUMOND *et al.*,1979). A wide spectrum was also evidenced for *Heterorhabditis* species (KHAN *et al.*,1976). These broad host ranges can be explained by efficient pathogenic properties of the bacterio-helminthic complexes to inhibit the insect defence reactions. In fact pathogenic action of both partners of these symbiosis cannot be separated : they act together.

2°/ Biology and Pathogenicity of *Xenorhabdus* spp.

The question is what kind of evasion system is involved to prevent the insect immunity? In most of the insects no immune responses are recorded neither against nematodes nor against *Xenorhabdus* living cells. The situation can be summarized in two strategies : avoidance of recognition and/or avoidance of the defence reaction itself. For instance in locusts, the surface of nematode cuticle is not recognized as foreign when *Steinernema* penetrates into haemocoele (BREHELIN & BOEMARE, 1988). When the nematode penetrates into the body of the host through the cuticle, tracheae or digestive tract, it makes wounds which induce the secretion of antibacterial proteins which could lyse the symbiotic bacteria released by the nematodes. **Steinernematidae** are able to secrete a factor which selectively inhibits the immune proteins produced for instance by *Hyalophora cecropia* (GOTZ *et al.*, 1981) or *Bombyx mori* (BREHELIN *et al.*, 1990). Works are in progress to compare this inhibiting factor with toxins and protease evidenced in *Steinernema* (BOEMARE *et al.*,1982; LAUMOND *et al.*,1989). Even when **Diptera** built up a capsule arround nematodes (*Aedes, Culex, Chironomus*), and consequently are recognized as non self, **Steinernematidae** and their bacterial guests are able to kill their insect prey. So these bacteria with the helping of their nematode hosts are capable to evade the insect immunity in two ways: evasion of non-self recognition and/or depression of the defence reactions.

No-recognition is essential for an holoxenic parasitism but not sufficient for predatism which must kill the insects. In fact each partner of these bacterio-helminthic complexes possess several "weapons" against insects. For the bacteria guests those are exotoxins, endotoxins and their proper virulence (BOEMARE & AKHURST, 1990) and, in addition, the contribution of the antimicrobial production to prevent any foreign contamination. For the nematode hosts those are immunedepressive factor, protease and toxins (LAUMOND *et al.*,1989). The significance of the interactions between these agressive elements depends on the target insects and the bacterio-helminthic complexes. An interesting example is the complex *Steinernema glaseri/Xenorhabdus poinarii* which is highly pathogenic for *Galleria mellonella* larvae although neither *S.glaseri* nor *X.poinarii* alone is pathogenic for this insect (AKHURST & BOEMARE, 1990).

3°/ Gnotobiology and nutritional supplies

Germ free rearing of nematodes was performed from axenization of the eggs to prevent any contamination (BOEMARE *et al.*,1982; 1983; EHLERS *et al.*,1990). Complex empiric media serve as a subsitute for the necessary nutrients provided naturally by the bacteria (BONIFASSI, 1987). Previously all the axenic cultures of nematode were prepared from axenization of L3 which always were doubtful due to possible contaminations from the digestive tracts. Rearing of axenic nematodes were very important to analyze the gnotobiology of the nematodes species. It was the way to combine in a second step several bacteria and to identify the most suitable strains on a medium cheaper than the complex medium used in axenic rearing. To probe this second medium have to be minimal and uncapable to provide by itself nutritional conditions for the nematode growth as the medium used for germ free nematodes.

Xenorhabdus are beneficial to obtain a good production of nematodes on economical diet but the specificity of the association with its host is not essentially required. Some other Gram negative bacteria can be sometimes good substitutes for this purpose (BOEMARE *et al.*,1983; EHLERS *et al.*,1990). Evenmore the best nutrient conditions for the nematodes are sometimes not necessarily produced by the natural symbionts of one species. In fact it is the transmission of *Xenorhabdus* which is determined at a higher level of specificity. Generally each nematode species is limited in its ability to retain the symbiont of another species of the same genus (AKHURST & BOEMARE, 1990).

4°/ Phase variation

Xenorhabdus spp. occur in two forms. The first is the bacterium isolated from L3 and named phase one (BOEMARE & AKHURST, 1988). Phase one is characterized by production of antibiotic compounds, adsorption of dyes (AKHURST,1980) and, according to strains and species, is pigmented, produces bioluminescence, lecithinase and protease. Phase one cellular populations are pleomorphic, larger than phase two cells, contain rods (80-90%) and spheroplasts (10-20%), have protoplasmic paracrystalline inclusions and fimbriae (BOEMARE & AKHURST, 1990).

During the stationary period of *in vitro* culture or during nematodes rearing on artificial diet, a secondary form, named phase two, appears spontaneously. After selection of these new clones, pure cultures of phase two do not evidence the previous properties or sometimes very weakly. However both phases are equally entomopathogenic and share commonly all the other bacteriological properties. DNA homology studies showed unequivocally that the two phases of each *Xenorhabdus* are homologous and that differences are not due to contamination (AKHURST *et al.*,1990).

In gnotobiological terms phase two provides less suitable nutrient conditions for the nematode, specially for the *Xenorhabdus-Heterorhabditis* associations. Although this phase variation phenomenon is spontaneous and the reverse shift is difficult to obtain specially during the nematode rearing on artificial diet, the most amazing feature is that the L3 harvested after the multiplication contain phase one symbionts.

Genetics studies are in progress to identify the mechanism involved in this phenotypic variation. Occurence of plasmids (XU *et al.*,1989; COUCHE *et al.*,1987; POINAR *et al.*, 1989) and megaplasmids (SMIGIELSKI *et al.*,1990) in a variety of *Xenorhabdus* species was reported. To date no functions have been related to the presence of these extrachromosomial DNAs. Genes of the bioluminescence function were identified in *X.luminescens* DNA but infortunally without any explanation about the regulation on phase variation (FRACKMANN & NEALSON,1990). Less than 1% of phase one light production was recorded with the phase two while the same quantities of mRNA were measured for both phases. This strongly suggest that the regulation is post-transcriptional.

5°/ Taxonomy

Taxonomic studies are essential to give the basis for a system of identification useful for registration purposes and to record data about different *Xenorhabdus* isolates. Five species have been described within the genus, four associated with *Steinernema* spp. and the fifth, *X.luminescens*, associated with *Heterorhabditis* spp. (AKHURST & BOEMARE,1988). 16s rRNA olinucleotide cataloguing data were in agreement with these phenotypic analysis (PUTZ *et al.*,1990). DNA homology studies showed that the described *Xenorhabdus* species fell into different homology groups confirming that they are valid species. Some symbionts from new described *Steinernema* spp. fell into different homology groups. Differences between DNA homology of the symbionts of *Heterorhabditis* spp. and those of the Steinernematidae suggest that they represent two genera (AKHURST *et al.*,1990). A paper describing these data is in progress (BOEMARE *et al.*,1991).

6°/ Conclusion

Studies on the interaction between nematode and bacteria, and also on regulatory factors acting possibly on the bacterial growth, are not fully explored. Investigations on food supplies provided by the *Xenorhabdus* spp., on significance of phase shifts and on mechanism of *Xenorhabdus* retention within L3 larvae have to be carried on.

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2. Meeting of the subgroup entomopathogenic nematodes.

Subgroup "Entomopathogenic nematodes"

Report of the discussion during the subgroup meeting and of future activities

Nematode taxonomy

On the meeting in Versailles, 1989, the difficulties with the identification of entomopathogenic nematodes were already expressed. A first result of the cooperative efforts of the subgroup was the poster presented in Wageningen on the identification of Heterorhabditis and their symbiotic bacteria. The results of the identification of steinernematid nematodes from Italy and Germany will soon be published in **Nematologica**.

Workshop on nematode taxonomy:
It was suggested that someone being skilled in the determination of nematodes would not be willing to identify the hundreds of isolates so far existing in European laboratories. So we decided on the performance of a workshop on the taxonomy of Steinernema and Heterorhabditis, which will take place at the laboratory of Ralf Ehlers in Kiel, Germany on thursday, 21st until saturday, 23rd of May 1992. Methods for the identification on the basis of morphometric characters of dauer juveniles and male nematodes will be presented. P. Smits and G. de Raay will introduce into the RFLP-method for taxonomic purposes. To distinguish between steinernematid and heterorhabditid and other nematode taxa associated with insects an introduction will be given by W. Sudhaus. Other specialists will be asked for a contribution. Every participant should bring some of his nematode isolates. The number of participants is limited due to laboratory capacity and equipment. For further informations please contact Ralf Ehlers.

On tuesday and wednesday, 19th and 20th (before the taxonomy workshop) the annual meeting of the project group "Entomopathogenic nematodes" of the "Deutsche Phytomedizinische Gesellschaft" will be held at the same place. International contributions to this meeeting are welcomed. Please contact Ralf Ehlers.

Cross breeding:
If colleagues already work on the identification of steinernematids the following reference species should be used for cross breeding experiments: S. carpocapsae (USS-DD136), S. feltiae (NLS-OBSIII), S. affinis (DS-D1), S. intermedium (USS-NC). All isolates can be obtained from Ralf Ehlers.

Data base and strain collection:
So far a strain collection is not necessary as everybody is busy with his own material. Techniques for long term preservation for Heterorhabditis spp. need further development. However, more data on the strains around Europe were requested in order to have an overview of what has been isolated and where. Nobody of the participants runs a data base, but Ray Akhurst, CSIRO, Australia has established a base several years ago and some of the participants have already sent their data. It was decides that the information should updated and colleagues who have not yet contributed to the data base are invited to send their infor-

mation. If this would mean too much effort, everybody is asked to give detailled information at least about those strains which are mainly used in the every days laboratory and biocontrol work. Sheets can be ordered from Ray Akhurst or Ralf Ehlers.

Codes and names for isolates
Everybody has his own codes. To prevent confusion and give additional data of origin we decided on an improved system. At first everybody should give the code of the national car number plate, then "H" for Heterorhabditis and "S" for Steinernema. After a dash your own code should appear. The Netherland HL81 would now be given as NLH-L81, because the "H" before all Netherland strains stands for Heterorhabditis already.

The EC COST action 812

Between the two workshops a meeting of the EC COST action 812 will be held on wednesday afternoon. P. Westerman gave a general view of the objectives of the COST action presented here:

COST - "Cooperation Europeenne dans le domaine de la Recherche Scientifique et Technique" - was founded some 17 years ago by the EC to stimulate co-operation between European countries in the field of scientific and technological research. 19 countries can participate in COST actions; the 12 member states of the EC, the 5 EFTA countries, Yugoslavia and Turkey. The EC can not provide financial support; it only finances the initial co-ordination costs. The COST organization has its own network of offices, representatives and ambassadors.

COST action 812 (Cold active lines of insect parasitic nematodes (IPN)) was initiated in December 1988 by The Netherlands, by Paula Westerman. Due to slow processing by Brussels bureaucracy, the proposal was not approved by the COST Senior officials Committee until mid 1989, and was thereafter opened for signing by the various COST states which wished to participate. A call for signing was placed in Nematology News and possible participants were contacted by local COST representatives. The first meeting took place in September 1990 and the second on February 22nd 1991 preceeding the IOBC meeting on Microbial Control.

Colleagues who would like to join this COST action are requested to contact Dr. A. Leonard, Commission of the European Communities, DGXII, SDM 2/1, Rue de la Loi 200, 1049 Brussels (tel. No. 32-22363224, fax No. 32-22355365) or to contact the local COST representative.

IOBC-COST Meeting in Kiel

COST cannot provide any finances and therefore the possibilities for organizing meetings are limited. The start of the COST action more or less co-incided with the foundation of a seperate IOBC subgroup on entomopathogenic nematodes, and we therefore took the opportunity to organize a combined meeting of COST and IOBC.

An important issue during the previous COST meeting was the development and standardization of bio-assays for IPN's. A meaningful comparison of existing bio-assays is very difficult, as the relation between results from (lab) assays and results in the field is unknown.

[* During the IOBC meeting in Wageningen a British S. carpocapsae was suggested as alternative for DD136. DD136 is an 'old' strain and it is likely that the material present in the different laboratories has diverged from the original material. The same could have happened with HW79, and therefore the Swiss reisolation of HW79 was suggested as an alternative.]

OBSIII is available in The Netherlands (P.R. Westerman, The Netherlands), the British S. carpocapsae can be obtained from Dr N.G.M. Hague, Crop protection research unit, University of Reading, Department of Agriculture, Earley Gate, Reading RG6 2AT, Tel.: 875123 Ext 8493, Fax.: 352421. The reisolation of HW79 can be obtained from Dr. W. Steiner (Switserland) and the Irish M145 is available in The Netherlands or can be obtained from Dr C.T. Griffin (Ireland).

Agreement on one standard bio-assay on this stage is therefore unlikely. The COST members decided to compare the techniques applied in their laboratory by testing four strains (DD136*, OBSIII, M145 and HW79*) at four different temperatures (6, 9, 12, and 20 C). All other conditions are free; test insects, medium, etc. The goal is to rank these strains, according to tests applied under local conditions in different laboratories.

The results will be presented and discussed during the COST meeting in Kiel. Anybody how is interested is invited to attend the COST afternoon.

Bio-assay

Beside the discussion of bioassays on the COST meeting a workshop on the same topic is planned for the Heidelberg meeting of the Society of Invertebrate Pathology (16th to 21st of August 1992).

Next meeting and organisation

The topics of the next meeting in Zürich 1993 will be on formulation of pathogenes and nematodes and on host-pathogen interactions. The subgroup meeting is opened for any contribution on entomopathogenic nematodes in biological control.

It was asked to form an official subgroup "Entomopathogenic nematodes" with a convenor. The election of Ralf-Udo Ehlers was realized by acclamation. I hope to be able to satisfy all demands and would like to call for participation to further activate our subgroup on entomopathogenic nematodes.

Ralf-Udo Ehlers

(subgroup convenor)

ECOLOGICAL CONSIDERATIONS FOR ENTOMOPATHOGENIC NEMATODES ACTIVITY UNDER SUB-OPTIMAL CONDITIONS

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Summary

The present paper reviews the factors effecting the activity of entomopathogenic nematode on exposed surfaces i.e. plant foliage. The ability to withstand low relative humidity (RH) conditions varies between different entomopathogenic nematode strains, under similar conditions. The survival of infective juveniles (IJs) of S. **carpocapsae** 'All' strain on foliage of bean plant at 50-70% RH was reduced to 20% within 4 h and to 0% after 8 h exposure. While the 'Mexican' and 'Pye' strains of S. carpocapsae survived twice longer under the same conditions. Exposing (IJs) of S. **carpocapsae** 'Mexican' strain to different relative humidity levels (30-80% RH) indicate that gradual reduction in their viability occurs when the (IJs) are exposed to levels higher then 55% RH while drastic mortality was recorded at lower levels of relative humidity. Nematode viability on leaf surface was also influence by the leaf type. When (IJs) of S. **carpocapsae** 'Mexican' strain were exposed to 60% RH on the surface of leaves with rough surfaces such as Tomatoes and Soy, their survival level was approximately two fold higher (60-75%) then the nematodes which were exposed to the same conditions on leaves with smooth surface (35-45%) like Cotton, Pepper, Beans. Substantial reduction in larval population and damage caused by three cotton foliage-pests **Earias insulana**, **Helicthis armigera**, Spodoptera **littoralis** was recorded when the S. **carpocapsae** 'Mexican' strain was applied on the canopy of plants at 70% RH in the glasshouse as well as simulated field conditions. Most effective results were obtained when the nematode suspensions were mixed with the antidesiccants Biosys 627 (15% and 20% wt/wt), 'Folicote' (6% wt/wt) and natural wax (18% wt/wt).

The third stage infective juveniles of the entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae are capable of infecting and killing a wide range of insects (Kaya, 1985; Poinar, 1986). These nematodes are mutualisticly associated with bacteria of the Xenorhabdus sp. (Akhurst & Boemare, 1990). Together they possess unusual virulence killing the insects within 24-48 h. The pathogenic process of the nematode-bacteria complex in the insects has been reviewed extensively (Dunphy & Thurston, 1990; Kaya, 1985; Poinar, 1986). On account of this capability, entomopathogenic nematodes offer a biological control alternative to chemical insecticides in growing number of applications (Gorgis 1990a,b). As nematodes inhabit the soil, most applications involved the targeting of insects which dwell in the soil throughout their life cycle or do so for at least part of it (Klein, 1990). Nonetheless, entomopathogenic nematodes can also be

applied to off-ground cryptic habitats such as tunnels caused by tree borers (Kaya, 1985; 1990).

The sensitivity of steinernematid and heterorhabditid IJs to inactivation by extremes of the physical environment, especially desiccation (Begley, 1990; Kamionek et al., 1974; Kaya, 1990), prevents them from reaching their full potential and produced generally erratic results in the field (Akhurst 1986, Gaugler 1981). Nematodes require adequate moisture for infectivity so that high ambient humidity (>90%) and free water on the leaves are important prerequisites for infection. However, the foliar environment often exposes the nematodes to unfavorable moisture conditions that result in their rapid desiccation and death (Begley, 1990; Kamionek et al., 1974; Kaya, 1990). Thus, environments with high humidity and moderate temperatures such as glasshouses are preferred environments for foliar applications of nematodes. In addition, evening applications, the incorporation of antidesiccants into aqueous nematode suspensions or oil formulations offer possibilities for increasing efficacy against foliar insects (MacVean et al. 1982, Webster & Bronskill 1968, for reviews see also Begley; Kaya 1985, 1986).

Indeed, field trails using nematodes as biological control insecticides against foliage-feeding insects have primarily involved Steinernema carpocapsae strains 'All' and DD-136, or to a limited extent, S. feltiae (= S. bibionis) and Heterorhabditis bacteriophora (Begley, 1990). Glazer & Navon (1990) showed that survival of infective juveniles of S. carpocapsae 'All' strain on foliage of bean plant at 50-70% RH conditions was reduced to 20% within 4 h and to 0% after 8 h exposure. Under similar relative humidity conditions the ability to withstand low RH conditions varies between different entomopathogenic nematode strains. Among six nematode strains tested, the 'Mexican' and 'Pye' strains of S. carpocapsae survived twice longer under low RH conditions.

Exposing infective juveniles of S. carpocapsae 'Mexican' strain to different relative humidity levels (30-80% RH) indicate that gradual reduction in their viability occurs when the infective juveniles are exposed to levels higher then 55% RH (50% nematode mortality within 6-8 h) while drastic mortality was recorded at lower levels of relative humidity (complete mortality within 3-4 h). Nematode viability on leaf surface was also influenced by the leaf type. When infective juveniles of S. carpocapsae 'Mexican' strain were exposed to 60% RH on the surface of leaves with rough surfaces such as Tomatoes and Soy, their survival level was approximately two fold higher (60-75%) then the nematodes which were exposed to the same conditions on leaves with smooth surface (35-45%) like Cotton, Pepper, Beans (Glazer, 1991).

In a recent study the systematic measures necessary to achieve successful reduction in larval population of the cotton pests Earias insulana, Heliothis armigera, Spodoptera littoralis, by using S. carpocapsae 'Mexican'strain on the canopy of plants were defined (Glazer et al., 1991). In a laboratory screening assay, the survival at low relative humidity (60% RH) of nematode IJs treated with the antidesiccants Biosys 627 (15% and 20% wt/wt), 'Folicote' (6% wt/wt) and natural wax (18% wt/wt) was three times higher than the control (25%). Non of the antidesiccants tested, with the exception of 'New

Film', had any adverse effect on nematode viability in aqueous solutions.

In greenhouse experiments, IJs at concentrations of 500 and 1000/ml in water was required to attain a >85% control of E. **insulana** and S. **littoralis**, respectively, when applied to the foliage of bean plants. Addition of the antidesiccants 'Biosys 627'(20% wt/wt), natural wax (18% wt/wt) or 'Folicote' (6% wt/wt) to the nematode suspension had a similar effect on insect mortality with lower nematode concentrations (125 and 250 IJs/ml for E. **insulana** and S. **littoralis**, respectively). In the case of H. **armigera**, nematode suspension at a concentration as high as 5000 per ml resulted in only 22% control when applied in water. Addition of the antidesiccants resulted in a fourfold increase, to 85-95%, in insect mortality.

In microplot experiments foliage application of the S. **carpocapsae** 'Mexican' strain (250 IJs per ml) mixed with 'Folicote' (6% wt/wt) resulted in a 61% reduction in the persistence of S. **littoralis** larvae on cotton plants. Furthermore, the level of damage to the foliage was reduced by 46% as compared with control. Substantial reduction (76%) of E. **insulana** larvae population on the plant's foliage was achieved by application of 125 IJs per ml mixed with 'Folicote' (6% wt/wt) . To a lesser extent, the treatment with nematodes alone also reduced insect persistence (66%) as compared to control.

The present findings support the notion that the use of nematodes on the foliage is feasible once the optimal conditions are identified (Kaya & Reardon 1982, Sikora et al. 1979). Additional work is required to evaluate nematodes effectiveness under natural field conditions and to study the nature of the interaction between the IJs and the target host on the plant surface.

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COMPARISON OF MIGRATION AND HOST SEARCHING ABILITY OF VARIOUS HETERORHABDITID SPECIES AND ISOLATES IN SAND COLUMNS

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Summary

Migration and host searching towards Galleria mellonella in sand columns was investigated for various species and isolates of Heterorhabditis. The nematodes behaved all differently; some isolates dispersed into the columns, while others remained near the point of application. Some isolates responded to the presence of the insect host at the bottom of the sand cylinders, others did not. There were also differences between isolates that belonged to the same species.

It is unknown to what extent the migration and host searching tested in the laboratory in sand accounts for activity in the field.

1. Introduction

Insect parasitic nematodes of the genus Heterorhabditis can be very effective against soil inhabiting insects, like various species of root weevils, e.g. Otiorhynchus sulcatus, the black vine weevil. The nematodes migrate into the soil and can actively find and infect insects, so that they have an advantage over chemical pesticides.

The host searching ability of the nematodes is considered to be one of the most important features of this biological insecticide (Gaugler et al., 1989a). As a result, dispersion and host searching ability have received a lot of attention in recent studies (Gaugler et al., 1989a, 1989b, Choo et al., 1989, Westerman & Godthelp, 1990). Westerman and Godthelp (1990) showed that H. bacteriophora behaved differently from Dutch heterorhabditids in sand columns. Apparently there is variation in migration within this genus.

Eight different heterorhabditid species and isolates originating from five different countries were compared with respect to migration and host searching towards larvae of the greater wax moth, Galleria mellonella.

2. Materials and methods

The species and isolates tested are listed in table 1. H. bacteriophora was obtained from Dr P.H. Smits (IPO, Wageningen), who received it from Dr H.K. Kaya (Univ. California, Davis). The North American H. megidis, the New Zealand isolate NZH3, and two Irish isolates K122 and M145 were obtained from Dr C.T. Griffin (St. Patrick's College, Maynooth), HP88 (USA) was send by Dr I. Glazer (The Volcani Center, Bet Dagan) and the British HUk by Dr P.B. Rodgers (AGC, Cambridge). The isolate HF85 originated from a soil sample from Flevoland in The Netherlands.

The experiments were conducted at 20 °C in PVC cylinders (4.5 cm ϕ, 9 cm high), made of six separate rings, connected with adhesive tape. The bottom of the cylinder consisted of a small petri-dish (5.5 cm ϕ), fixed to the bottom ring with synthetic modelling clay. The cylinders were filled with fine sterile sand (93% < 180-240 µm, approx. 220 gr/cylinder), moistened with demin water (8% w/w, pF ≈ 1.4). A small petri-dish was used as a lid on top of each cylinder. A series of one treatment cylinder, with a last instar larva of G. mellonella at the bottom, and a control cylinder were inoculated simultaneously with approximately 2000 nematodes in 0.5 ml water. After a number of hours at 20 °C the rings were separated and the sand of each ring was rinsed in 50 ml water. The rinse water for rings 2 to 5 was combined and the number and percentage of nematodes in ring 1, rings 2 to 5 and ring 6 were estimated by counting four samples of 3 ml of the rinse water. The average distance covered by the nematodes was

Table 1. Average distance [cm] covered by various Heterorhabditis species and isolates in the absence (-) or presence (+) of 2a larva of G. mellonella (G.m.) after 2, 4, 6 and 8 hours at 20 °C in sand cylinders. (Figures followed by the same letter are not significantly different within the group of data per nematode (Tukey, $\alpha = 0.05$)).

species/ isolate	n	G.m.	hours 2	4	6	8
HF85	4	-	--	1.94 a	2.08 a	2.08 a
		+	--	6.71 b	7.62 b	7.69 b
H. bacteriophora [1]	4	-	--	1.00	1.06	1.27
		+	--	0.81	1.01	0.94
H. bacteriophora HP88	2	-	1.1 a6	1.1 a	1.6 a	2.9 ab
		+	1.3 a	2.1 ab	2.7 ab	4.5 b
H. megidis	4	-	2.2 a	2.8 a	3.2 ab[2]	3.6 abc
		+	3.5 abc	5.2 bc	5.0 bc	5.5 c
HUk	2	-	1.1 a	1.5 ab	1.5 ab	1.6 ab
		+	2.3 ab	2.8 b	4.5 c	4.6 c
NZH3	2	-	1.1 ab	0.9 ab	1.0 ab	1.6 b
		+	0.9 a	1.0 ab	1.2 ab	1.4 ab
K122 [1]	2	-	2.5	2.5	2.5	2.3
		+	2.8	2.6	2.9	3.6
M145	2	-	0.8 ab	1.3 abc	1.6 bc	1.7 c
		+	0.8 a	1.4 abc	1.9 c	1.8 c

(1) no significant differences
(2) one missing plot, n=3

calculated [(# ring 1 * 0.75cm + # ring 2-5 * 4.5cm + # ring 6 * 8.25 cm)/ total # of nematodes]. Experimental data were analysed statistically using analysis of variance (ANOVA; l.s.d., $\alpha = 0.05$) and Tukey's test for comparison of means ($\alpha = 0.05$).

3. Results

The results are listed in table 1. The results for HF85 are typical for all Dutch isolates tested so far (Westerman & Godthelp, 1990). Dutch isolates seem to belong to the most active migrators among Heterorhabditis. The nematodes from H. bacteriophora all stayed near the point of application, although the isolate HP88 responded well to the presence of a larva of G. mellonella and showed an increase in activity over time. The British isolate HUk resembled HP88; in the control cylinders the nematodes stayed at one level, but in the presence of the insect host they responded significantly. The Irish isolate K122 did not respond to G. mellonella and the nematodes stayed at one level during the whole experiment. The other Irish isolate M145 resembled K122. NZH3 showed a similar behaviour as H. bacteriophora; all nematodes could be recovered from the first ring.

4. Discussion

There is variation in migration and host searching ability between nematodes of different species and isolates of Heterorhabditis. Some isolates dispersed into the sand columns, while others stayed near the point of application (H. bacteriophora and NZH3). It is unknown to what extent migration in the laboratory accounts for activity in the field. Georgis and Poinar (1983a,b) tested some species of Steinernema in a number of soil types, like sand, sandy-loam and clay, and they observed that the nematodes migrated best in sand. May be the sand column test will yield a kind of the maximum activity or migration potential of the isolates. Some nematodes responded to the presence of G. mellonella at the bottom of the cylinders, while others did not. G. mellonella seems to be one of the most attractive insects to these nematodes (Westerman & Godthelp, 1990), but it is possible that some isolates will favour and therefore respond better to other insect species. There were also differences between isolates that belonged to the same species (H. bacteriophora). Up till now the West European isolates have not been identified to the level of species, so it is possible that some of the Dutch, Irish and British isolates are also related.

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Interactions in the entomopathogenic nematode-bacteria complex *Steinernema/Heterorhabditis-Xenorhabdus*

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The relationship between the nematode and the associated bacterium is considered to be mutualistic (POINAR & THOMAS, 1966) as well as symbiotic (POINAR & HANSEN, 1986). Different species may be associated in an ecological (mutualistic, phoretic) or trophic (symbiotic, commensalic or parasitic) relation (BAER, 1952). Which of these conceptions best describe the nematode-bacteria complex can only be considered when their behavioural and physiological interactions in the different phases of the nematode's life cycle are better understood. Three phases of the life cycle shall be distinguished for the consideration of the characteristics of the interaction:

1. Free-living phase
2. Infective phase
3. Propagative phase

Interaction in the free-living phase

Dauer juveniles of *Steinernema* spp. contain about 10 to 200 cells of their specific *Xenorhabdus* spp. in a nematode-originated vesicle in the ventricular portion of their intestine (BOVIEN, 1937; POINAR, 1966). Inside this vesicle the bacteria are supposed to be carried monoxenically (BIRD & AKHURST, 1983). The selective conditions during incorporation of the symbiotic bacteria are not fully understood. However, specific morphological and physiological interactions of the surface structure of the vesicle with the bacteria envelope are suggested. Bacteria cells are also found in the intestine. Isolation of non-symbiotic bacteria from dauer juveniles (POINAR, 1966; BOEMARE, *et al.*, 1983) indicate that these bacteria may be non-symbiotic.

Heterorhabditis spp. do not form this vesicle and bacteria are distributed in the lumen of the pharynx and the intestine (POINAR *et al.*, 1977; POINAR *et al.*, 1979, ENDO & NICKLE, in press). Taxonomic studies of *X. luminescens* from different *Heterorhabditis* spp. and strains show that the species consists of at least 5 subspecies, which could as well be elevated to species level (EHLERS & STACKEBRANDT, unpublished). The species-specific association found in the complex *Steinernema* sp.-*Xenorhabdus* sp. (PÜTZ *et al.*, 1990) was not found in the *Heterorhabditis-X. luminescens* complex (EHLERS & STACKEBRANDT, unpublished). Within the species *H. bacteriophora* identified by comparision of RFLP patterns (SMITS *et al.*, in press) different nematode strains carry for instance different bacteria subspecies. The same results were obtained when the bacteria of the NW-European nematode group (SMITS *et al.*, in press) were studied. The majority of the nematode strains carried one specific bacterium. However, the symbionts of the strains NLH-E87.3 and the NLH-FR could be

distinguished from the common symbiont species of this group (EHLERS & STACKEBRANDT, unpublished). In comparison to the steinernematid nematode-bacterium complex, the affinity of the intestine of the *Heterorhabditis* dauer juveniles towards their symbionts seems to be less specific. The high variability of *X. luminescens* bears the possibility for host-specific virulence selections and the creation of new combinations of *Heterorhabditis-X. luminescens* complexes which are better adapted to pest control purposes.

The incorporation of the bacteria into the dauer juvenile is of important ecological significants for the *Xenorhabdus* spp. Inside the nematode the bacterium is protected against the aggressive soil environment. A survival of *Xenorhabdus* in the soil seems to be impossible as it has never been isolated directly from soil samples. How long the bacteria stay alive inside the dauer juvenile influences the virulence of the antagonistic complex and its possibilities to propagate in the host insect. In the free-living phase of the life cycle the bacterium seems to be the only part of the complex that benefits from the association which can only be characterized as being mutualistic.

Tripartite interaction in the infective phase

In this phase of the life cycle the insect host is interacting with the invading nematode-bacterium complex from the moment of the penetration of the dauer juvenile until the death of the insect. The nematode-bacterium association is mainly characterized by their joint attempts to overcome the insects humoral and cellular defense mechanisms. A detailled contribution on the tripatite interaction during the infective phase of the nematode-bacterium complex is given by DUNPHY & THURSTON (1990).

At the moment of the first contact of the nematode with the haemolymph the nematode may be identified as nonself and, depending on the host insect, either humoral or cellular defense reactions or both lead to the encapsulation of the invading nematode (POINAR & LEUTENEGGER, 1971; ANDREADIS & HALL, 1976; EHLERS & GERWIN, 1990). This defense reaction may help the insect to survive an attack by the nematode-bacterium complex. However, partially encapsulated parasitic stages of *S. feltiae* (syn. *S. bibionis*) were found in dead *Tipula paludosa* larvae and *X. bovienii* had been released before the nematode could be killed by the insect's defense (EHLERS & PETERS, unpublished).

The nematode may also not be identified as non-self (DUNPHY & WEBSTER, 1987) and no defense reaction against the nematode is activated. Evidences for a surpression of the identification or encapsulation reactions by the nematode have so far not been reported. The role of the nematode toxin (BURMAN, 1982; BOEMARE *et al.*, 1982) in its interaction with the insect defense mechanisms is still unknown.

When the symbiotic bacteria are released into the haemolymph, humoral and cellular defense reactions are activated. DUNPHY & WEBSTER (1988ab) described the activation of the plasmotocytes of *Galleria mellonella* by lipopolysaccharides (LPS) of the bac-

terial envelope. *Xenorhabdus* spp. cells are bound to the haemocytes of *G. mellonella* and eventually damage the haemocytes, causing the collapse of the insect's cellular defense. The toxic factor was identified as the fatty acids of the lipid A moiety of the LPS. BREHELIN *et al.* (1990) evidenced an activation of the pro-phenoloxidase system (humoral factor increasing the recognition of foreign bodies) by the bacterial LPS. On the other hand a still unidentified factor of the bacterium triggered the activating reaction. If this phenomenon is caused by one of the exoenzymes excreted by *Xenorhabdus* spp. (see below; SCHMIDT *et al.*, 1988) is still unknown. In immune insects the antibacterial protein cecropin is able to lyse cells of *X. nematophilus* (GÖTZ *et al.*, 1980). This reaction is inhibited by a protein secreted by the nematode (GÜLZOW, 1986).

The combined reactions of the antagonistic complex to overcome the host defense in the infective phase can only be regarded as being mutualistic. Further investigations on the interaction of the nematode-bacterium complex with the insect's defense mechanisms could help to select hostspecific and virulent pathogenes for pest control.

Interaction in the propagative phase

Steinernema spp. and *Heterorhabditis* spp. are not able to reproduce in a host insect unless their associated bacteria *Xenorhabdus* spp. are present. The physiological mode of action of this symbiotic relation is not fully understood. It may be suggested that certain enzymes released by *Xenorhabdus* spp. digest the haemolymph thus enabling the nematode's reproduction.

For biological insect control the *in vitro* propagation of entomopathogic nematodes is a prerequisite. From these cultures phase variants of *Xenorhabdus* spp., designated as primary and secondary form, have been isolated and were further described (AKHURST, 1980; BOEMARE & AKHURST, 1988). BEDDING (1981) suggested a detrimental effect of the secondary form on nematode reproduction, which was not confirmed for steinernematid *in vitro* cultures (EHLERS *et al.*, 1990). However, the mortality of heterorhabditid nematodes increased when they were grown on secondary form cultures and growth was totally inhibited on a yellow pigmented variant of *X. luminescens* of strain NZ. Pure cultures of the secondary form of steinernematid symbionts are easily obtained by several selective subcultures, whereas *X. luminescens* is not stable in the secondary form and intermediate (EHLERS *et al.*, 1990) and other variants were found (HURLBERT *et al.*, 1989). Factors inducing the variants have so far not been identified. Considering possible negative effects on the propagation of *Heterorhabditis* spp., the mechanisms of phase variation are of great importance for the *in vitro* culture and investigations are focussing on the interaction of the nematode-bacterium complex in the propagative phase of the nematode's life cycle. Some observations during experiments to optimize the liquid culture of *H. megidis* (strain DH-SH1) will be described:

When *X. luminescens* was cultured in a modified WOUTS medium (24 g/l bacto-nutrient-broth, 3 g/l pepton, 2 g/l yeast extract and

1% v/v vegetable oil in 1000 ml water) red, yellow and orange pigmented cultures were observed. An influence of the age of the inoculum culture was suggested and to prove this hypothesis the following experiments were conducted: *X. luminescens* strain DH-SH1 was isolated from infected *G. mellonella* and subcultured four times before storage at -30°C in 15% glycerol (v/v). This stock culture was propagated again in YS broth and stored in the deep freezer before 2 ml of the melted bacteria suspension were inoculated into YS broth and shaken at 180 rpm at 25°C. From this culture WOUTS media (30 ml) were inoculated after the preculture had been agitated for 12, 24, 36, 48 and 60h. For every time interval 10 flasks (100 ml) were inoculated.

All 10 replicates of the 12 and 24h batches were red pigmented after two days incubation. The 36h batch was yellow and the 48 and 60h batches were always orange pigmented. The different pigmentation influenced by the age of the inoculum culture was reproduced four times. The fourth replicate was used to assess the exoprotein content of the culture supernatant by the method of BRADFORD (1976). The results are given in figure 1.

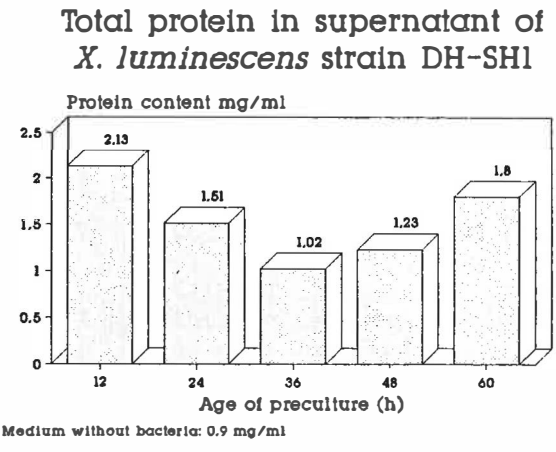

Figure 1. Total protein in the culture supernatant of *X. luminescens* strain DH-SH1 inoculated from one preculture at different incubation times after 120 h incubation (Medium 0 h: 0,9 mg/ml)

When 1 µl of the sterile filtrated supernatant was injected into last instar larvae of *G. mellonella* all insects injected with supernatant of the red variant died immediately, turned black and were flaccid. All larvae injected with the supernatant of the yellow culture died within a 16 h period, did not loose turgescens and did not change colour.

To determine the influence of the variants on the nematode reproduction the bacteria cultures of the fourth replicate were

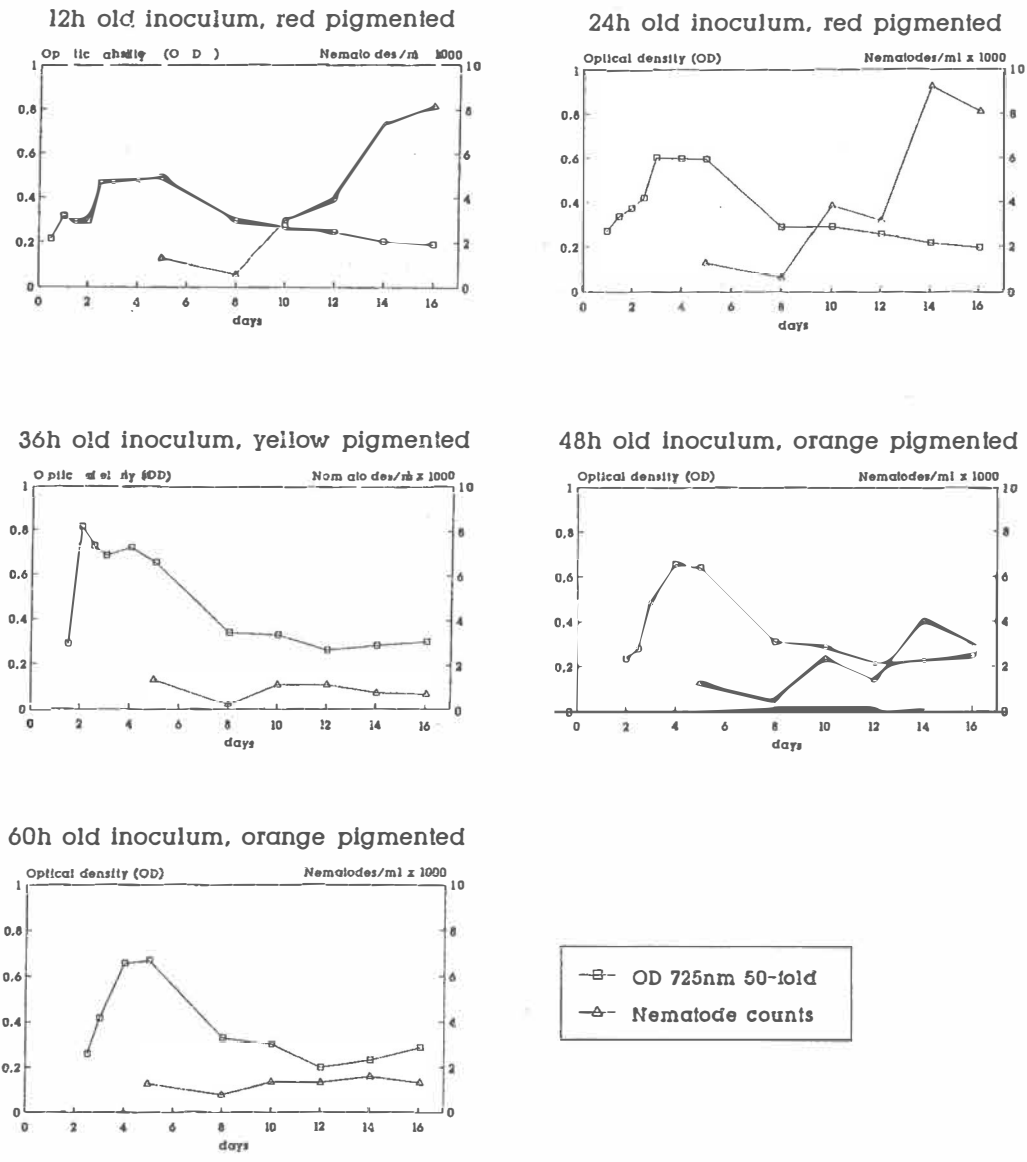

Figure 2: Growth profiles of monoxenic nematode-bacterium cultures (n=10) *H. megidis-X. luminescens* strain DH-SH1 in modified WOUTS medium inoculated at different incubation time of one bacterial inoculum culture in YS broth.

inoculated with 1300 dauer juveniles/ml medium after 5 days incubation. The bacterial population was determined by detection of the optical density at 725 nm in a 50-fold delution. The nematode population was counted. The mean density of the bacterial and nematode populations are presented in Figure 2. A high nematode reproduction was observed only in the red pigmented cultures with about 90% dauer juveniles in the nematode's stationary growth phase. In yellow pigmented bacteria cultures nematodes developed into small adult females, however, the growth of the second generation offspring was totally inhibited. In the orange pigmented cultures dauer juveniles were produced in a lower concentration than in the red variant cultures.

When the bacteria clone was once more or several times subcultured, the pigmentation could not be related to the age of the preculture as determined in the previuos experiments and yellow, orange and red cultures were evenly distributed in the batches of the different ages of the preculture. When a different clone subcultured only twice after isolation was used, the 12, 24 and 36 h variants were red pigmented and the 48 and 60 h variants were yellow. However, the detrimental effect of the yellow pigmented culture on the nematode propagation was still observed.

The presented results indicate a positiv correlation of the nematode reproduction potential with the content of exoproteins in the *X. luminescens* cultures. The yellow pigmentation and the shorter generation time (Figure 1) is a character of the secondary form (BOEMARE & AKHURST, 1988). However, paralell plating on NBTA-agar revealed no secondary characters in BTB uptake, activity on Tween 20, 40, 60, 80, gelatin and casein agar, cell size, inclusion bodies and bioluminescens.

When *X. luminescens* is repeatedly grown in axenic cultures, we always deal with mixed populations of the primary and secondary forms. Thus the orange pigmented cultures are mixed populations of the red and yellow variant. So far it was suggested that the secondary form occurrs after prolonged subculturing. Applying only the characters pigmentation, exoprotein content and support of nematode propagation, we can define the yellow variant as a secondary form. We were able to repeatedly induce the occurrence of this secondary form by varying the age of the inoculum culture. This observation leads to the conclusion that the physiological state we observe in the cultures may be reached already in the preculture and does not change when the bacteria are subcultured. The yellow pigmented variant occurs when the inoculum is taken in the stationary growth phase of the inoculum culture.

If this conclusion is transfered to the situation under *in vivo* conditions, an explanation for the secondary form phenomenon is possible: In the infective phase the bacteria produce secondary metabolites to overcome the insect's defense and to provide conditions for the nematode's propagation. When this function is fulfilled the cease of the metabolic pathway for the production of exoproteins saves available resources for the continuing cell replication in order to supply enough cells for the nematode nourishment. The secondary form utilisation of amino acids which have not been assimilated by the primary form (BOEMARE & AKHURST, 1990) supports this theory. The occurrence of primary

form cells in the dying phase supplies primary cells for the incorporation into the dauer juvenile.

The effect of the age of the preculture on the occurrence of the secondary form depends on the number of subcultures after isolation of the clone from the nematode-bacterium complex. When *X. luminescens* is subcultured more than five times, the secondary form could not be induced any more at a specific age of the inoculum culture. It seems as if some kind of regulation may be missing which might be active only in a symbiotic relation with the nematode. If we suppose an interaction of the nematode with its symbiont during the propagative phase, the axenic bacterial culture will than exhibit a different growth physiology than a monoxenic culture or a reproduction under *in vivo* conditions.

The presented results need further confirmation. It still has to be proven if the occurrence of the secondary form is expressed already in the preculture. Any nematode factor with influence on the physiology of the bacterium has also not yet been identified. However, the presented theory is a possible explanation for the ecological and physiological function of the phase shift into the secondary form. The nematode-bacterium complex is a dynamically interacting system, which will hardly be understood if only the physiology of the single components in axenic culture are studied.

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3. Meeting of the subgroup entomopathogenic Fungi.

IOBC/WPRS Working Group: "Insect Pathogens and Insect parasitic Nematodes"

IIIrd European Meeting on Microbial Control,
Wageningen, 24-27 February 1991

Discussion of the Subgroup "Fungi"

Report of the Organizer, B. PAPIEROK

List of Participants: W. ANDERSCH, I.S. BEN-ZE'EV, T.M. BUTT, K. CHARNLEY, C. CHASTEL, J. EILENBERG, B. EKBOM, J. FRANSEN, S. HENNING, L. JOSHI, S. KELLER, R. KLEESPIES, G. LATTEUR, J. v. LENTEREN, D. MOORE, E. MULLER, B. PAPIEROK, J. PELL, J. PELSENEER-COREMANS, C. PRIOR, P. ROBERT, M. ROUGIER, H. SERMANN, N. SMITS, F. TILLEMANS, R. VERWEIJ, F.L. WÄCKERS, N. WILDING, G. ZIMMERMANN.

The discussion is open by B. PAPIEROK, who pointed out the individualization of a Subgroup "Fungi" within the Working Group "Insect Pathogens and Insect parasitic Nematodes". There is quite enough people working on entomopathogenic fungi in Europe, for the most part with complementary field of interest (ecology, systematics, production...); it is essential indeed to further any opportunity of reinforcing existing collaborations or arousing new ones.

The discussion was mainly devoted to biology, ecology (persistance) and systematics of entomopathogenic fungi. Other topics concerned the mode of action of these pathogens and the defence reactions of the host, in relation with host specificity as well as problems dealing with production, formulation and registration of fungal preparations. Concerning the main topic, special emphasis was on the need to search for pathogens of "neglected" insects or pathogens of neglected insect stages (e.g. eggs) and on the question of strains circulation. Some participants are maintaining collection of strains of Hyphomycetes only (K. CHARNLEY, C. PRIOR, P. ROBERT, H. SERMANN) whereas anothers are keeping cultures of Hyphomycetes and Entomophthorales (J. EILENBERG, S. KELLER, B. PAPIEROK, J. PELSENEER-COREMANS, N. WILDING, G. ZIMMERMANN). Excepting a very few of these collections, for which an agreement is required, there is no restriction for circulation of these strains. Furthermore, the drawing-up of lists of entomopathogenic fungal species recorded in the different countries should be encouraged, as already done in Israel. In the field of the persistance of fungal propagules, Hyphomycetes are considered (T. BUTT, J. PELSENEER-COREMANS, G. ZIMMERMANN) as well as Entomophthorales (J. EILENBERG, S. KELLER, G. LATTEUR, N. WILDING). Efforts should be made when studying the role of edaphic factors by using a given type of soil as a standard.

The participants were in agreement on the proposal that B. PAPIEROK acts as convenor of the subgroup "Fungi". Next meeting could take place in Heidelberg, august 1992, given the opportunity of the XXV SIP Annual Meeting.

ENTOMOPHTHORALES ON ADULT CABBAGE ROOT FLIES (DELIA RADICUM)

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The fungus species Entomophthora muscae and Strongwellsea castrans are both recognized as pathogens of adult cabbage root flies (Delia radicum) (Smith, 1927, Batko & Weiser, 1965, MacLeod et al., 1976, Keller, 1984)

Few studies on the interactions are, however, published. In studies focusing on the prevalence on S.castrans in the field (Nair & McEwen, 1973, Lamb & Foster, 1986) flies captured in water traps were used as samples for analysis of the prevalence of this fungus during the growth season.

During 1988 and 1989, a study of the prevalence of fungal pathogens on adult cabbage root flies was undertaken in a field of white cabbage ("Korsbjerggaard", Roskilde).

FUNGUS SPECIES OCCURRING IN THE FIELD

Adult flies were swept with a net and incubated in the laboratory by use of a method previously described for field studies of the occurrence of fungal pathogens on adult carrot flies, Psila rosae (Eilenberg & Philipsen, 1988).

The overall results are shown in table 1. As seen, several fungal pathogens were recognized. The most common species were E.muscae and S.castrans, both being frequent during the two seasons. Other pathogens occurred much more rarely: Conidiobolus (two species) and Zoophthora radicans.

Flies dying of E.muscae or S.castrans developed either conidia or resting spores. Except for one male containing resting spores of E.muscae, only female flies were found with resting spores. Fungus-killed flies found on plants in the field always contained only conidia, thus sweeping and incubation of living flies are necessary tools to obtain information of resting spore occurrence in fly populations.

The frequency of fungal infection in the fly population during the season showed that several epidemics could be detected: The beginning of June, and again during July. In 1989, a high level of infection occurred towards the end of the season (August-September).

		Total	E.muscae conidial stage	E.muscae resting spore stage	S.castrans conidial stage	S.castrans resting spore stage	Conidiobolus spp.	Z.radicans
1988	Males	635	155	0	44	0	0	0
	Females	2158	370	98	423	173	3	2
1989	Males	445	112	1	10	0	0	0
	Females	386	96	13	37	3	0	0

Table 1.

Occurrence of fungi from Entomophthorales on adult cabbage root flies (Delia radicum) in "Korsbjerggaard" during the seasons 1988 and 1989.

	E.muscae	S.castrans
June	1.4 %	0 %
July	22.3 %	19.4 %
August	24.5 %	44.7 %
Sept.	56.5 %	0 %
October	-	6.9 %

Table 2.

Frequency of resting spores among female cabbage root flies infected with Entomophthora muscae and Strongwellsea castrans 1988. The figures are the percentages of the infected females containing resting spores.

Resting spores of either species were not found in any flies during the first epidemic in June. During July and August 1988, a still higher proportion of the infected female flies contained resting spores (table 2).

CULTIVATION IN VITRO

In vitro cultures were obtained of all four fungal species occurring on cabbage root flies. S.castrans has never before been isolated in vitro, but it was possible to obtain cultures using liquid growth media (Eilenberg et al., 1991).

For other strains of fungal species from the E.muscae-complex we have developed a method for spore production in vitro on a limited scale (Eilenberg et al., 1990). At the moment, attempts are being made to induce spore production of E.muscae strains isolated from cabbage root flies by modifications of this method.

BIOLOGICAL CONTROL OF CABBAGE ROOT FLIES WITH ENTOMOPHTHORA ?

The two common species of Entomophthora both contain the basic property to establish epidemics and act as important mortality factors in the field.Thus, E.muscae and S.castrans should be investigated further for possible use in biological control.

In an ongoing study, relevant aspects for a further evaluation of Entomophthorales as tools in biological control in cabbage are studied: factors governing resting spore formation in vivo, factors governing epidemic development in the field, characterization of strains in vitro, host range etc.

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SURVIVAL OF SPORES OF *ASCHERSONIA ALEYRODIS* EXPOSED TO HIGH TEMPERATURES

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SUMMARY
The entomopathogenic fungus *Aschersonia aleyrodis* can be used as a microbial-control agent against whitefly in greenhouses. The spores of the fungus are the infective units and the whitefly larvae are killed after spores have germinated and penetrated the host cuticle. The spores have to be mass-produced and applied in high quantities to obtain an immediate effect. The development of a product may involve techniques like spray-drying of sporesuspensions using high temperatures. The heat resistance of spores of *A. aleyrodis* was investigated. Germination of spores exposed to 40°C for 300 seconds was still 98.4% after a 48 hr incubation at 25°C. Spores from suspensions exposed to 45°C for 60 seconds and to 50°C for 10 seconds survived for 97.1 and 95.6%, respectively. Addition of 1 and 5% Dextran or 0.01 and 0.05% Kelzan increased the heat resistance of the spore suspension. After exposure of the sporesuspension with 0.05% Kelzan to 55°C for 10 seconds still 64.3% of the spores germinated, whereas only 0.6% germinated after treatment of the sporesuspension without Kelzan.

1. Introduction

The fungus *Aschersonia aleyrodis* is a selective entomopathogen infecting whiteflies like greenhouse whitefly, sweet-potato whitefly and citrus whitefly (Fransen, 1990). The fungus infects the whitefly larvae and produces orange-coloured sporemasses in pycnidia on the host insect as well as on semi-artificial media like rice or millet. Research has been carried out on different aspects of *A. aleyrodis* as a microbial-control agent of greenhouse whitefly (Fransen, 1987).
In addition to the introduction of the parasitoid *Encarsia formosa* its use is compatible, and, therefore, can be used in situations where the parasitoid is not able to suppress the pest below the economic damage level (Fransen, 1987, Ramakers & Samson, 1984).

To achieve good control of whitefly the entomopathogen has to be introduced in an inundative way to result in immediate effect. Generally, about 10^7 spores/ml are needed to obtain 90% infection of whitefly larvae in a greenhouse situation (Fransen *et al.*, 1987).

Before *A. aleyrodis* can be applied on a commercial base, topics considering mass production, storage and formulation need to be investigated. In this paper results on the heat resistance of the spores are presented. This information is of importance when considering different techniques of drying and formulating the pycnidiospores harvested from cultures on artificial media. Also, several application devices, for instance ultra low volume spraying equipment, produce high temperatures which may interfere with the viability and infectivity of the spores in the product.

2. Materials and Methods

Germination of *Aschersonia aleyrodis* spores

A suspension of spores (2.0-2.5 X 10^6 spores/ml) was applied to water agar. The Petri dishes were kept at different temperatures ranging from 11^o to 33^o C. Artificial light was provided for a 16 hr photoperiod. Observations were made 12, 24, 48 and 72 hr after incubation. The percentage germination was determined by recordings of over 300 spores per agar plate. Three agar plates were used per temperature.
Spores were considered to have germinated when the length of the germ tube was equal to or exceeded the breadth of the spore.

Heat resistance of *Aschersonia aleyrodis* spores in suspension

Spores suspended in water were exposed to different temperatures by the use of a warm-water bath. Sterilized water, 2.5 ml per glass tube was warmed to the required temperature. An amount of 0.2 ml of spore- suspension was added. Exposure to a temperature of either 40, 45, 50, 55 or 60^oC took place for 0, 5, 10, 30, 60 or 300 seconds. Afterwards the suspension was poured into another glass tube and put in cool water to decrease the temperature until room temperature was reached.
The spore suspensions were applied to water-agar plates (six per treatment). The germination was recorded 24, 48 and 72 hr after application. The Petri dishes were kept at 25^oC and a 16 hr photoperiod.

Another experiment was carried out exposing sporesuspensions to temperatures of 55^o and 60^oC for 0, 2, 5, 10 and 30 seconds. In some treatments certain compounds were added. The polysaccharids Dextran and Kelzan were used. Dextran is a maltodextrin composed of glucose components. Kelzan is a gel-forming polysaccharid produced by the bacterium *Xanthomonas campestris*, and is also known as Xanthan Gum.
1% and 5% Dextran (1 gr/l and 5 gr/l) were used and 0.01% and 0.05% Kelzan (10 mgr/l and 50 mgr/l).

3. Results and discussion

The germination of spores takes place under temperature conditions ranging from about 11^o to 33^oC (Figure 1). The optimum for germination lays between 25^o and 30^oC. Higher temperatures will cause a delay in germination or germination will not even take place.
Exposure of the spore suspension to 40^oC for up to 300 seconds does not cause a reduction in spore germination (Table 1). However, a temperature of 45^oC has a negative effect and germination is reduced to 3.8%. An exposure time of 60 seconds at 45^oC does not effect germination. Treatment at 50^oC for 30 seconds results in 3.0% germination whereas after a treatment for 10 seconds still 95.6% of the spores germinates. Most of the spores did not survive a heat treatment at 55, 60, 85 and 90^oC.

The addition of Kelzan or Dextran to the suspension resulted in an increase of survival of the spores after heat treatment at 55^o and 60^oC (Table2). Even after exposure of the suspension with 0.05% Kelzan for 10 seconds at 55^oC still 64.3% of the spores germinated whereas only 0.6% survived when no additives were used. However, heat treatment at 60^oC is detrimental to spores of *A. aleyrodis* even in addition with Dextran or Kelzan.

The protection of spores for short instances may be crucial when preparing a product, for instance, by the use of a spray-drying method. The heat resistance of the spores can depend on the environmental conditions.

Table 1. Mean percentage germination of *Aschersonia aleyrodis* spores after a 48 hr incubation at 25°C after exposure to different heat treatments.

exposure (seconds)	temperature (°C)	% germination	stand. dev.
0	-	98.7	0.7
5	40	98.9	0.4
	45	97.9	0.7
	50	98.2	0.8
	55	0.9	1.0
	60	6.6*	1.8
	85	0.0	-
	90	0.0	-
10	40	97.6	1.0
	45	97.5	1.2
	50	95.6	1.9
	55	0.8	0.9
	60	2.4	2.1
30	40	99.1	0.9
	45	97.1	1.0
	50	3.0	3.3
	55	0.1	0.3
	60	2.7	3.1
60	40	98.4	0.5
	45	97.1	1.2
	50	1.6	1.7
	55	0.1	0.1
	60	0.0	-
300	40	98.4	0.5
	45	3.8	2.7
	50	2.4	1.4
	55	0.0	-
	60	0.0	-

Thus, spores suspended in water may be more susceptible to temperature influences than dry spores. Zimmermann (1982) found that in a suspension the thermal death point of *Metarhizium anisopliae* for 30 min exposure was near 50°C and for conidia between 50° and 55°C at 100% RH. At 33% RH, however, the thermal death point increased to 80°-85°C. Still about 75% of the spores germinated after exposure for 30 min at 65°C at 33% air humidity. The spores of *M. anisopliae* are produced in dry spore masses and, therefore, may stand low humidity and high temperature conditions. *A. aleyrodis* spores are produced in a mucilaginous matrix and also this mucus may contain compounds that protect the fungus from detrimental effects of low humidity and high temperature. When suspending the spores in water this matrix is diluted and probably loses part of its function. Analysis of the compounds of this matrix may result in finding substances that can contribute to improval of product development of these microbial products.

Table 2. Mean percentage germination of *Aschersonia aleyrodis* spores after 48 hr incubation at 25°C after exposure to different heat treatments with addition of Dextran and Kelzan.

temp. °C	exp. sec	water	1%dextran	5%dextran	0.01%kelzan	0.05%kelzan
	0	99.1(0.2)*	99.3 (0.7)*	98.2 (1.7)*	99.2 (0.2)*	99.0 (0.3)*
55	2	79.9(7.3)	97.3 (0.9)	94.2 (2.3)	80.1 (4.0)	98.7 (0.4)
	5	10.0(9.9)	40.7(20.6)	26.2 (9.8)	50.1(10.5)	95.3 (2.6)
	10	0.6(0.7)*	0.0 (-)	0.0 (-)	6.4 (8.6)*	64.3(12.3)
	30	0.0(-)*	0.1 (0.2)*	0.3 (0.6)*	1.3 (1.2)*	2.3 (1.5)*
60	2	1.6(2.3)	0.0 (-)	3.6 (5.8)	2.7 (2.3)	12.6 (6.8)
	5	0.0(-)	0.0 (-)	1.0 (1.4)	2.8 (1.9)	1.3 (1.7)
	10	0.4(0.5)*	0.4 (0.5)*	0.0 (-)	0.4 (0.6)*	1.1 (1.2)*
	30	2.1(2.0)*	1.1 (1.4)*	0.0 (-)*	0.4 (0.6)*	1.2 (1.1)*

* mean % germination over 3 agar plates instead of 6
( ) standard deviation

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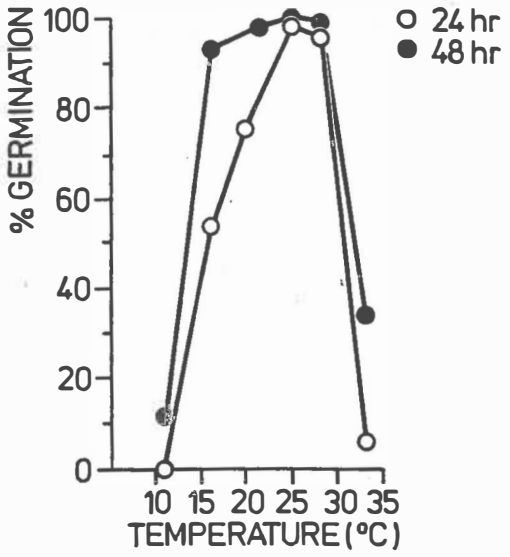

Figure 1. The germination rates of spores of *Aschersonia aleyrodis* on water agar after incubation at different temperatures.

Combination of Metarhizium anisopliae against Diabrotica undecimpunctata and of VA-mycorrhiza on potted corn

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Already for some time the interest in the entomopathogenic fungus Metarhizium anisopliae and its use in biological control of various soil pests has increased. In Germany, research activities resulted in the development of a not yet registered biopreparation 'BIO 1020'produced by the industrial company BAYER. So far, this preparation has mainly been tested against the black vine weevil, Otiorhynchus sulcatus. At the same time, research on vesicular-arbuscular mycorrhizal (VAM) fungi, their various beneficial effects on plants and their practical use has been intensified. Both organisms, M. anisopliae as well as VAM fungi, are applied in the soil especially in the root area of ornamentals and other crops.

As both agents may be used together, the following questions had to be clarified:

(1) Has M. anisopliae any side effects on the colonization of corn roots by a VAM fungus
(2) Is it possible to combine M. anisopliae with a VAM fungus in connection with control of the southern corn rootworm Diabrotica undecimpunctata.

In addition, it was of interest whether the technique used for applying M. anisopliae is suitable for control of the southern corn rootworm.

The experiments were conducted in the greenhouse on potted corn in common compost soil. As mycorrhiza inoculum, particles of expanded clay containing spores of Glomus etunicatum were used. In the first trial these particles were equally covered with dry conidia of M. anisopliae and subsequently mixed with the soil (20 g VAM fungus particles + 1 x 10^{10} conidia = 1 g BIO 1020 per 1 l soil). In a second trial different dosages of M. anisopliae (BIO 1020) were applied together with different amounts of the VAM fungus to a soil layer of about 5 cm under the corn seeds (20, 10, 5 g VAM fungus + 1, 0,5, 0,25 g BIO 1020 in 300 ccm soil). Eggs of D. undecimpunctata were distributed in the upper soil layer after the emergence of corn. Three to four weeks later the number of larvae of the pest was counted, and the colonization of corn roots by the VAM fungus and the root length were recorded.

The following results were obtained from both experiments:

(1) No side effects of M. anisopliae on the VAM fungus G. etunicatum and its colonization of corn roots were noticed.
(2) M. anisopliae caused a high mortality of D. undecimpunctata amounting to about 90 %, irrespective of the presence or absence of the VAM fungus.
(3) The application technique does not impair the efficacy of M. anisopliae in controlling the southern corn rootworm.

The results demonstrate that a combination of both organisms is possible without any visible or measurable interactions.

THE IIBC-IITA-DFPV COLLABORATIVE RESEARCH PROGRAMME ON THE BIOLOGICAL CONTROL OF LOCUSTS AND GRASSHOPPERS

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SUMMARY

The integration of biological control methods into spraying programmes for locusts and grasshoppers will reduce the dependence on chemical pesticides which is causing concern both to the afflicted countries and the supporting donor agencies. The joint research programme being carried out by IIBC, IITA and DFPV is developing entomopathogenic fungi in the genera Metarhizium and Beauveria as mycopesticides formulated in oil for application at ultra-low volume rates. The isolate of Metarhizium flavoviride adopted for further testing will kill >90% of adult Schistocerca gregaria in 5 days at 30°C and 35% RH when inoculated topically in an oil diluent at a dose of 50000 conidia/insect. Application under a similar regime but using a rotary atomiser on an experimental track sprayer resulted in >90% mortality in 5 - 8 days depending on conidial concentration and droplet size. This oil formulation was applied using a hand-held rotary atomiser and caused 83% mortality due to infection within 8 days in adult S. gregaria in field trials at Cotonou, Republic of Benin. Addition of compounds which absorb ultra-violet radiation can double the survival time of formulated condidia when exposed to simulated tropical sunlight. Further work is needed to improve the stability of formulated conidia at high temperatures.

INTRODUCTION

Locusts (particularly the Desert Locust *Schistocerca gregaria*) and grasshoppers (*Oedaleus senegalensis*, *Kraussaria angulifera* and many other species) are the most serious insect pests of agriculture in Sahelian agriculture (Geddes, 1990). In plague years swarms of the Desert Locust may attack crops from West Africa to India (Steedman, 1990). Both grasshoppers and locusts frequently require chemical control measures. The campaign against the Desert Locust outbreak which began in 1985 had cost US$150 million by 1988, with approximately US$70 million of this cost borne by the afflicted countries (Brader, 1988). In 1988 alone, US$100 million was spent on Desert Locust control (Anon., 1990). Costs for grasshopper control in 1986 - 87 amounted to US$62 million (Brader, 1988).

The insecticide formerly favoured for Desert Locust control was dieldrin, which is persistent for months after application. This was very effective at very low doses per hectare, but is now banned in most countries where control is required. The last plague was fought with a variety of less persistent pesticides, of which the most frequently used were fenitrothion and malathion. These are less

effective precisely because they are less persistent, and may give inadequate control (Brader, 1988). As a result of the switch to these somewhat more environmentally acceptable pesticides, control costs have risen tenfold (Brader, 1988). The amounts of pesticide applied have also greatly increased, because several applications may now be necessary instead of only one of dieldrin.

The very high cost of these control operations and the concerns expressed about their effectiveness have led to unease among the donor community, especially in the USA which provides 20% of the donor support (Anon., 1990). Brader (1988) noted that the ban on dieldrin should be an incentive to review control strategies and to carry out research on new control methods, including biological control. This approach was reviewed by Prior and Greathead (1989). They discussed the range of biological agents that may attack the Desert Locust, and concluded that the only viable approach was to develop the entomopathogenic Deutermycotina *Metarhizium* and *Beauveria* spp. as biological insecticides. The reasons for this are reviewed below.

BIOLOGY OF LOCUSTS AND THEIR NATURAL ENEMIES

During recession periods between plagues, Desert Locusts occur as very sparse populations of individuals in the solitary phase, which cause no damage. When conditions for breeding become more favourable, usually as a result of rainfall which provides more areas of soft soil for egg laying, populations increase and the insects switch to the gregarious phase. Hopper bands and adult swarms then form and the latter migrate very large distances by flying, following well-documented wind patterns. Because of the great fecundity of locusts, huge numbers may appear very rapidly when breeding conditions are favourable: a swarm may contain up to 50 million adults per square kilometre, and in 1988 there were swarms in Sudan covering 150 square kilometres (Brader, 1988). A major invasion of swarms may weigh 100000 tonnes and eat this much green vegetation every day.

If breeding areas were both predictable and accessible, treatment of egg fields or hopper bands would be the preferred approach to control. When dieldrin was used, swaths were laid down in the desert which persisted for many months and these effectively controlled hoppers which marched into them. Now that non-persistent chemicals must be used, if hoppers are to be treated it is essential either to treat the whole area or to find and treat every band, but neither of these approaches is feasible. The areas involved are too great for economic treatment and the environmental damage would also be unacceptable. Surveys for hoppers cannot normally hope to detect more than a small fraction of the bands in a given area, but >90% must be killed to give useful control (Brader, 1988). In addition many breeding areas are not accessible due to remoteness or military activity. It is therefore often necessary to adopt a "firefighting" approach which involves detecting adult swarms and treating them before they arrive at agriculturally important areas.

If adult swarms are the target then a suitable biological agent must have certain properties to be effective against them. Prior and Greathead (1989) noted that although the arthropod predators and parasites of locusts had a major role in hastening the end of plagues they were unsuitable for manipulation, and migratory pests such as the Desert Locust were poor targets for classical biological control. Pathogens showed more promise, because some of these could be

manipulated as biological pesticides. This allows the possibility of using conventional application technology, including aerial spraying, which is already highly developed for locust control.

The known pathogens of locusts and grasshopper include: entomopoxviruses of unknown host range and pathogenicity, a single record of a baculovirus in Desert Locust, several protozoa including the well-studied *Nosema locustae*, mermithid nematodes, several opportunistic, wound-infecting bacteria such as *Serratia marcescens* and *Pseudomonas aeruginosa* with a wide host range including mammals, the fungus *Entomophaga grylli* sensu lato and deuteromycete fungi in the genera *Metarhizium*, *Beauveria*, *Nomuraea*, *Paecilomyces* and *Verticillium* (Prior and Greathead, 1989).

To be effective as a biopesticide, the properties of a pathogen must include: cheap and easy production; safety and host specificity; application using existing technology; rapid action. Only the entomopathogenic deuteromycetes, especially *Metarhizium* and *Beauveria* spp., fulfill these requirements. They also have the additional advantage of penetration directly through the cuticle, whereas all other agents have to be ingested. These fungi can therefore be used as contact pesticides, and there is no need for them to persist in the harsh desert environment for the target insects to find and eat them before infection can occur.

FORMULATION AND APPLICATION OF FUNGAL PATHOGENS FOR LOCUST CONTROL

In 1988/89, IIBC developed a joint proposal with the Biological Control Programme Centre of the International Institute of Tropical Agriculture (IITA) at its station in Cotonou, Republic of Benin, and the Département de Formation en Protection des Végétaux (DFPV) in Niger for the biological control of locusts and grasshoppers. The core of this proposal was research on the development of formulations of the conidia of entomopathogenic deuteromycetes which would be effective under arid conditions and suitable for application using controlled droplet application (CDA) technology at ultra-low volume (ULV) rates.

The most commonly recorded fungi in this group which attack locusts and grasshoppers are *Metarhizium anisopliae*, *M. flavoviride* and *Beauveria bassiana*. Conidia, the natural units of dispersal, and blastospores, which are thin-walled spores produced in submerged culture, are both pathogenic but conidia are more stable. The conidia of *Metarhizium* and *Beauveria* are hydrophobic and difficult to disperse in water, but they are lipophilic and can be suspended easily in oils. The insect cuticle is also hydrophobic, but oils spread readily over the epicuticle which contains a high proportion of waxes. A suspension of conidia in oil would therefore adhere readily to the cuticle. The oils may also have the additional advantages of protecting the conidia from desiccation, thus encouraging germination when ambient humidity is low, and spreading them to the intersegmental membranes where penetration may occur most easily. Some evidence for this was obtained by Prior et al. (1988), who found that the LD50 of conidia of *Beauveria bassiana* for the cocoa weevil pest *Pantorhytes plutus* was over thirty times lower if the conidia were suspended in a vegetable oil instead of water.

There was no evidence from the work on *Pantorhytes* that such an effect could be demonstrated at low humidities. However, Marcandier and Khachatourians (1987) showed that when the grasshopper *Melanoplus sanguinipes* was inoculated with a aqueous suspension of *Beauveria bassiana*, the rate and level of mortality was unaffected by the relative humidity during subsequent incubation, and the LT50 was 7 - 8 days whether the RH was 100%, 33% or 12%.

It thus appears possible to kill grasshoppers by topical application of *B. bassiana* even at very low relative humidity, and to greatly reduce the dose of fungus required by formulating in oils instead of water. Oil formulation would have the additional advantage that conventional CDA equipment could be used, at ULV rates. CDA is a more efficient way to apply pesticides than conventional methods of atomisation, but it is essential to formulate in non-volatile diluents to avoid evaporation of the very small spray droplets. Oils are frequently used for this purpose.

CDA techniques are well-adapted to ULV application rates and are essential where the formulation must be applied by air, as is often the case for Desert Locusts. The technology of ULV application was developed for locust control and the logistical infrastructure already exists. There are also good prospects for controlling a variety of economically important grasshoppers by ULV spraying. Unlike locusts, it would be feasible to tackle grasshoppers by ground based-spraying. The technology for fungus production and application would be in place for grasshopper control at times when locusts are in recession and this would also ensure its availability for locust control in plague years.

Four international donor agencies agreed to contribute to a three year research programme to demonstrate the feasibility of this approach to locust and grasshopper control. These are the Canadian International Development Agency (CIDA). the Netherlands Directorate General for Development Cooperation (DGIS), the UK Overseas Development Administration (ODA) and the USA Agency for International Development (USAID). Work began in October 1989.

RESEARCH PROGRESS OCTOBER 1989 - FEBRUARY 1991

The use of fungi to control an insect pest under the arid climatic conditions of the Sahel poses a considerable challenge, since naturally occurring fungal infections of insects are normally associated with humid conditions. The previous arguments suggest that formulation may be the key to overcoming this constraint. Research has been directed initially to answering four questions, as follows.

1. Are suitable fungal pathogens available?

Surveys are being undertaken in W. Africa, Oman and Pakistan for new isolates of suitable fungi, and existing isolates from culture collections are also being tested, using an assay technique described in 2. (below). All isolates highly virulent to *S.gregaria* which have been tested to date are in the genus *Metarhizium* and have originated from Orthoptera. The isolate adopted as a standard and used in all assays for comparison is *M. flavoviride* IMI 330189 which originated from the grasshopper *Ornithacris cavroisi* collected in Niger. Other isolates which are highly virulent to *S. gregaria* are: *M. flavoviride*

IMI 324673 ex *Zonocerus elegans*, Tanzania; *M. anisopliae* IMI 168777ii ex *S. gregaria*, Ethiopia; *M. anisopliae* ARSEF 324 ex *Austracris guttulosa*, Australia. The standard strain IMI 330189 did not appear to be pathogenic to *Zonocerus variegatus* in preliminary studies in Benin.

No highly virulent isolates have yet been found originating in non-orthopteran hosts, although some coleopteran isolates showed moderate virulence. No isolates of *Beauveria* have yet been found which are more than weakly virulent to *S. gregaria*, even those which originated from the host itself or other acridids.

It would appear from the limited evidence to date that *Metarhizium* spp. are more likely to be virulent than *Beauveria* spp. and isolates from Orthoptera are more likely to be virulent than those from other orders. However, many cases are known of an isolate from one order showing virulence to an insect in another order (Prior, 1990) and it would be premature to draw any conclusions yet on the most favourable sources of useful genotypes. It is clear that highly virulent isolates are available, both from *S. gregaria* and from taxonomically related hosts, even if the latter are geographically isolated from it.

2. Can infection occur at low humidity and high temperature using suitable diluents?

An assay technique has been developed using topical application of a standard dose of 50000 conidia/insect in oil to adult *Schistocerca gregaria*. After inoculation the insects are held unfed in plastic boxes at 30°C and mortality measured over a 7 day period. This method discriminates satisfactorily between the majority of isolates which cause no mortality during this period, and a few which cause mortality beginning on day 4 and rising to >90% by day 6.

The relative humidity in the boxes is approximately 35% and it would therefore appear that these results support the claim by Marcandier and Khachatourians (1987) that infection can occur at low RH following topical application. Mortality also occurs under these conditions following inoculation with an aqueous suspension of conidia, but the mortality peak is delayed by about 2 days. Comparative LD50 determinations for oil and water have not yet been carried out, but it would appear that there is a reduction in LD50 with oil formulations similar to that observed by Prior et al. (1988).

3. Are the formulations stable at high temperatures and high incident levels of ultra-violet radiation?

Studies have been carried on the effect of temperature on the survival of conidia of isolate IMI 330189 suspended in various oils. In the most benign oils, there was only a 5% loss of viability after 20 weeks at 5° and 15°C, but a similar decline occurred in 2 - 4 weeks at 25°C and in <1 week at 35°C. No attempt was made in these experiments to improve stability by the use of additives or by pre-treating the conidia, although several approaches are feasible. It appears that long-term storage will require cooling. Conidia are probably sufficiently stable to allow spraying under desert conditions, but more research is required to improve temperature tolerance.

Ultra-violet radiation in the UVB range is lethal to many fungal spores. A range of compounds is available which absorb UVB and have been used to improve the stability of biological pesticides. Those which are compatible with oils have been investigated in an attempt to improve the survival of conidia of IMI 330189 on exposure to tropical sunlight. Oil suspensions of conidia with added chemical sunscreens have been exposed to the UV component of tropical sunlight using an "Oriel" brand sunlight simulator.

Oils themselves have a considerable absorbance for UVB but conidial suspensions in oils without added sunscreens decline to approximately 25% of their initial viability after 60 mins exposure. The addition of a single sunscreen chemical can increase survival to approximately 45% after 60 mins, and a combination of two chemicals can increase it to approximately 65%. Care must be taken in selecting combinations of additives, because some combinations have a cancelling effect and the conidial survival is worse than in the unamended suspension.

4. Can the required lethal dose of spores be delivered to the target?

Under field conditions the biopesticide will impact on the target as very small droplets, probably approximately 75μ Volume Median Diameter. The lethal dose must therefore be applied in a large number of very small droplets rather than the single large drop used in the laboratory assay. In order to study the deposition of formulated conidia under more realistic conditions, an experimental track sprayer has been built in which the target insects are passed on a conveyor belt at controlled speed through a cloud of spray droplets produced by a conventional CDA rotary atomiser. The technique can also be used to study the effects of varying the droplet size by adjusting the speed of rotation of the atomiser. The experiments are carried out at 30°C and 30 - 35% RH but these environmental conditions can also be varied. Mortality occurs in 5 - 8 days depending on dose and droplet size. This suggests that the formulation is suitable for field testing using similar rotary atomisers, which are available commercially as hand-held sprayers.

Preliminary field tests have been carried out on *S. gregaria* in Cotonou, Benin at the IITA Biological Control Programme Centre using oil suspensions of IMI 330189. Mortality due to infection reached 83% by 8 days at a distance of 5 m from the spray path. Further studies are now under way to improve mortality by optimising formulations, dose rates and droplet spectra.

CONCLUSIONS

The results of this research programme have shown that it is possible to formulate the conidia of *M. flavoviride* in oil diluents suitable for CDA using rotary atomisers and to apply the formulations successfully in the field to kill locusts. Research will now concentrate on improving the formulation, particularly with regard to its stability at high temperatures and its resistance to ultra-violet light. Since isolates of *Metarhizium* and *Beauveria* show considerable specificity it will also be necessary to continue screening for isolates with virulence to other target acridids.

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4. Persistence of insect pathogens.

PERSISTANCE DE LA VIRULENCE DES CONIDIES D'*ERYNIA NEOAPHIDIS* REMAUD. ET HENN SUR DE LA TERRE NON STERILE.

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Summary

The authors studied the persistence of infectivity of conidia of *Erynia neoaphidis* Remaud. & Henn on the surface of unsterilized soil, kept wet at + 18°C., + 10°C. or + 5°C., slowly dessicated at + 18°C. or kept at - 5°C. On wet soil, the infectivity persists for one to two weeks at + 18°C., at least eleven weeks at + 10°C. and ten weeks at + 5°C. The dessicated soil is very favourable to the persistence of the infectivity during four weeks. As for the frozen one it keeps it for at least mine months.

1. Introduction

Le rôle joué par le sol dans la conservation d'entomophthorales telles que *Conidiobolus obscurus* Hall et Dunn et *Erynia neoaphidis* Remaud. et Henn a été démontré précédemment (LATTEUR, 1977).

Nous avons aussi observé que les conidies primaires de ces entomophthorales appliquées sur du sol humide non stérile étaient capables d'engendrer des conidies répétitives pendant plusieurs semaines ou plusieurs mois selon les conditions auxquelles elles étaient soumises. (LATTEUR, 1980; LATTEUR et RANDALL, 1987). Cependant, si l'on sait que les conidies répétitives de *C. obscurus* produites dans de telles conditions s'avèrent infectantes (LATTEUR, 1980) cela n'avait pas été vérifié pour *E. neoaphidis*, ce qui a motivé le travail dont les résultats sont présentés ci-après.

2. Matériel et méthode

La terre utilisée est un loess contenant 5 % de sable, 15 % d'argile et 80 % de limon. Elle est desséchée jusqu'à poids constant à 20°C. Avant de l'utiliser pour l'expérience, on lui ajoute 25 % de son poids en eau et on la répartit dans des boîtes de Petri en matière plastique de 5 cm de diamètre à raison de 20 g de terre humide par boîte. La surface de la terre est bien égalisée avant de placer au centre de chaque boîte une momie sèche d'*Acyrtosiphum pisum* (Harris) adulte aptère tué par *E. neoaphidis*. Le couvercle de la boîte de Petri est maintenu à 1,5 cm de la terre grâce à un anneau de plexiglas de 4,5 cm de diamètre placé sur la terre afin que les conidies produites par la momie retombent sur la terre au lieu de se coller sur le couvercle. Les boîtes ainsi préparées sont placées dans les conditions de l'expérience à l'intérieur d'un sachet en matière plastique bien fermé et l'eau qu'elles perdent est rajoutée, après pesée, tous les trois jours sauf les échantillons soumis à la dessiccation.

2.1. *Facteurs étudiés*

2.1.1. Effets des températures positives

Les échantillons sont placés à + 5°C., + 10°C. ou + 18°C. sous 12 heures de photopériode à 3.000 lux. Les trois objets sont réalisés en même temps.

2.1.2. Effet du gel

Les échantillons sont d'abord placés à + 5°C. pendant 96 h. pour permettre la conidiogenèse puis ils sont transférés à - 5°C.

2.1.3. Effet de la dessiccation

Le couvercle de la boîte de Petri est pourvu de 4 trous de 2 mm de diamètre pour permettre la dessiccation lente de la terre. Le poids constant est obtenu après 2 semaines. Les échantillons sont conservés à + 18°C., pendant 12 heures de photopériode et à 80 % d'humidité relative. Un essai sur terre humide est réalisé en même temps.

2.2. *Mesure du pouvoir infectant des inoculums*

La mesure du pouvoir infectant des inoculums est réalisée avec des larves de pucerons du 4e stade à ptérothèques appartenant à l'espèce ***A. pisum***. Quinze pucerons sont placés sur l'inoculum dans une petite cage sans fond constituée d'un anneau en plexiglas de 2 cm de diamètre et de 3 mm de haut recouvert d'une fine toile de nylon. Ils y sont maintenus pendant 12 heures dont 6 à l'obscurité et 6 à la lumière, à 18°C., puis transférés sur de jeunes féveroles comme décrit précédemment (LATTEUR et al. 1985).

Six répétitions sont réalisées pour chaque objet et les échantillons ne sont utilisés qu'une seule fois.

Afin que les pucerons soient au contact de l'inoculum pendant la période où un maximum de conidies répétitives sont émises, des essais préalables ont montré qu'ils devaient être placés sur les terres humides ayant séjourné à + 5°C., + 10°C. et + 18°C., dès la période de conservation écoulée, tandis que sur celles stockées à - 5°C. et sur celles desséchées, ils devraient l'être respectivement juste après leur dégel pendant 12 heures à + 5°C. et juste après leur réhumidification.

3. Résultats et discussion

Tableau 1. Evolution du pourcentage moyen de mycoses selon la température de conservation de l'inoculum sur terre humide.

Semaine	+ 18°C	+ 10°C	+ 5°C
1	80 %	94 %	97 %
2	1 %	95 %	99 %
3	0 %	96 %	97 %
4	1 %	97 %	92 %
5	0 %	56 %	81 %
6	-	-	-
7		17 %	31 %
8		16 %	14 %
9		11 %	6 %
10		12 %	3 %
11		54 %	0 %

Tableau 2. Evolution du pourcentage moyen de mycoses sur sol humide et sur sol sec réhumidifié avant l'essai d'infection

Semaine	Sol humide	Sol sec
1	71 %	80 %
2	9 %	62 %
3	0 %	73 %
4	0 %	44 %
5	-	-
6	0 %	0 %
7	0 %	0 %

Tableau 3. Evolution du pourcentage moyen de mycoses obtenues à partir d'inoculums conservés sur sol à - 5°C

Mois	Mycoses
1	100 %
2	98 %
3	96 %
4	100 %
7	21 %
9	12 %

Aux températures positives et sur sol humide, les résultats confirment ceux obtenus précédemment dans les mêmes conditions et dans les mêmes limites de temps avec des conidies de *C. obscurus* (LATTEUR, 1980). Sur sol humide, à + 18°C., après 7 jours, l'inoculum conserve un pouvoir infectieux important. Il diminue rapidement ensuite pour s'avérer presque nul après 2 semaines. Ces résultats sont relativement comparables à ceux obtenus par BROBYN et al (1985) à partir d'inoculums déposés sur des feuilles de féverole et conservés à 70 % d'humidité relative ou plus.

Aux températures inférieures, + 10°C. et + 5°C., l'inoculum s'avère très infectant pendant 4 semaines.

Cependant, à + 5°C. la diminution de l'infectivité de l'inoculum s'amorce après la 4e semaine et s'avère nulle après 10 semaines tandis qu'à + 10°C., après une diminution de l'infectivité plus rapide qu'à + 5°C., les pourcentages moyens de mycoses obtenus sont comparables de la 7e à la 10e semaine, puis on observe une augmentation très nette en fin d'expérimentation.

La conservation des inoculums à - 5°C. est très favorable à leur survie puisque les pourcentages de mycoses obtenus lors des 4 premiers mois sont égaux ou proches de 100 et qu'ils sont toujours infectieux après 7 et 9 mois. Les conidies résistent donc beaucoup mieux aux températures négatives que les corps hyphaux frais ou secs car ceux-ci sont tués par le gel excepté dans l'azote liquide (LATTEUR, observations non publiées).

Enfin, un inoculum déposé sur de la terre humide soumise à une dessiccation lente conserve à un niveau élevé (> à 40 %) son pouvoir infectieux nettement plus longtemps qu'un inoculum non desséché (quatre semaines au lieu d'une). Cela peut signifier que la sécheresse inhibe l'émission des conidies répétitives mais sans la compromettre définitivement pour autant que la terre soit réhumidifiée au plus tard 5 ou 6 semaines après leur séjour à + 18°C.

4. Conclusions

A la lumière des ces résultats et de ceux des études citées précédemment on peut conclure que :

1°. l'évolution du pouvoir infectieux d'un inoculum constitué au départ de conidies primaires est parallèle à celle de la dynamique de la production de ses conidies répétitives ; autrement dit, qu'un tel inoculum conserve son pouvoir infectieux tant qu'il est capable de produire des conidies répétitives ;

2°. les conidies de *C. obscurus* et de *E. neoaphidis* sont potentiellement capables de résister aux rigueurs de nos hivers et d'être à l'origine d'infections printanières au sein de populations aphidiennes ;

3°. si ces entomopathogènes sont un jour produits et formulés en vue de leur application au champ, leur faculté de persister 1 à 2 semaines sur sol humide et de résister fort bien à la dessiccation peut permettre d'être optimiste quant à leur efficacité dans la lutte préventive contre les pucerons.

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PERSISTENCE OF METARHIZIUM ANISOPLIAE AND BEAUVERIA BASSIANA IN FINNISH AGRICULTURAL SOILS: PRELIMINARY RESULTS

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MATERIAL AND METHODS:

The persistence of Metarhizium anisopliae and Beauveria bassiana was studied in four different soil types and in plots sown with barley or turnip rape, managed with direct drilling or conventional ploughing. The fungal strains used in the experiments were of Finnish origin (Vänninen et al. 1989, Acta Entomol. Fennica 53: 60-71). The spores of M. anisopliae were produced on sterile rice and those of B. bassiana on sterile wheat flakes.

1. Soil type experiment

The soil types were sand (sand1 with 30 % and sand2 with 22 % of fine sand), clay (sandy) and Carex-peat. Three of the sites (sand2, clay and peat) were located within 10 kilometres from each other in Jokioinen, south-western Finland. The fourth site (sand1) was situated approx. 65 kilometres from others in Vihti, southern Finland. The weather patterns and amount of rainfall, however, were practically similar in all four sites.

32 holes (diam. 25 cm) were dug in the soil on each site in July 1988. The soil from the holes was mixed and air dried for 2 months. After this the soil from each site was divided in 32 metal net cylinders that were taken back to the respective site and inserted in the holes. The fungal spores were poured onto the surface of the soil in water and covered with a thin layer of soil. Each cylinder received 10^8 spores/cm^2. There were 8 cylinders treated with a weakly pathogenic M. anisopliae strain, 8 treated with a pathogenic strain and 8 treated with a pathogenic strain of B.bassiana (pathogenicity was tested with Tenebrio molitor larvae). 8 cylinders were left untreated as controls.

The cylinders were sampled once in a growing season in 1989 and 1990. From four cylinders per treatment at a time a soil core was taken with a borer (diam. 2.5 cm, depth 25 cm, vol. 122 cm^3) and divided in three parts: 0-5, 5-10 and 10-25 cm measured from the surface. The soil from each depth was mixed well and 10 grams were weighed for a dilution series. OAES-agar (Schmittener & Williams 1958, Ohio Agr. Exp. Sta., Botany Plant Pathol. Mimeo. Ser. No. 29) with 200 mg of cycloheximide/l was used as a semiselective medium to assess the number of colony forming units (CFU) in the soil. Five plates served as replicates from each dilution. The dry weight of the soil was measured to facilitate proper comparison of CFU's in different depths and soil types.

In counting the percentage survival of the fungi the number of spores applied on an area of 4.9 cm^2 (the cross section area of the borer) was used as the starting point. The average number of CFU's per g of dry wt soil in each depth was multiplied by the total dry wt of the soil in that particular depth. This number was then related to the amount of spores originally applied on the area of the borer, resulting in percentage of applied spores that had migrated to and survived in each depth at any particular sampling time.

2. Cultivation experiment

In this experiment the soil was sandy clay. Two plots of the size of 20 x 20 meters were sown with barley and two similar plots with turnip rape. One of the two plots under same cultivar was managed with direct drilling, the other one was ploughed conventionally in the autumn and prepared for sowing in the spring. In each plot in July 1988 a subplot of 1 x 3 meters was treated with spores of M. anisopliae, and a similar subplot with spores of B. bassiana. One subplot was left untreated. The dosage was 5 x 10^6 spores/cm^2.

Five samples from each subplot were taken with a soil borer in May 1989 (10 months from application) before preparing the soil for sowing, and in September 1989 after harvest but before ploughing (15 months from application). In 1990 samples from the conventionally managed plots were taken in May and from the direct drilling plots in August (22-25 months from treatment). Dilution series were prepared as described above

15 Tenebrio molitor larvae divided in five pots were exposed to soil taken from the treated and untreated subplots on each sampling occasion to verify the infectivity of the fungi from the soil.

RESULTS:

1. Persistence of fungi in four soil types

B. bassiana survived very poorly in all four soil types. 12.4 and 3.3 % of the fungus could be isolated from sand1 and sand2, respectively, one year after the application. In clay and peat the survival was only 0.6 and 0.1 %. The variation between the replicates was big, however, and only sand2 differed significantly from controls which harboured 0-33 000 CFU/g of soil compared to 500-100 000 in sand2.

In the second year only 0-4000 (0-0.1%) CFU of B. bassiana/g of soil was isolated from any of the sites.

Metarhizium persisted considerably better than Beauveria. Averaged over all soil types 63 and 30 % of the weakly pathogenic strain was recovered from the cylinders in 1989 and 1990, respectively. In 1989 the percentage survival was best in both sands (78 and 80 %). In clay this strain survived almost equally well (61 %), and even in peat the survival was as high as 33 %. In 1990 the differences between the soils had almost disappeared: survival was 22 and 25 % in sand1 and sand2, 41 % in clay and 32 % in peat. Thus the amount of fungus had not declined in peat anymore during the second year, but proportionally more of it was recovered in 5-10 cm layer than in the previous year. In other soils this strain of Metarhizium remained in the uppermost layer.

The decline of the pathogenic strain followed a similar pattern. The recovery averaged over all soil types was 27 % in 1989 and 13 % in 1990. This strain survived best in clay (54 % in 1989 and 41 % in 1990) (18 and 21%). In peat a higher proportion of this strain was recovered in 5-10 cm layer than in sands, although in clay a large proportion of this strain was also found in 5-10 cm in 1989. In 1990, however, only the uppermost layer contained Metarhizium in clay. Only 4, 7, and 2 % of the original amount was recovered from sand1, sand2, and peat in 1990.

2. Persistence of fungi in the cultivation experiment

10 months after the treatment 34%, averaged over both cultivars, of Metarhizium could be recovered in the conventionally managed plots and 20% in the direct drilling plots. The CFU counts were in the order of 25 to 200 thousand/g of soil. Two years after the treatment the percentages had declined to 13 and 3, respectively.

In direct drilling plots the proportional amount of fungus recovered was quite even in turnip rape and barley, but in plouhged plots the fungus survived better in the barley plots, especially during the first year (Fig. 1).

The vertical distribution of the fungus in direct drilling plots and ploughed plots differed, as expected. Ploughing and preparing of the plots relocated the fungus from the surface layers deeper into the soil. During the first winter after treatment the overall survival of Metarhizium was better in ploughed plots than in direct drilling plots. From spring 1989 to autumn 1989 there was no decline in the amount of Metarhizium in the ploughed plots, whereas in direct drilling plots the fungus declined during both winter and summer (Fig.1).

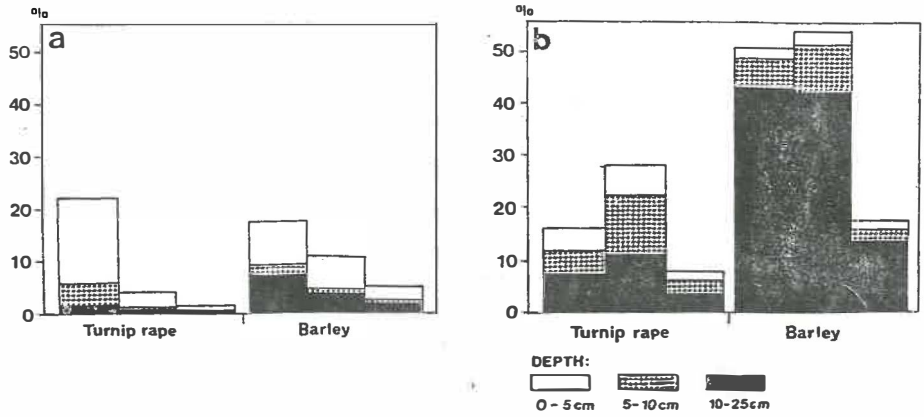

Fig.1 Percentage recovery of M. anisopliae in direct drilling plots (a) and in plots managed with ploughing (b), with turnip rape or barley as the cultivar. The bars from left to right above the name of the cultivar show recovery after 10, 15 and 22-25 months after treatment of the plots with spores.

Of the Tenebrio molitor larvae that were exposed for the soil taken from the Metarhizium subplots 45% died from the fungus in 1989 and 17% in 1990. Thus 25-30 thousand CFU/g of soil was still enough to cause infection in 1990. No larvae died from B. bassiana. In the control plots and Beauveria plots some larvae died from Metarhizium, although the CFU counts were only in the order of 0-5000/g of soil.

B. bassiana survived very poorly in the cultivation experiment. Practically no fungus could be isolated in 1989 or in 1990.

DISCUSSION:

Clearly it is not possible to expect a reliable effect from Metarhizium in intensively cultivated fields for more than one growing season. It was of interest that in ploughed, i.e. disturbed plots the overall survival of Metarhizium was better than in direct drilling plots. This may be explained by the fact that ploughing relocates the fungus deeper in the soil where the physical processes of frost, and biological processes as well, are milder or less active than in the surface layers. The fact that the fungus did not decline in the ploughed plots during the period from spring 1989 to autumn 1989 speaks also for this supposition.

If the soil is not disturbed, Metarhizium remains in the uppermost layers of 0-5 cm of soil, where also larvae of many pest insects occur. The large size and hydrophobic character of Metarhizium spores probably was responsible for their remaining in the upper layers and not migrating downwards with water. In peat, however, where the soil was less compact, even Metarhizium migrates downwards to some extent. The good survival of Metarhizium in clay corresponds to the fact that it was often recovered in clay soils in survey done in Finland (Vänninen et al. 1989).

As for Beauveria, it is a very poor candidate for situations where long term survival is expected. Beauveria's poor survival without hosts is of interest, because it is considered to be a more saprophytic species than Metarhizium (Dr. Nina Lappa, pers. comm.), which should facilitate its survival outside hosts. In nature the ecological strategies of these two fungal species are obviously very different from each other.

Persistence of the entomopathogenic nematode *Heterorhabditis megidis* HE-87.3.

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Summary

The persistence of *Heterorhabditis megidis* HE-87.3 in sandy soils was studied in the laboratory and the field. Following inundative release of 1 million nematodes/m² on irrigated grassland nematode numbers dropped to a level of 20-40.000 per m² within a week. At 25°C in containers filled with moist sand also 60-80% of the nematodes died within a week. The survival of *H. megidis* HE-87.3 under these conditions therefore seems limited to a period of only one or two weeks. A small proportion of the nematodes however persisted for several weeks and at former release sites small *H. megidis* HE-87.3 populations could still be found two years after application.

Introduction

The insect pathogenic nematode *Heterorhabditis megidis* HE-87.3 has been tested in laboratory and field trials for its potential to control grass grubs, in particular *Phyllopertha horticola* (Coleoptera: Scarabaeidae) in grassland and lawns (Smits, 1991). An important feature of the control potential of a nematode is the ability to persist for longer periods in the environment in which it has been applied.

It is known that following application the persistence of large numbers of *H. bacteriophora* is limited to a period of weeks rather than months (Molyneux, 1985; Kaya, 1990; H.Bathon, J. Curran, pers. comm.). Despite the rapid decrease in numbers during the first weeks following the long-term persistence of *Heterorhabditis* over periods of more than one year reportedly good (Rovesti, 1991). Entomopathogenic nematodes of the genera *Steinernema* and *Neoaplectana* generally maintain higher numbers for more extended periods than *Heterorhabditis* spp. (Molyneux, 1985; Kaya, 1990).

The persistence of nematodes of the strain *H. megidis* HE-87.3 was studied on grassland in the field and in experiments in the laboratory in small containers with sterilised sand under various moisture and temperature regimes.

Materials and methods

Nematodes. *H. megidis* HE-87.3 was isolated in 1987 from parasitised *P. horticola* grubs collected on a golf course on sandy soil in the south of Holland. The isolate was characterised as *H. megidis* based on DNA-RFLP (Smits et al., 1991) and morphological parameters (Smits & Ehlers, 1991). The nematodes used in the experiments were produced in waxmoth, *Galleria mellonella*, larvae and used within one month after production. The nematodes were stored until use at 5°C in water and showed good fat-reserves and less than 10% dead individuals.

Field experiments. Experiments were carried out in the "rough" of a golf course on sandy soil. Plots of 2 by 2 metres in a latin square design with 5 replications were treated with *H. megidis* HE-87.3 nematodes in early August. Dosages of 1 and 0.3 million nematodes/m² were applied after sunset by an adapted sowing machine, which injected the nematode suspension at 3-5 cm depth with 10 cm row interval using a volume of 10 l of water per plot. Following application the plots were irrigated overnigth with a large volume of water (ca. 20 l/m²). Soil samples were taken just before application and at day 1, 7, 14 and 28 after application. From each plot random 36 samples were taken with a soilborer (length 10 cm, ϕ 1 cm). Samples of one date were pooled per plot forming one sample of 700-1000 g of soil.

Galleria trap method. The whole soil sample taken from the field experiment (see above) was placed in a beaker and five *G. mellonella* larvae were placed in the middle of the sample. After five days at 25°C the larvae were collected, the red-coloured ones were dissected and the number of nematodes inside each larva was counted. The data in Figure 1 represent the averages of the numbers found in each of the five fields per treatment.

Container experiments. Small plastic containers (50 ml, ϕ 36 mm) were filled with sterilised silver sand to which 0, 4, 8, 12, 16 or 20% w/w water was added. On top of the sand 100 nematodes in 1 ml of water were applied. The pots were closed with a plastic lid and incubated at 25°C in the dark. After 1, 7, 14 and 28 days the number of nematodes was counted. The nematodes were extracted from the sand using Cobb's modified decanting and sieving method ('s Jacob & Van Bezooijen, 1983). For each combination of mositure and time 10 containers with sand and nematodes were used and analyzed. The data in Figure 2 represent the averages of the survival in 10 containers.

Results

Figure 1 shows the persistence of nematodes following application to grassland. The results show that one day after application ca. 15% of the released number of nematodes was recovered both in the plots treated with 1 and 0.3 million nematodes/m². After one week the numbers further decreased to 20.000 nematodes/m² or less. The real number of nematodes actually present may have been larger as the recovery rate of the *Galleria* trap method we used is much lower than 100%. In experiments done under similar circumstances we measured an efficiency of 10-20 % (Smits, unpubl. data). More important than the exact numbers however, are the developments over time. It is clear that after one week very few nematodes are still available or able to parasitise grubs. Where they have gone, whether they are dead, inactive or moved downwards or away is not clear. Small

numbers, those which have found good sites in the soil or have chosen a good survival strategy, could still be found 30 days following application. Similar studies in earlier years on the same site showed that even after 2 years low numbers can still be found in the release plots, though these certainly represent offspring of the original nematodes that were released.

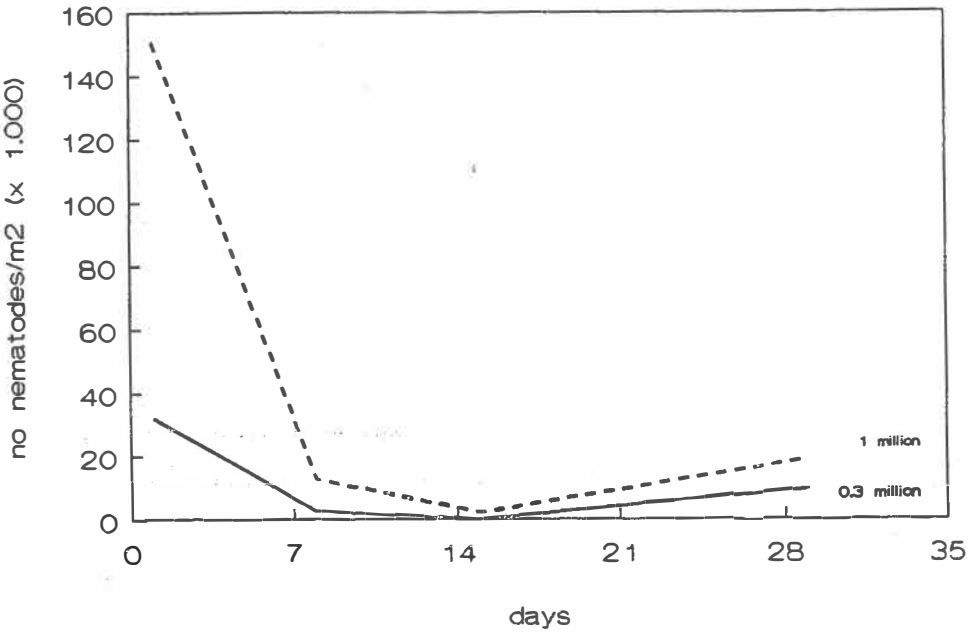

Figure 1. Number of *Heterorhabditis megidis* HE-87.3 nematodes recovered with the Galleria-trap method during 4 weeks following application of 1 and 0.3 million nematodes/m² on grass with an adapted sowing machine.

Figure 2 shows survival of *H. megidis* HE-87.3 nematodes at 25°C in small containers filled with sterile sand with various moisture levels. The experiment was done to see whether physical circumstances could be responsible for the rapid decrease in nematode numbers as found and shown in Figure 1. The results show that the percentage surviving nematodes rapidly decreased to 20-40% within a week. The rapid decrease occured at all moisture levels but least so at 20% water. In the second week the number of living nematodes further decreased in all treatments but at a much lower rate. The higher the moisture level the higher the survival though the differences are not spectacular. Similar experiments at temperatures of 15°C, 20°C and 30°C showed that the higher the temperature the more rapid the decrease in survival appears to be. At the lower temperatures in particular the 16% moisture level gives the highest rate of survival, 70% after 30

days at 15°C. At 30°C the moisture level does not have much influence and most nematodes are dead after 14 days. It appears that the optimum moisture level for *H. megidis* HE-87.3 lies somewhere near 16% water whereas the higher the temperature the shorter the period of survival tends to be.

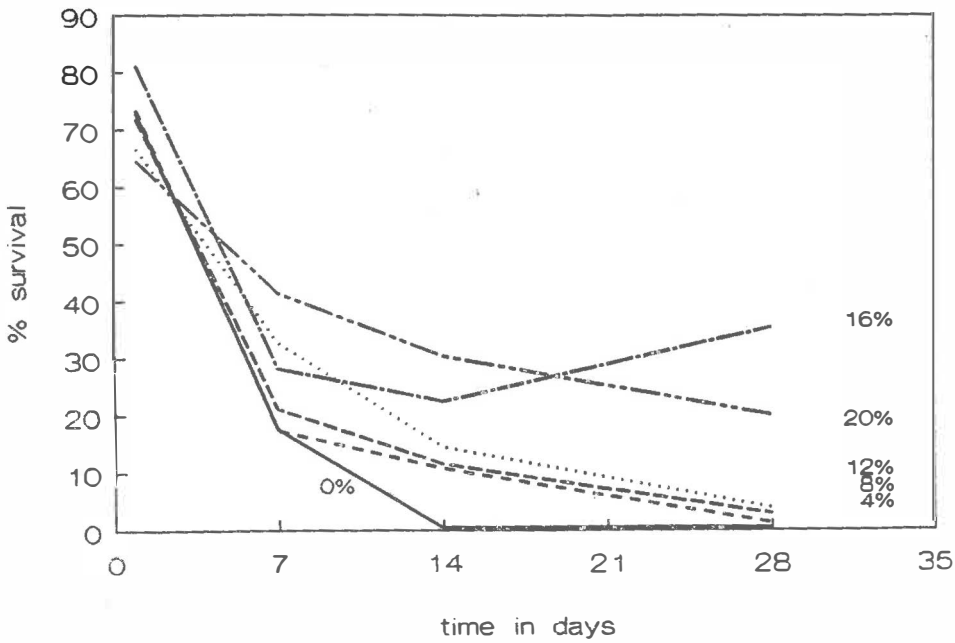

Figure 2. Percentage surviving *Heterorhabditis megidis* HE-87.3 nematodes over time at 25°C in small containers filled with dry sterilised silversand moistened with 0-20% water.

Discussion

In the field experiments the soil temperatures in the daytime in the toplayer of 10 cm were measured to be 25-30°C as it was a beautiful August. Comparing the survival of nematodes both in the field (Fig. 1) and in small containers with sterile sand (Fig.2) the similarity in the graphs is obvious. Although biological factors such as predation may have played an additional role in reducing the nematode population in the field it appears that the survival of the nematodes was mainly influenced by temperature and moisture conditions. As may be clear from this study as from other studies with *Heterorhabditis* species (Molyneux, 1985; Kaya, 1990; H. Bathon & J. Curran, pers. comm.) that we cannot expect long persistence at high numbers in the soil. The number of *Heterorhabditis* nematodes soon decreases to a level of 20-40.000 nematodes/m² after mass release. This

level was also found in natural *Heterorhabditis* populations in Australia (J. Curran, pers. comm.). It could mean that there is only a limited number of good sites for survival of nematodes in the soil, although one would then expect large differences between soil types. It may also suggest that there is interaction between the nematodes and that at higher population densities a larger part of the population will either move away or use up its energy in search for hosts so that the population falls back if there are not enough hosts present. Part of the population will always choose the strategy of waiting for a host to pass and saving its energy until then. One thing is clear for a nematode: you only can use your fat reserves once, either you spend it quikly moving around in search for a host or you spend it slowly and sit and wait until a host comes near. Both strategies give you a chance to survive and reproduce and which is the best strategy depends largely on the environmental conditions and on the density and behaviour of the potential hosts. The relatively short persistence of *H. megidis* HE-87.3 in large numbers may well explain the disappointing control effects in the last two hot summers of 1989 and 1990 (Smits, 1991) in contrast with the relatively good control effects in the wet and cold August of 1988. The other factor which probably also played an important role is the grub density and the associated stress factors which influences the susceptibility to nematodes. If several nematodes rather than one are required to kill a larva, which seems to be the case with healthy *P. horticola* grubs (Smits 1991), short persistence of high numbers of nematodes leads to very low chances of parasitisation.

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ASSESSMENT OF THE PERSISTENCE OF PINE BEAUTY MOTH, *PANOLIS FLAMMEA*, NUCLEAR POLYHEDROSIS VIRUS

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Summary
Persistence was studied in relation to pine beauty moth, *Panolis flammea*, and its NPV in lodgepole pine forests in Scotland. The quantity of virus (in terms of polyhedral occlusion bodies, PIBs) produced as a result of an epizootic induced in 1985 was estimated to be 1.7 to 9.25 x 10^{14} PIBs per hectare. Levels of NPV infection in the year after spraying ranged from 26 to 71%. A significant relationship was found between the level of inoculum produced in 1985 and the maximum level of NPV infection measured in 1986. Investigation of the possible routes of virus persistence revealed that viable virus could survive on the pine foliage over winter and was able to persist in the soil at least three years after spraying with NPV. Host-mediated persistence was estimated in three unsprayed plots. A low level of NPV infection was found in all three instances.

1. Introduction

Persistence is one of the key factors involved in the epizootiology of baculoviruses as it allows them to survive when the host passes through quiescent or non-infective stages or, perhaps, even longer periods of population decline. However, although persistent virus is often referred to as a means of host population re-infection surprisingly little is known about the interaction of persistent baculovirus populations and their hosts in the majority of insect populations. This could be of particular relevance in an applied context as residual populations of viable virus could influence the nature (and the destructive capacity) of any further pest outbreaks. More detailed information is needed in three broad areas: the measurement of the affect of persistent virus, the elucidation of the dynamics of the baculovirus:host relationship and more accurate assessment of the major routes of transmission.

The insect described in these investigations is the pine beauty moth, *Panolis flammea* (D & S) a major pest of lodgepole pine (*Pinus contorta*) in the UK (Watt and Leather, 1988). A nuclear polyhedrosis virus (NPV) was isolated from a *P. flammea* outbreak in Scotland in 1979. Since then it has been developed as a control agent (Entwistle and Evans, 1985, Cory and Entwistle, 1990a, Entwistle *et al.*, 1990). Although field trials have invariably been geared towards assessment of the control potential of PfNPV, and thus were not necessarily ideal for ecological studies, we have tried where possible to use them to gain an understanding of the interaction between PfNPV and its host. More recently ecological research of this nature has become particularly relevant in relation to risk assessment studies for the release of genetically modified baculoviruses (Entwistle *et al.*, 1986, Cory and Entwistle 1990b).

2. Materials and Methods

All the work described was carried out in lodgepole pine plantations situated in the Sutherland and Caithness regions in northern Scotland.

1985/1986 persistence studies:
In 1985 one hectare plots of lodgepole pine planted in 1973 were sprayed by helicopter using ULV equipment with two doses (2.2 x 10^{11} and 4.4 x 10^{11} polyhedral inclusion bodies (PIBs) per hectare) of PfNPV. Spraying took place at approximately 95% *P. flammea* egg hatch and the plots were sampled weekly thereafter. Sampling was carried out by randomly selecting 7 trees each week and collecting all the larvae on the tree by beating the branches over a white sheet. The larvae were counted and then frozen in individual containers. At a later date a sub-sample of 25 larvae were assessed for the presence of NPV occlusion bodies by smearing each larva onto a microscope slide, staining with Giemsa and diagnosing under x1000 oil immersion on a light microscope. Instar was also estimated at this point by measuring the head capsule.

Infection in the following season was measured by carring out a similar sampling regime in five selected plots in 1986. The body of virus inoculum produced in each plot as a result of the 1985 NPV epizootic was estimated by working out the numbers of each instar which were infected on each sampling date and then multiplying this value by the number of PIBs which should be produced if an insect dies in that instar, using the data of Evans *et al.*, (1981) for the closely related cabbage moth, *Mamestra brassicae*.

Routes of *P. flammea* NPV persistence:
Baculoviruses can theoretically persist by two main routes; via the environment or via the host (or hosts). PfNPV persistence was studied in the Scottish lodgepole pine ecosystem. In order to investigate environmental persistence pine foliage and soil were assessed as reservoirs for viable virus. NPV was recovered from lodgepole pine foliage, collected after the 1985 control trials, by sonication and bioassayed in *M. brassicae* larvae using the technique described in Carruthers *et al.*, (1988). Virus in soil samples collected from sites which had been sprayed with PfNPV some years earlier, together with soil from an unsprayed site, was bioassayed by incorporation into semi-synthetic insect diet which was then fed to *M. brassicae* larvae (Stark, unpublished data).

If host-mediated persistence is to be investigated the effects of any virus in the environment must first be removed. This was done by collecting eggs from several sites and rearing them through away from any contaminating influences. In this first trial eggs were collected in early spring from three unsprayed sites. All the eggs were separated from the foliage on which they had been laid, half the eggs were then surface-sterilized to remove any external sources of virus by washing in 0.2% chloros followed by 10% formalin and the remainder were left untouched. Both batches of eggs were reared through individually in a virus free laboratory to assess residual levels of NPV infection within these populations. Two of these plots were also monitored during the following summer for NPV infection (the third required control measures due to the high population levels) using the techniques descibed above.

3. Results

The *P. flammea* populations in all the plots chosen for follow-up studies exhibited a characteristic two-peaked response to PfNPV application, although this varied in shape depending on treatment. The first infection peak is thought to result from the death of early instar larvae after NPV application. The virus liberated from these larvae after their death then provides inoculum for older larvae which form the second peak of infection. This phenomenon has been recorded in other Lepidopteran species (e.g. Woods and Elkinton, 1987). The total quantity of PIBs

produced in the plots varied from 1.7 x 10^{14} to 9.25 x 10^{14} PIBs per hectare. The plot treated with a double dose of NPV (4.4 x 10^{11} PIBs/ ha) produced less virus than any of the single dose treatments. This was thought to be because the higher dose of virus initiated infection earlier than the single dose treatment resulting in infection in earlier instars which produce less progeny virus. *P. flammea* in all plots developed NPV infection in the following season with maximum infection levels ranging from 26% to 71% per tree, although an obvious bi-modal response was absent. There was a significant relationship between the quantity of virus produced in the first year and the maximum level of NPV infection attained in the second.

Viable PfNPV was found on lodgepole pine foliage throughout the winter months and up until the hatch of the next *P. flammea* generation (Carruthers *et al.*, 1988). Viable NPV was also found in the soil collected from several sites at least three years after NPV application. In the egg-sterilization experiment NPV infection was found in both sterilized and unsterilized eggs. Levels of NPV infection in unsterilized eggs ranged from 0 to 2.44% infection per tree and that in the sterilized eggs ranged from 0.185 to 0.5% per tree. In both instances the infection usually resulted from only one or two egg batches. Maximum NPV infection in two of the plots during the summer reached 20% and 49% per tree whereas the original unsterilized level of NPV infection in the eggs had been 2.44% and 0% respectively.

4. Discussion

The data presented here are a preliminary analysis of the effect of persistent NPV in the *P. flammea*/ lodgepole pine ecosystem. Virus infection was found in all the plots which were studied in the year after spraying. Although a relationship was found between the virus produced in one year and NPV infection in the subsequent generation, other aspects of this relationship are still poorly understood. Viable virus was found to persist on both pine foliage and in the soil. Although environmental persistence via the foliage is the most likely route for NPV persistence, as has been shown in other systems, in particular the detailed work on gypsy moth, *Lymantria dispar* (Murray and Elkinton, 1990) there was also some evidence of a low level of host-mediated NPV transmission. Other factors affecting NPV persistence such as degradation by ultra violet light and physical removal from the host environment are not covered here but must form an important component in the system. Given that in excess of 10^{14} PIBS were produced as a result of the 1985 spray trials, 1000 times more virus than was originally applied, the response in the subsequent season is low. Thus the virus must have been inactivated or removed from the environment: this removal needs to be estimated in order to gain some insight into the dynamics of the process.

Although more is probably known about the persistence of NPV in forests than in any other ecosystem (e.g. Kaupp, 1983, Olofsson, 1988) our understanding of its role in the host-virus interaction is far from complete. More detailed experimentation is needed in order to elucidate the dynamics of the process. The use of molecular tools, such as the sensitive polymerase chain reaction for analysis of environmental samples and baculoviruses which have been 'marked' with a small non-coding piece of DNA, may well become important in unravelling some of the more difficult areas in virus persistence studies. In particular, in the monitoring of persistent virus populations for more than one year. In this way we shall gain a better understanding of the role baculoviruses play in insect populations: information which will also have a more practical application in the design of more effective, long-term pest control systems.

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5. Ecology of insect pathogens.

ECOLOGIE ET SPECIFICITE PARASITAIRE DES ENTOMOPHTHORALES PATHOGENES DE DIPTERES EN ZONE TEMPEREE

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Summary

Considering the data provided by the literature and their own respective results, gained in several European countries, the authors emphasize the variety of habitats and host ranges of Entomophthorales pathogenic to Diptera. These fungi were found in Europe only on adults. According to fungal species, infected Diptera occurred near the water level or floating on the water, on the underside of leaves or on bark at the border of forest, on plants in open fields or in premises (houses, cellars, cowsheds or poultry buildings). Concerning host specificity, most of the Entomophthorales species pathogenic to Diptera are only found on these insects. A few species have a wider host range, covering several insect orders.

1. Introduction

Près de la moitié des espèces d'Entomophthorales (Zygomycètes) à potentialité entomopathogène sont trouvées sur Diptères, Nématocères et Brachycères, en zone tempérée. Un certain nombre d'entre elles ont été mises en évidence dès la seconde moitié du XIXe siècle. Jusqu'à ces dernières années cependant, il était difficile d'avoir une vue globale sur les exigences écologiques et la spécificité parasitaire de ces champignons car très peu d'auteurs s'étaient en fin de compte attachés à rechercher d'une manière exhaustive les cas de mycose à Entomophthorales chez les Diptères pris dans leur ensemble. Aujourd'hui, en confrontant les résultats des observations effectuées respectivement par le second auteur en Suisse (Keller, 1987, 1991) et par le premier dans d'autres pays d'Europe (Papierok et Dumas, 1991) aux données de la littérature, notamment celles de Nowakowski (1883), de Thaxter (1888) et de Gustafsson (1965), nous pouvons distinguer, au sein des Entomophthorales pathogènes de Diptères, des espèces ou groupes d'espèces en fonction des caractéristiques écologiques des stations où elles évoluent préférentiellement et de leurs spectres d'hôtes.

2. Types de stations où évoluent les Entomophthorales pathogènes de Diptères

La quasi-totalité des Entomophthorales s'attaquant aux Diptères sont trouvées sur adultes, dans le milieu aérien. L'exception la plus remarquable est représentée par **Erynia aquatica**, qui, découverte sur larves et nymphes de Culicidae en Amérique du Nord, a été observée en Europe uniquement sur adultes, de Chironomidae ou d'autres Nématocères, mais jamais à ce jour de Culicidae.

Selon l'espèce fongique, les Diptères adultes morts d'entomophthorose se rencontrent dans différents types de stations, qui reflètent les exigences écologiques du champignon. Ces types sont également fonction à la fois du cycle biologique du pathogène et de celui de l'insecte hôte. C'est ainsi que nous pouvons distinguer les groupes suivants, selon que les cadavres sont trouvés:

- juste au dessus de la surface de l'eau, éventuellement flottant sur l'eau: **Entomophaga conglomerata** / Culicidae ou Chironomidae, **E. papillata** / Chironomidae ou Simuliidae, **Entomophthora culicis** / Chironomidae, Simuliidae ou Culicidae, **Erynia aquatica** / Chironomidae, **E. conica** et **E. curvispora** / Chironomidae ou Simuliidae, **E. ovispora** / Psychodidae, **E. variabilis** / Nématocères de petite taille,

- dans des clairières ou en lisière de forêt, le plus souvent à la face inférieure des feuilles, beaucoup plus rarement sur rameaux, tiges ou troncs: **Conidiobolus apiculatus** / différentes familles dont Psilidae, **Entomophaga limoniae** / Limoniidae, **Erynia dipterigena**, **Zoophthora lanceolata** et **Z. radicans** / différentes familles,

- en zone ouverte, sur des plantes basses, dans un environnement pouvant être assez sec: **Entomophthora israelensis** (= **brevinucleata**) / Cecidomyiidae, **E. schizophorae** / Anthomyiidae ou Psilidae, **Strongwellsea castrans** / Anthomyiidae, ou dans un environnement assez humide: **E. trinucleata** / Sciaridae, **Entomophaga gigantea** et **Eryniopsis caroliniana** / Tipulidae,

- dans des locaux: **Erynia** sp. / Culicidae hibernant dans des caves.

La plupart des Entomophthorales pathogènes de Diptères se rangent dans l'une ou l'autre de ces grandes catégories. Cependant, la présence de certaines espèces n'apparaît pas strictement limitée à tel ou tel type de situation écologique. C'est le cas, par exemple, de **Entomophaga domestica**, trouvé sur Sciaridae dans les serres ainsi que sur Chloropidae en zone ouverte, sur des plantes basses, de **Entomophthora culicis**, pouvant être rencontré relativement loin de l'eau ou de **E. muscae**, trouvé sur Anthomyiidae, Scatophagidae, Syrphidae en zone ouverte, sur des plantes basses ou dans des clairières ou en lisière de forêt, à la face inférieure des feuilles, ainsi que sur Muscidae, à l'intérieur des locaux.

Il faut enfin signaler que n'ont pas été prises en considération, dans ce classement, les espèces de **Conidiobolus** à tendance saprophytique occasionnellement isolées de Diptères: **Conidiobolus coronatus, C. osmodes, C. stromoideus** et **C. thromboides**. Leur mise en culture à partir de cadavres d'insectes est en effet un phénomène accidentel. Ces champignons se comportent comme des microorganismes opportunistes, envahissant secondairement des insectes tués par une Entomophthorale à tendance parasitaire ou morts d'une tout autre cause.

3. Spécificité parasitaire des Entomophthorales pathogènes de Diptères

Compte non tenu des espèces de **Conidiobolus** à tendance saprophytique, pour lesquelles il est difficile de parler de spécificité parasitaire, la majorité des Entomophthorales trouvées sur Diptères sont connues pour n'être pathogènes que d'insectes de cet ordre. Seules quelques espèces (**Conidiobolus apiculatus, Entomophaga domestica, Erynia conica** et **Zoophthora radicans**) infectent également des insectes d'autres ordres mais il est probable qu'existent chez celles-ci des races physiologiques adaptées aux différents insectes hôtes. Parmi les Entomophthorales inféodées aux Diptères, on rencontre à la fois des espèces attaquant des insectes de plusieurs familles ou des espèces inféodées à une famille, comme **Entomophaga limoniae** sur Limoniidae, **Entomophthora israelensis** sur Cecidomyiidae, **Entomophaga gigantea** et **Eryniopsis caroliniana** sur Tipulidae ou **Strongwellsea castrans** sur Anthomyiidae.

4.Conclusion et Perspectives

Au sein des Entomophthorales, l'ensemble des espèces pathogènes de Diptères est remarquable par la variété des situations écologiques où elles évoluent et par celle de leurs spectres d'hôtes. Un certain nombre de ces champignons infectent des insectes d'importance agronomique ou médicale; plusieurs espèces, étant régulièrement responsables d'épizooties, se comportent même comme des ennemis naturels particulièrement actifs. Cependant, l'étude écologique de ces mycoses n'a été abordée que dans de rares cas: **Strongwellsea castrans / Delia** spp. (Anthomyiidae) (Lamb et Foster, 1986), **Entomophthora muscae / Delia antiqua** (Carruthers et Haynes, 1985, 1986; Carruthers *et al.*, 1985), **E. muscae /Psila rosae** (Psilidae) (Eilenberg, 1987; Eilenberg et Philipsen, 1988). On ne sait, ainsi, pratiquement rien des facteurs conditionnant l'établissement et la propagation de la mycose à **Entomophthora culicis** ou de celle à **Erynia conica** dans les peuplements de simulies en zone tempérée. Il y a là tout un domaine d'investigation qui mériterait d'autant plus d'être exploré que les résultats obtenus permettraient de mieux saisir le potentiel éventuel offert par ces champignons en lutte biologique. Une autre voie de recherche intéressante à développer concerne les

Entomophthorales pathogènes de Diptères, qu'ils soient d'importance agricole ou médicale, en zone tropicale; ces champignons y ont en effet été très peu étudiés jusqu'à présent.

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Simultaneous utilization of an entomopathogenic fungus and nematodes against larvae of the black vine weevil and the influence on plants

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INTRODUCTION

Black vine weevils are serious pest in ornamental plants, nurseries and strawberries field for larvae and adults develop resistance toward insecticides. Biological control by fungi or nematodes achieve good results in laboratory tests or small scale trials. The hyphomycete Beauveria brongniartii seems to be an effective biological agent against the larvae of the black vine weevil Otiorrhynchus sulcatus. The entomopathogenic fungus has been successfully tested as compatible with some phytosanitary products The conidia remain for more than 3 years in the soil and as deep as 40 cm. Heterorhabditis nematodes live in symbiosis with a bacterium Xenorhabdus which transform the host after penetration by the nematodes into a diet suitable for further development of the infective nematode third instar larvae (L3) into adult and for reproduction but their persistance in the soil is till now unknown.

MATERIAL AND METHOD

B. brongniartii (BB) (strain 4792 from laboratory collection at U.L.B.) is collected after 2 weeks growth on Sabouraud's medium at 25°C. Two strains of nematodes, one unidentified and one Herorhabditis spp. (Canadian isolate H88 from Webster) are used in the test as N1 and N2 respectively. Stomatal conductance and foliar potential are recorded on a porometre from Delta T Device and on a pressure chamber, foliar fluorescence and proline contents complete the data. During the measurements, the soil is kept at his field capacity, in such conditions, comparison of hydric status of the different modalities can be used as criterion of efficiency for the larvae feed on roots. Two experiments are carried out on 70 plants each, in greenhouse and phytotron (16h photoperiod, P.A.R. 785 µE/cm², Hr 90%, T°day 25°C, T°night 20°C, T°soil 22°C). The plants were split into 6 treatments and a control for 10 repetitions. Five 5th instar larvae O.sulcatus a plant are added at begin of experiment 1, and 100 hatching eggs in experiment 2. Treatment consist in pouring 2 10^{+7} conidia of B. brongniartii 4792 (BB) or 8,000 nematodes per liter soil. Combinations BB, N1, N2, BB+N1, BB+N2, and larvae alone (OS) are assayed.

RESULTS

For both experiment on Fragaria sp. and Acer groserii, we record same evolution

On plant

Plants with nematodes alone do not differ from the control, on the contrary infested plants OS show higher potential and resistance, the final small recovering can be explained by molting of the larvae into pupae which suspend feeding and therefore enable the plant to build up new roots. Potential and stomatal resistance of plants inoculated with B. brongniartii (BB, BB N1, BB N2) are lower than the other treatments. Statistical analysis performed on the data show even that a synergy exist between B. brongniartii and nematodes treatment if the untreated plants (Control and OS) are excluded.

The results of the measurement show that B. brongniartii, regarding the way of adjunction, enhances the resistance to water stress so that after a week of suspending water supply only the plant inoculated with B. brongniartii avoid wilting. Microscopic preparations of roots show differences between plants who receive B. brongniartii and the others. Roots hairs are less numerous and when present surrounded by mycelium, roots are shorter and larger than normal ones. Sporulation of B. brongniartii on the root is observed. This in conjunction with the better water uptake suggest first stage of a mycorhizal association.

On Otiorrhynchus

Pupae were amorphous and slightly yellow to brownish. Outbreak of mycosis appears in quarantine. Sporulation form small aggregates and rarely cover the body, production of red pigment is also noticed. Larvae treated with the N2 strain were quickly killed. The multiplication of the bacterium confer a red colour to those larvae and after a week of quarantine the L3 offspring of nematodes emerged from stigmates, mouth and anus of the larvae. Efficiency vary from 100% for the N2 regardless to the presence of the fungi, and reached 98 and 96% for B. brongniartii alone or with N1. No larvae seems to be parasitized by the N1 strain.

CONCLUSION

Physiological measurements are correlated with efficiency and status of the roots. Mycosis is recorded and fungi is reisolated. The fungus enhance water uptake in dry conditions and has an influence on the shape of the roots. The strain of B. brongniartii and of H88 Heterorhabditis spp achieve good control of Otiorrhynchus sulcatus larvae, and both treatments, if considered from plant view, are effective.

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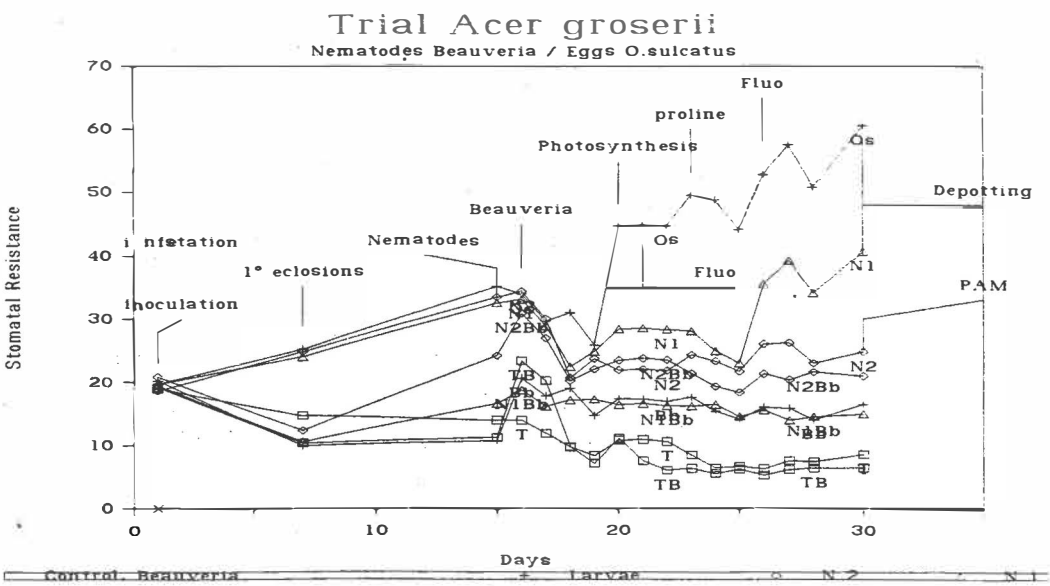

THE KNOWLEDGE OF FUNGAL ENTOMOPATHOGENS IN SPAIN

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SUMMARY

Three entomophthoralean species news for Spain, *Entomophthora muscae* complex, *Erynia gammae* and *E. radicans* and some host-pathogens combinations unrecorded elsewhere e.g. *Beauveria bassiana* on the orthopters *Dociostaurus maroccanus, D. genei*, and *Calliptamus italicus*; the coleopters *Cleonus mendicus* and *Aubeonymus mariaefranciscae* and the lepidopter *Ocnogyna baetica*; *Entomophthora planconiana* on *Israelaphis lambersi* and *Erynia gammae* on *Spodoptera littoralis* have been found during our survey period.

The records of entomopathogenic fungi in Spain are very scarse throughout the period of time comprising the beginning of the study of Insect Pathology and the first half of this century. As far as we know awareness of these entomopathogens began at the end of the 19th-century, when a member of the Entomophthorales, *Entomophthora grilly* (= *Entomophaga grilly*), was recognized as the disease agent of the Spanish locust *Dociostaurus maroccanus* (NAVARRO 1901). The next entomophthoralean fungus recorded was a misidentified species (REMAUDIERE and HENNEBERT, 1980), *Entomophthora aphidis* (SALA i PONS, 1924), thought to be responsible for an epizootic on *Aphis sp.* infesting *Chenopodium ambrosioides*. The author was dealing with a species of the genus *Erynia* which was insufficiently described to identify correctly. Finally, fifty years after the first recording, *Empusa grilly* (= *Entomophaga grilly*), was cited again on *D. maroccanus* (BENLLOCH, 1949).

In relation to Fungi Imperfecti, the only documented record refers to *Beauveria effusa* (= *B. bassiana*). This species was thought to be the causative disease agent on *Leptinotarsa decemlineata* (ALFARO, 1943).

In any case the fungi recorded were chance encounters than a well defined study. Not until the second half of this century, when the basic and applied research on Insect Pathology was stabilized, and forest and agricultural entomologist began to rely on microbial control, was a clear interest in entomopathogenic fungi developed.

This paper is a preliminary report on fungi encountered in Southern Spain.

FUNGI IMPERFECTI:

Most of the insects infected by the fungi of this group were infected by the genus *Beauveria*. The ubiquitous species *Beauveria bassiana* has been encountered in a great variety of insect species from different orders, Orthoptera, Hemiptera, Coleoptera and Lepidoptera (TABLE, 1). These records considerably broadened the *B. bassiana* host range e. g. the orthopters *Dociostaurus maroccanus, D. genei* and *Caliptamus italicus* ; the coleopters *Cleonus mendicus* and *Aubeonymus mariaefranciscae* and the lepidopter *Ocnogyna baetica* are hosts unrecorded elsewhere.

ENTOMOPHTHORALEAN FUNGI

The recorded species of the Entomophthorales coincides with the diverse insect species surveyed (TABLE, 2), because each insect order has a typical set of entomophthoralean fungus species. It is expected that as the survey progresses more fungi species and host combinations will be recognized as just shows a more detailed study in the Entomophthorales infecting aphids (BARREIRO and SANTIAGO-ALVAREZ, unpubl. data). The species *Entomophthora muscae* complex, *Erynia gammae* and *E. radicans* are cited for the first time in Spain.

On the genus *Entomophthora* it is interesting to note that *E. planchoniana* has been observed to infect the aphid *Israelaphis lambersi* (SANTIAGO-ALVAREZ, 1990). This was the first record of an entomophthoralean fungus on a species of the genus *Israelaphis*. *E. muscae* on *Musca domestica* and *Delia platura* shows differences in the number of nuclei in the conidia. Insufficient fungal material was examined for a final identification; the *E. muscae* on *M. domestica* shows 4-5 nuclei in the conidia is near to *Entomophthora schizophorae* (KELLER, 1987) or included in the *E. muscae* "forma 5" as defined by BEN-ZE'EV et al. (1988). On the contrary *E. muscae* on *D. platura* must be included in *E. muscae* "forma 8" defined by BEN-ZE'EV et al. (1988).

On the genus *Erynia* the most striking report is of *E. gammae* on *S. littoralis*. This species regularly produces an epizootic at the end of the summer which continues through autumn. The resting spores and the conidial stage of the fungus appear separately in different host larvae. This host-pathogen combination is unrecorded elsewhere.

The seasonal occurrence of the entomopathogenic fungi in this warm and dry part of Spain as well as the epizootiology of *B. bassiana* on *C. mendicus* and *A. mariaefrenciscae*; *E. gammae* on *S. littoralis* are now under research in our laboratory.

TABLE 1. IMPERFECTI FUNGAL SPECIES, HOST-PATHOGENS COMBINATIONS AND SEASONAL OCCURRENCES.

HOST INSECTS	FUNGUS GENUS			Place	Date	Ref.
	Beauveria	Verticilium	Aspergillus			
O. ORTHOPTERA						
Fam. Acrididae						
Dociostaurus maroccanus	B. bassiana *				May-July 1990	This paper
D. genei	B. bassiana *				"	"
Caliptamus italicus	B. bassiana *				"	"
Malacolus sp.			Aspergillus sp.	Córdoba	22/ 9/83	12
O. HEMIPTERA						
Fam. Pentatomidae						
Aelia sp.	B. bassiana			Sierra de Grazalema (Cádiz)	23/2/78	This paper
Fam. Miridae						
Lygus sp.	B. bassiana			Burgos	Sep.1982	12
				Burgos	14/11/84	12
Fam. Coccidae						
Saissetia oleae		V. lecanii		Valencia	1975	11
O. COLEOPTERA						
Fam. Carabidae						
Unknown	B. bassiana			Córdoba	5/10/90	This paper
Fam. Curculionidae						
Cleonus mendicus	B. bassiana *			Lebrija (Sevilla)	19/ 6/78	This paper
Rhodocnymus mariaefranciscae	B. bassiana *			Ecija (Sevilla)	3/12/82	This paper
					1984	"
				Espejo (Córdoba)	7/ 6/89	"
O. LEPIDOPTERA						
Fam. Noctuidae						
Heliothis armigera	B. bassiana			Córdoba	4/ 8/83	12
				Sevilla		
Heliothis virescens	B. bassiana				14/11/84	12
Fam. Arctiidae						
Ocnogyna baetica	B. bassiana *			Córdoba	25/ 1/91	This paper
Fam. Lymantriidae						
Lymantria dispar	Beauveria sp.		Aspergillus sp.		1959-1965	8
Fam. Tortricidae						
Lobesia botrana			Aspergillus sp.	Valencia	3/ 5/79	This paper

* New host-pathogen combination.

TABLE 2. ENTOMOPHTHORALEAN FUNGUS SPECIES, HOST-PATHOGEN COMBINATIONS AND SEASONAL OCCURRENCES.

HOST INSECTS	FUNGUS GENUS Entomophtora	Erynia	Tarichium	Place	Date	Ref.
O. HEMIPTERA						
Fam Aphididae						
Israelaphis lambersi	E. planchoniana + *			Córdoba	April 1988	10
Myzus sp.		E. neoaphidis		Sta. Amelia (Badajoz)	1984	12
Myzus persicae		E. neoaphidis		Ciudad Real	1984	12
				Córdoba	15/10/84	This paper
Acyrtosiphum pisum		E. neoaphidis		Badajoz	1984	12
				Córdoba	14/ 4/78	This paper
					19/ 4/79	"
					20/ 6/79	"
					11/ 4/80	"
Therioaphis trifolii		Erynia sp.		Córdoba	29/ 9/86	This paper
					1/10/86	"
O. LEPIDOPTERA						
Fam. Noctuidae						
Spodoptera littoralis		E. gammae + *		San Ignacio del Villar (Sevilla)	15/ 9/78	This paper
				Córdoba	15/10/82	This paper
					5/11/82	"
					5/ 9/87	"
					26/10/90	"
					9/11/90	"
Agrotis segetum			T. megasperma	Badajoz		4
O. DIPTERA						
Unknown		Erynia sp.				
Fam. Muscidae						
Musca domestica	E. muscae +			Badajoz	Oct. 1988	This paper
Fam. Anthomyiidae						
Delia platura	E. muscae +			Badajoz	Oct. 1988	This paper
O. HYMENOPTERA						
Fam. Ichneumonidae						
Unknown		E. radicans +		Córdoba	13/ 9/87	This paper

+ New species for Spain; * New host-pathogen combination

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ECOLOGY OF NOSEMA MELIGETHI (MICROSPORIDA) AS A PARASITE OF MELIGETHES AENEUS (COL., NITIDULIDAE)

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Summary
221 samples of Meligethes aeneus F. were collected from southern and central Finland in 1989-90, and 10 152 beetles individually examined to determine the occurrence of Nosema meligethi I. & R. The disease possibly occurs everywhere in Finland together with the host beetles, but at levels of any practical importance only among Meligethes populations subsisting on noncultivated hosts. This suggests that N. meligethi may not be compatible with the current use of pesticides on oilseed rape. The level of infection steadily increases during the season, and horizontal transmission appears to take place in flowerheads where the beetles aggregate to feed. Despite serious effort, no infection in the larvae was detected. In the autumn infected beetles migrate to overwintering sites earlier than healthy beetles, and infected beetles weigh less than healthy ones. This may drastically affect the overwintering survival of infected beetles.

1. Introduction

Meligethes aeneus F., the rape blossom beetle, ranks as the number one pest of oilseed crucifers in Europe, occasionally supplemented by several other, locally important insect pests such as the weevils Ceutorrhynchus spp., the flea beetles Psylliodes chrysocephala and Phyllotreta spp, or the gall midge Dasyneura brassicae (Bromand 1990). Consequently, any natural antagonists of this pest are of great interest as potentially valuable biological control agents. Recently Nosema meligethi, a microsporidian disease of Meligethes, has been discovered (Issi & Raditscheva 1979), and some information about its distribution, life-cycle, and host range have been gathered since (Hokkanen & Lipa, in press a, b; Lipa & Hokkanen, in press a, b).

Despite extensive surveys in different parts of Europe, N. meligethi has only been recorded from samples collected in eastern Europe and Finland; one infected beetle has also been recorded from Denmark and one from Austria (Lipa & Hokkanen, in press a).

The aim of this paper is to discuss some ecological features of N. meligethi and in particular sevaral puzzling questions about the parasite.

2. Material and methods

Samples of adult M. aeneus were collected from a total of 145 different locations in southern Finland. 50 samples were taken in the autumn of 1989 (new generation of beetles), 71 samples in the spring-summer of 1990 (overwintered, old generation beetles), and 100 samples in the autumn of 1990 (new generation beetles). As from each sample approximately 30-60 beetles were examined, the total

number of individually examined adult beetles was 10 152. Additionally 40 samples of M. aeneus larvae were collected mainly from the rape growing area, but also from all the locations where the disease had previously been detected. A series of samples throughout the season was collected at seven localities, where the disease was known to occur.

In the autumn the beetles aggregate to feed on pollen in the flowers of Sonchus arvensis. This may facilitate an efficient horizontal transmission of the disease as the beetles, often 20-50 of them packed in one flowerhead, crawl around and feed. One infected beetle could possibly contaminate the whole flowerhead and thus infect the other beetles. A total of 76 individual Sonchus flowerheads were collected, and all the beetles in each flower were inspected for the presence of infection. Altogether 1086 beetles were examined, and the distribution of the diseased individuals among the flowerheads was determined and tested for randomness vs. contagiousness.

Beetles were also collected at overwintering sites from the flowers of Leontodon autumnalis and Hieracium spp. These beetles were not yet overwintering, but had decided to leave the fields and to enter the overwintering area. Samples were collected at 5-7 day intervals between August 31st and September 22nd. At the same time a sample of beetles was collected from the breeding and feeding site in the field.

To assess the effect of Nosema infection on the weight of adult beetles entering the winter diapause, 243 air dried beetles were individually weighed with the accuracy of 10^{-5} g (Mettler AE 163), and examined for the presence of infection.

3. Results and discussion

Nosema meligethi has now been found at 25 locations in Finland, eight of which are within the main growing area of oilseed rape, and 17 outside of it (Figure 1). Specifically there were 91 distinct sampling sites where rape was cultivated, i.e. the beetles apparently had been subjected to the normal agronomic practices related to rape growing. N. meligethi was found in 14 (15.4 %) of these samples. In contrast, 41 sampling sites were areas where no rape was grown in the vicinity, i.e. the beetles obviously subsist on cruciferous weeds among the cereals, which seldom have to be treated with insecticides. The disease was found in 23 (56.1 %) of these Meligethes samples. This difference is highly significant (X^2 = 23.22, $p < 0.001$), and proves that N. meligethi is poorly compatible with the agronomic practices related to rape growing. When the disease did occur, however, there was no significant difference in the level of infection per sample: 8.3 % for rape growing areas, and 8.6 % for rape non-growing areas.

N. meligethi apparently occurs everywhere in Finland, but has yet to be detected in most of the the main rape growing area (Fig. 1). No doubt an intensive sampling effort would reveal the presence, but the fact remains that currently in most of this area N. meligethi does not occur at levels which could be of practical importance.

The infection level normally remained rather low, being 5.0 % for all the samples. Locally, however, the infection levels often reached 20-25% in the non-rape-growing areas. In large areas in central Finland the overall infection level exceeded 10 %. For an

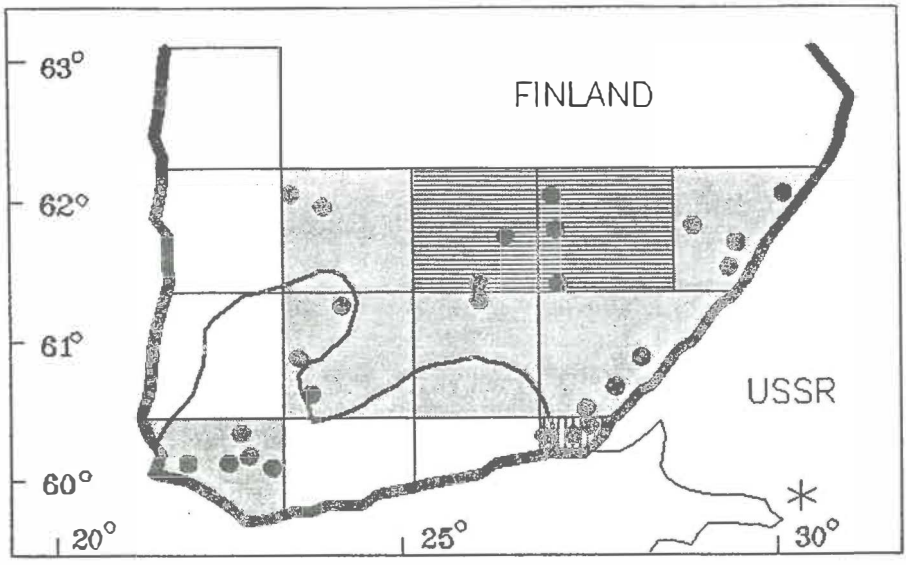

Fig. 1. Locations in Finland where Nosema meligethi has been found (black dots), and the mean infection percent of Meligethes aeneus (shading). Southwest of the curve is the main oilseed rape growing area (over 5% of field area on rape).

effective, permanent suppression of the host population this is too low - for pathogens such as Nosema the ideal infection level may be about 30-50 % (see Andersson 1982). Artificial augmentation might be a solution, provided that suitable methods are found and that the compatibility with pesticide treatments is achieved.

During the season the level of infection roughly doubles from what it was in the spring (Fig. 2). The increase is rather steady and clear also among the old generation beetles (first two sampling dates of 1990), despite the likelihood of differential mortality among healthy vs. infected beetles towards the end of their lives. This would indicate efficient horizontal transmission of the disease at that time. The same phenomenon seems to occur also after the emergence of the new generation adults (16.8-13.9, Fig. 2); by that time all old generation beetles have died.

Overwintering mortality in M. aeneus due to the presence of N. meligethi could not be directly assessed on our data. The combined data from all sampling sites does not reveal any differences in infection level between autumn 1989 and spring 1990 (Fig. 2). At the most intensively sampled areas, however, there was about 50-100% decrease in infection level over the winter. This suggests that winter mortality of M. aeneus is greatly increased by the presence of Nosema infection also in Finland, as reported by Issi & Raditscheva (1979) for Leningrad, and as known for many other insects in similar situations (see Brooks 1988).

Nosema meligethi was not detected in the larvae of M. aeneus, despite intensive efforts. Over 3000 larvae were inspected, most from sampling sites where at the same time the infection level of adult beetles was 10-20 %. At present we have no explanation for this result. It is particularly puzzling, because right after the emergence of new generation adults, infection levels are "normal". Somehow the disease must be present in the larvae, but it will not replicate before pupation is over. As such behaviour is not common for protozooan diseases, this has to be studied separately in greater detail (cf. Weiser 1981, Brooks 1988).

In the autumn horizontal transmission between the adult beetles seemed to take place: the distribution of the disease in Sonchus flowerheads was contagious rather than normal. There were more flowerheads than expected, where infection was not present at all, or at a very low level, and again more flowerheads than expected with high infection levels (Fig. 3). On the other hand, flowerheads where the infection level was approximately the average, were less numerous than expected. The distribution differs significantly from the normal (Fig. 3: chi-square = 19.93, df = 7). The contagiousness parameter of Taylor's power law (Taylor 1961, 1965) is 1.69. The index of dispersion (see Southwood, 1978, p. 39) differs greatly from that produced by Poisson randomness (I_D = 409.2), indicating significant aggregation.

It is possible that the horizontal transmission is somewhat more effective than indicated by this comparison, because newly infected beetles are likely to escape detection in the microscopic assessment. In fact, most of the examined beetles were only lightly infected, indicating that the infection was rather recent.

In the autumn infected beetles fly to overwintering sites clearly earlier than healthy beetles (Fig. 4). This behaviour may be a direct response to the infection: it is probably better to enter the winter diapause before the newly acquired disease has a chance to replicate excessively, thus further lessening the chances of survival during the winter. Healthy beetles seem to delay migartion until the maximum amount of fat reserves for the winter have been accumulated. Beetles under the present practice of growing only spring oilseed crucifers in Finland appear to have difficulties in properly preparing for the winter (cf. Hokkanen, in press).

Even a light, but detectable, infection in the autumn significantly reduce the weight of the beetles ($t = 2.62$, $p = 0.013$, $df = 34$), which is crucial for winter survival (Hokkanen, in press). The reduction in weight is about 13 %, which depending on the general nutritional status of the beetles may have a great effect on winter mortality.

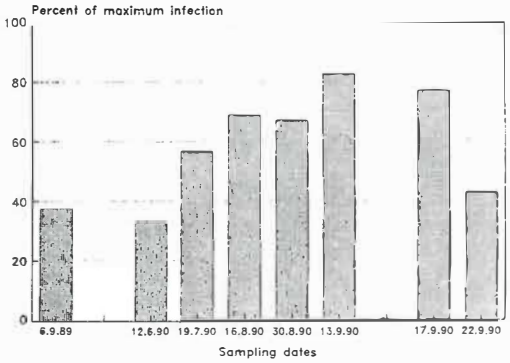

Fig. 2. Dynamics of Nosema meligethi infection over the season at seven locations, shown as the mean percent of the maximum infection at each site.

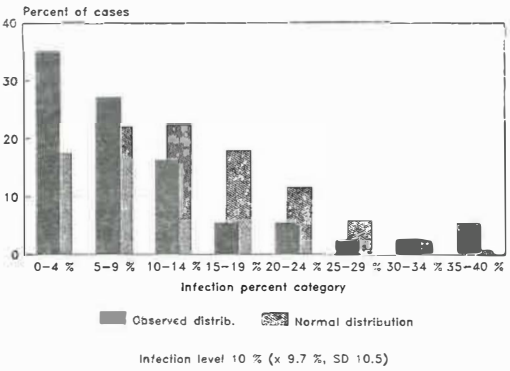

Fig. 3. Distribution of the infection level in the subpopulations of M. aeneus located on one flowerhead of Sonchus arvensis, September 1990. "Cases" refers to Sonchus flowerheads with a M. aeneus subpopulation at the time of sampling.

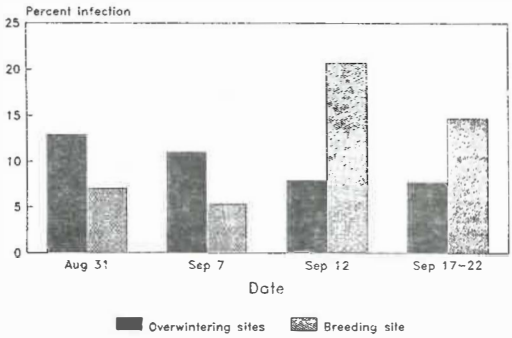

Fig. 4. Comparison of the overall infection levels of M. aeneus subpopulations at a given date at their breeding site (fields), and at the overwintering sites (three sites in the woods).

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ECOLOGY OF PATHOGENS ON CUTWORMS (AGROTIS SEGETUM)

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Abstract

In Denmark a total of 20.000 cutworms (larvae of turnip moth, Agrotis segetum) were sampled from 1984 to 1990. Generally, about 5-10 % of the larvae were parasitised and about 10 % were infected by insect pathogens (12 species have been recorded). In many cases larval samples were taken up to 10 times on the same fields in july-october. An analysis by simple epizootiological parameters indicates that pathogens reduce cutworm populations with 25 to 50%. An Entomophthoralean fungus (Erynia virescens), Baculoviruses and a microsporidium (Plistophora sp) contribute mainly to this reduction

1. Introduction

The turnip moth Agrotis segetum Schiff. (Lep. Noctuidae) is distributed in Europe Asia and Africa. The present investigation was made in order to obtain detailed information on the presence of diseases in danish cutworm populations and the impact on the host population during the period of larval activity (June-October).

2. Materials and methods

The cutworm populations were studied by sampling larvae in selected areas. In some years (1984-1986, 1990) the cutworms were sampled in relation to field trials with AsGV (Agrotis segetum Granulosis virus) and in some years (1987-1990) the cutworms were sampled in public areas containing 100-200 small garden plots of about 200 m^2. Sample sizes close to 100 cutworms were taken if possible. This gives more representative samples and besides, smaller statistical variation on the prevalence values obtained. After sampling the cutworms were size-categorised and reared individually in small cups (30 ml) in the laboratory at room temperature until pupation or death.

3. Results

During the seven-year period from 1984 to 1990 a total of 20.000 cutworms were sampled. The parameters investigated and the data recorded in relation to sampling were not the same for all years. Therefore, some factors can not be analysed for all seven years. In field trials with AsGV the natural prevalence of viruses can not be evaluated. In such field trials the prevalences of other pathogens are not affected besides the population-reducing effect of the virus. In plots with low effect of viruses the prevalence of other pathogens have been recorded. The overall sum of sampled cutworms and the level of diseases is shown in table 1. The pathogens involved are listed in table 2.

TABLE 1. The total numbers of cutworms sampled and the total numbers died from diseases.

YEAR	NUMBER sampled	ERYNIA nos	%	VIRUSES nos	%	MICROSP nos	%	MISC. nos	%	TOTAL nos	%
1984	1890	56	3.0	n.r.		n.i.		n.i.		n.i.	
1985	1063	55	5.2	n.r.		n.i.		n.i.		n.i.	
1986	3341	144	4.3	9	0.5	n.i.		n.i.		n.i.	
1987	76	4	5.3	0	0	n.i.		n.i.		n.i.	
1988	2112	42	2.0	n.f.		n.i.		n.i.		n.i.	
1989	6325	135	2.1	92	1.5	40	0.6	148	2.3	415	6.5
1990	5193	103	2.0	195	3.7	113	2.0	88	1.7	499	9.6
SUM	20.000	539	2.7	-		-		-		-	

n.r = not recordable because of field trials with virus.
n.a = not investigated.
n.f = not finished, not all deceased larvae are diagnosed.

TABLE 2. Cause of death for 914 diseased cutworms in 1989 and 1990.

microorganism	diseased cutworms nos.	percentage	
Erynia virescens*	238	26.0	(* syn.: Tarichium
Granulosis viruses	281	30.7	(Entomophthora)
Nuclear polyhedrosis	6	0.7	megaspermum)
Plistophora sp**	153	16.7	(** Microsporidia)
Miscellaneous total:	236	25.9	
Miscell. includes:			
Hyphomycetes***	21	2.3	(***P.fumosoroseus;
Mixed infections	20	2.3	P.farinosus; B.bas-
Yeast	38	4.2	siana; M.anisopli-
Nematodes****	12	1.3	ae)(****N.bibionis;
unidentified	145	15.8	(Mermis sp.

These data gives very little information besides showing the amount of cutworms sampled and the number dying from various diseases. To obtain some idea on the mortality during a prolonged period data from successive sampling on the same field are shown in figure 1 and 2. These figures focuses on data
from 1989 and 1990 only. The level of disease are compared for fields treated by three types of agricultural practices: private gardening, ecological farming and traditional agriculture.

No obvious epizootic trends couid be recorded during these two year for the total pathogen levels. The data for each pathogen did not show epizootics either. Only for short periods did the level of disease exceed 10-15%.

In figure 3 the mortalities are shown for the different size-categories of cutworms. Obviously, the disease level are much higher among smaller cutworms than among larger ones.
The density of cutworms varied between the years and the different fields, globally as well as locally. On most of the localities the cutworm density did not vary greatly in 1989 and 1990. It was usually close to 5 specimens/m².

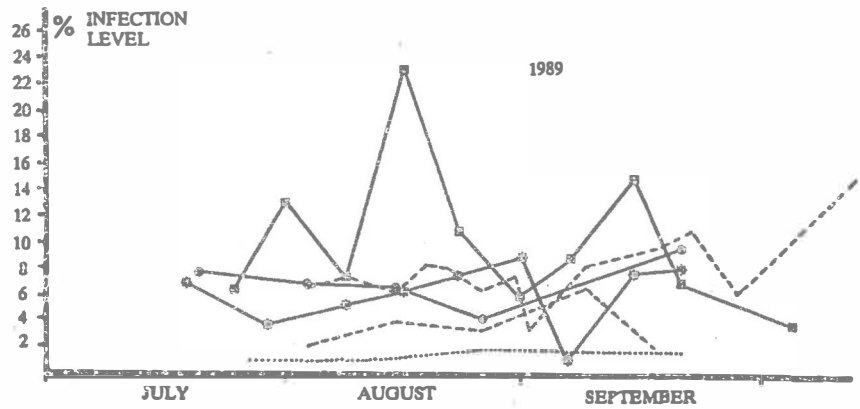

FIGURE 1. Total infection level of pathogens in cutworms from gardens (solid lines), chemically treated fields (broken lines) and ecological farming fields (dotted lines). 1989

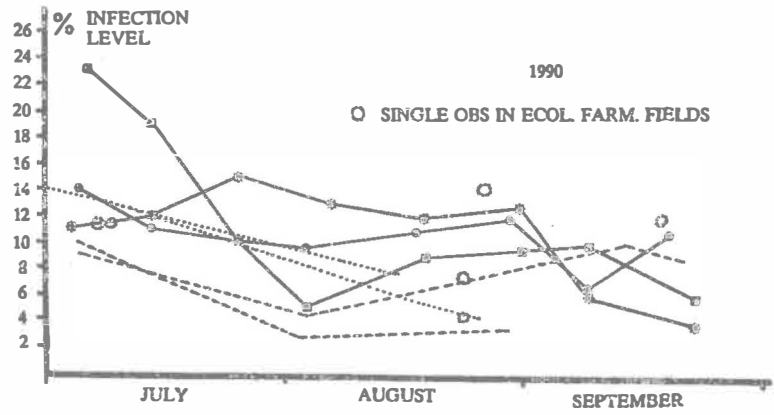

FIGURE 2. Total infection level of pathogens in cutworms from gardens (solid lines), chemically treated fields (broken lines) and ecological farming fields (dotted lines). 1990

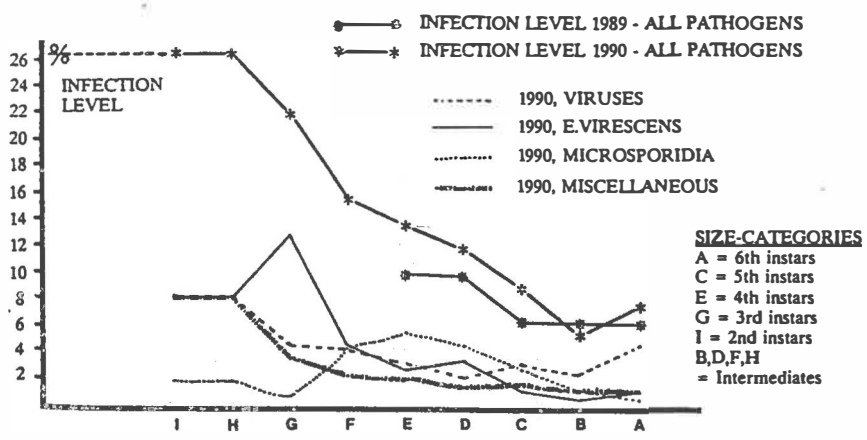

FIGURE 3. Infection levels related to cutworm size.

4. DISCUSSION

It is difficult to give precise information on the general mortality from diseases during all the season. However, some information can be obtained by a relatively simple epizotiological analysis of the prevalence data.
One way of analysing the data is demonstrated using simplified data on the basis of the observed data. The development of diseases must be considered for a total period of larval activity, appr. 120 days from middle of June to middle of October.

The prevalences (P) for this period may be estimated from the observed data in figure 2 and 3.

The lethal time (LT) must be estimated for each pathogen in order to calculate the daily mortality (DM). DM for each pathogen can be summarised to show the total daily mortality from diseases (TDMD).

DM is calculated from the formula: DM = P/LT

In these calculations a daily mortality from other, non-pathogenic causes (NPC) must be considered. The higher mortality from NPC the lower total impact of pathogens. Two examples demonstrates this relationship.

6. Epizootiology of insect pathogens.

THE EPIZOOTIOLOGICAL IMPACT OF A PERSISTENT PATHOGEN, THE NUCLEAR POLYHEDROSIS VIRUS OF *GILPINIA HERCYNIAE* (HYMENOPTERA: DIPRIONIDAE).

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Summary

A positive relationship was found between the size of the polyhedral inclusion body (PIB) population of the nuclear polyhedrosis virus of *Gilpinia hercyniae* (GhNPV) in host generation 'n' and the levels of GhNPV surviving on spruce tree (*Picea* spp) foliage immediately before generation 'n + 1'. The latter quantity governed the speed of onset of infection in *G.hercyniae* generation 'n + 1. Normalising the sigmoid growth of infection curve provided the parameter 'b' (of the straight line equation, y = a + bx) as a measure of the rate of infection growth in larval populations (n + 1). Infection growth rate (b) was found to be greatest in generation 'n + 1' when PIB populations were greatest in 'n'. Conversely, the relationship could apparently lead to infection extinction before host population extinction. This is discussed. A similar relationship was detected for another sawfly, *Neodiprion sertifer*, and its NPV. The different NPV replication strategies in sawflies and in Lepidoptera appears to account for the smooth growth of infection in the former and its biomodal nature in the latter.

1. Introduction

Gilpinia hercyniae is a diprionid sawfly of mainland Europe accidentally introduced into both North America and the British Isles in the 20th Century. In both these areas very severe *G.hercyniae* outbreaks have apparently been largely controlled by natural epizootics of GhNPV. The present report arises out of a study of a GhNPV epizootic in Central Wales, UK. *Neodiprion sertifer* (also a diprionid) outbreaks are of regular occurance on *Pinus* spp throughout Europe and beyond and are usually eventually attended by decimating epizootics of an NPV disease. Data reported here for *N.sertifer* are from Kaupp (1981).

2. Materials and Methods

Three, one hectar, *G.hercyniae* study plots were demarcated (in Hafren forest, Wales), *Picea sitchensis,* Sitka spruce (2 plots) and *Picia abies,* Norway spruce. Each plot was subdivided into 50 smaller plots each of 225 trees. Egg numbers were sampled (June/July) and larval populations were measured weekly (July/October) each by carefully severing branches from several canopy strata and placing in plastic sacs prior to examination. NPV was diagnosed in larvae by optical microscopy. Knowledge of the PIB production in each larval instar and the numbers of each instar infected in each sample permitted construction of PIB population growth curves for each *G.hercyniae* generation. From these, total PIB production per generation was calculated. In addition, in each year immediately before the anticipated time of adult emergence (about June), 200 small sprigs of foliage were collected from each plot and bioassayed for NPV presence employing first instar larvae from a laboratory culture. Similar methods were followed for *N.sertifer* in study plots in England, Wales and Scotland.

3. Results

The overwintering of GhNPV on foliage

Foliar sampling provided a biological index to the amount of GhNPV surviving the winter/spring. The foliar samples were taken from the lower canopy of trees $\leq$ 10 metres tall. *G.hercyniae* larvae feed and die of GhNPV infection, in the upper canopy but studies showed that during the winter GhNPV PIB populations

are mobile and come to be fairly evenly distributed over the whole tree. These studies consisted of :
(i) bioassay of foliar samples from a series of canopy height strata
(ii) *in situ* sodium hypochlorite surface sterilisation of some lower branches
(iii) replacement of some lower branches with others from non-*G.hercyniae* infested areas. (Foliar turgidity was maintained by wrapping wet cotton wool around the cut branch end and sealing this in a plastic bag).

In 'ii' and 'iii', samples of 'treated' and untreated foliage were taken for bioassay at intervals of two months between December and April. The results revealed the movement of NPV onto the sterilised and the introduced branches which is at least indicative of intra-tree movement of inoculum (Evans & Entwistle, 1981). Studies on bird dispersal of GhNPV during the larval period (Entwistle *et al.*, 1977a) and more particularly its continuance during the non-larval period of November to May (Entwistle *et al.*, 1977b) indicated the likelihood of inter-tree distribution of virus. Such active movement of inoculum tending to a more even distribution over the canopy appeared to justify the use of lower foliar samples as a realistic indicator of PIB and host population interactions.

Relationships of PIB populations, persistence and infection onset: There was a curvilinear relationship between the proportion of foliar samples infective just prior to *G.hercyniae* generation 'n + 1' and the PIB population produced in generation 'n'. More GhNPV appears to have been retained over winter on *P.abies* than on *P.sitchensis* probably due to the denser foliation of the former species.

A relationship was sought between the proportion of foliar samples infective and the course of development of infection in host generation 'n + 1'. The parameter selected to represent this was the time (days) between maximum larval population density and the development of 50 per cent of the eventual total level of infection expressed in 'n + 1'. It was felt this best represented a primary, and therefore causal, response to residual foliar inoculum with least confusion from the development of secondary infections. This parameter was therefore designated 'primary infection growth' (PRG). PRG decreased very rapidly from 60-20 days as infective foliar samples increased from about 2 to 10 percent, but thereafter was fairly independent of increasing foliar inoculum levels.

Relationship between the rate of infection growth, PIB populations and host populations: The proportion of infected larvae against time in any one *G.hercyniae* generation is invariably describable by a smooth sigmoid curve easily normalised by $\log_{10} \frac{x}{1-x}$, where x is the proportion of larvae infected.
The gradient 'b' of this line is an indication of the rate of infection growth. A relationship was detected between 'b', the size of the PIB population produced in generation 'n' and the density of the host larval population in 'n + 1'. Field data for *N.sertifer* were also inspected in this way. In both sawfly species, as might intuitively be anticipated, infection grew fastest with high populations of host and of residual foliar NPV and slowest where these were both low.

4. Discussion
The overall implicit relationship underlying these two approaches is that the PIB population of generation 'n' influences both the speed of onset of infection growth (PRG) and the rate of growth of that infection: however, earlier and faster infections lead to lower host populations in a following generation tending, eventually, to very low sawfly populations and apparent extinction of infection. The latter must be qualified for *G.hercyniae* because of the possiblity that an adult mediated vertical transmission pathway could lead to the persistence of larval infections at very low host population densities (Neilson & Elgee, 1968).

Finally, it is interesting to compare the pattern of NPV infection growth in diprionid sawflies with that in Lepidoptera. In sawflies infection growth is smoothly sigmoid whilst in Lepidoptera, eg *Panolis flammea* (Doyle & Entwistle, 1988), *Lymantria dispar* (Woods & Elkinton, 1987) & *Euproctis chrysorrhoea* (Kelly *et al*., 1988), it is biomodal. This difference almost certainly reflects the replication strategies of NPV's in these two taxa. In sawflies NPV's are mid-gut diseases and infective virus is excreted before death leading to a smooth progression of secondary infections. In Lepidoptera the level of NPV replication in mid-gut seems generally too low to lead to release of inoculum from this source. Virus from the early phase of population infection is released as a 'pulse' following death and disintegration of larvae and there is then a second infection response in which this inoculum is probably strongly involved.

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MODELLING THE EPIZOOTIOLOGY OF PATHOGEN-PEST-NON-TARGET HOST INTERACTIONS

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Summary
A model has been investigated of the dynamics of the interaction between two hosts which are both attacked by a common pathogen, where the pathogen has free-living infective stages. A wide range of dynamics is generated, including a number of patterns quite unlike those found in the one-host pathogen case, and also behaviour contingent on initial densities in the system. Attention is focused on the case where one host is a pest, the pathogen is a potential microbial control agent, and the other host is a non-target host which it is undesirable to harm. The model suggests, broadly, that non-targets are unlikely to be seriously threatened in such cases, and also that non-targets, far from undermining pest control, are quite likely to contribute to its efficacy.

1. Introduction

Historically, models in population ecology have focused on single species or interactions between two species. Recently, however, there has been a growing literature on the dynamics generated by multi-, usually three-species interactions (see references in Bowers & Begon, 1991). Here, a model is examined of an interaction between two hosts and a common pathogen, where the hosts do not interact with one another except by sharing the pathogen, where the pathogen has free-living infective stages the population dynamics of which must be modelled explicitly, and where the hosts have characteristics (lack of acquired immunity, for instance) which make it convenient to think of them as invertebrates.

The model is of interest as an investigation of a particular three-species system (see Bowers & Begon, 1991, and also Begon et al., 1991). But it is also of fairly direct practical importance in its relation to microbial pest control. One understandable fear in this context is that a pathogen released to control a pest (i.e. a target host) might have an adverse effect on some other, non-target species to which it is less pathogenic, and that this adverse effect may be accentuated by, or may even be entirely dependent on, the primary interaction between the pathogen and the pest. Conversely, the existence of non-target species with which the pathogen can interact might ameliorate the pathogen's effect on the pest and perhaps render it ineffective as a control agent.

2. The host-host-pathogen model

The model is as follows:

$$\frac{dH_1}{dt} = r_1H_1 - \alpha_1Y_1, \tag{1}$$

$$\frac{dY_1}{dt} = \nu_1W(H_1 - Y_1) - \Gamma_1Y_1, \tag{2}$$

$$\frac{dH_2}{dt} = r_2H_2 - \alpha_2Y_2, \tag{3}$$

$$\frac{dY_2}{dt} = \nu_2W(H_2 - Y_2) - \Gamma_2Y_2, \tag{4}$$

$$\frac{dW}{dt} = \lambda_1Y_1 + \lambda_2Y_2 - (\mu + \nu_1H_1 + \nu_2H_2)W. \tag{5}$$

Here H is the size of a host population of which Y individuals are infected by a pathogen , which itself has free-living infective stages, population size W. In addition, r is a host's intrinsic rate of increase, α is the pathogenicity of the pathogen, ν is the transmission efficiency of the pathogen between the free-living infective stages and a host, Γ is the overall rate of loss of infected hosts (natural and pathogen-induced mortality plus recovery), λ is the rate at which free-living infective stages are produced by a host, μ is the natural death rate of free-living pathogens, and the subscripts refer to the two hosts.

3. The Anderson & May model

The present model is derived from Anderson & May's (1981) model G (appropriate reduced forms of equations (1), (2) and (5)). In order to appreciate the behaviour of the present model, it is necessary to understand the simpler model first. There are three, mutually-exclusive broad classes of possible outcome in terms of dynamical behaviour, as follows.

(i) 'Pathogen extinction', where the pathogen is unable to maintain itself in the host population, which grows exponentially (since is not subject to limitation, other than by the pathogen). This occurs when

$$\lambda < \Gamma + r. \tag{6}$$

(ii) 'Host regulation', where the host is regulated by the pathogen to either a stable point equilibrium or a stable limit cycle (suitably high values of α and λ). Here point and cycle regulation are not distinguished from each other. Thus, regulation in this broad sense occurs when

$$\lambda > \frac{\alpha\Gamma}{(\alpha - r)} > 0. \tag{7}$$

(iii) 'Pathogen persistence', which occurs at parameter values intermediate between those for extinction and regulation, and means that H continues to increase exponentially, but at a reduced rate, ρ, also exhibited by Y, where

$$\rho = r - \alpha y_p \qquad (8)$$

where y_p is the prevalence of the disease.

4. Behaviour of the host-host-pathogen model

Various types of combinations of these three species may be distinguished from one another on the basis of the dynamics that would be displayed by each host if it interacted with the pathogen alone. All possible combinations have been examined (Bowers & Begon, 1991), i.e. regulation-regulation (both hosts are regulated by the pathogen when they interact with it alone), regulation-persistence, regulation-extinction, persistence-persistence, persistence-extinction and extinction-extinction. For convenience, a host species that would be regulated if it interacted with the pathogen alone may be referred to as an R-species and the subscript 'reg' applied to its parameters, referring similarly to P-species (subscript 'pers') and E-species (subscript 'ext').

The behaviour of this model, in its totality, is complex. A wide range of dynamics is generated, including a number of patterns quite unlike those found in the one-host pathogen case, e.g. persistence in one host, elimination of the other host; symmetric (both hosts increase at the same rate) and asymmetric persistence of pathogen in both hosts; and also behaviour contingent on initial densities in the system. The particular dynamics exhibited depends on the particular combination of parameters. Here, therefore, attention is focused only on those regions of parameter-space which are of most relevance to microbial pest control.

5. Application to microbial pest control

To begin with the case in which both pest and non-target are R-species, it is found in general that with two R-species, one always eliminates the other, the 'winner' being the species which has the higher W^* (equilibrium abundance). A high W^* is particularly likely to be associated with a relatively low α, which is likely to apply to the non-target species rather than the pest. Encouragingly, therefore, a pest that would otherwise have been regulated will now be eliminated (controlled more effectively), with the non-target species acting as a reservoir for the pathogen, available to eliminate further pest outbreaks. The non-target species itself would be unaffected in comparison with the absence of the pest (but the presence of the pathogen), and only slightly affected compared to the absence of the pathogen (i.e. not seriously threatened by the biological control agent).

With one R- and one P-species, it seems likely that the pest is the host that would be regulated. $W^*_{p\text{-}pers}$ (equilibrium abundance for the P-species) will typically exceed W_{reg}, leading to the elimination of the R-species. Thus, the results are again encouraging: the pest would not merely be regulated but eliminated, and the non-target would experience persistence as before.

With an R- and an E-species, it is not so straightforward to decide which is more likely to be the pest. One might argue that a pathogen being considered for biological control would be characterised by its ability to regulate the pest, But one might also argue that it would be characterised by very high pathogenicity - especially if control is to be achieved by periodic inundation (a microbial pesticide). Pathogen extinction is often a consequence of the pathogen being highly (in fact 'too highly') pathogenic.

If the pest is the R-species, the likely outcome is somewhat discouraging: 'symmetric persistence', in which both hosts increase at a common rate, ρ, which is less than either r because the pathogen persists.

Rather more encouragingly, however, the common ρ is likely to be only slightly below r for the non-target but far below r for the pest.

If, however, the pest is the E-species (and the non-target the R-species), the likely outcome is far more encouraging: pest eliminated, non-target regulated as when alone. Thus, the non-target host and the pathogen combine in the elimination of the pest: the efficacy of a pathogen can positively depend on the presence of non-target species, which themselves are only negligibly affected by the pathogen.

For persistence-extinction, it seems clear from the foregoing argument that the E-species is most likely to be the pest. Here, the model suggests behaviour contingent on initial densities: either persistence-elimination, or pathogen-extinction with unrestricted growth of both hosts, or symmetric persistence. Thus, if pest numbers are high, as in an outbreak, then the pathogen, even in combination with the non-target, is likely to be ineffective. But if the pest can once be brought under control, presumably by some other means, then the pathogen and non-target host, together, should be effective at eliminating the pest and preventing further outbreaks.

The remaining cases have little realistic application in this context.

Overall, therefore, the results presented here paint an encouraging picture of the likely outcome when a pathogen is released to control an invertebrate pest in the presence of other, non-target species which it is undesirable to harm. They are encouraging, too, in suggesting that data from the component two-species interactions can, if used correctly, be crucial in planning such releases. This does not mean, of course, that the results provide grounds for complacency. Caution remains essential in the planning of any release. Nonetheless it is possible to suggest that such planning can proceed more rationally and with more confidence within a framework of understanding of the underlying dynamical interactions, which has not been available hitherto, but which the work described in this communication has sought to help construct.

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A COMPREHENSIVE SIMULATION MODEL OF THE EPIDEMIOLOGY OF *Spodoptera exigua* NUCLEAR POLYHEDROSIS VIRUS ON BEET ARMYWORM IN GLASSHOUSE CHRYSANTHEMUMS

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SUMMARY

We describe a comprehensive simulation model of the epidemiology of *Spodoptera exigua* multiply enveloped polyhedrosis virus (SeMNPV), a pathogen of beet armyworm, *S. exigua*. The model applies to a population of beet armyworm which feeds on a glasshouse crop of chrysanthemums which is sprayed with SeMNPV. The model calculates the likely evolution of the insect-virus-host system by integrating available knowledge of the biology of *S. exigua*, plant growth, spray deposition, virus infectivity and the pathways of virus transmission. The model is used to evaluate various application scenarios of SeMNPV.

INTRODUCTION

Spodoptera exigua is a lepidopterous species of the family Noctuidae. It occurs worldwide in many species of field crops in tropical and subtropical regions and is also found in glasshouse crops in temperate regions like the Netherlands. *S. exigua* has a pest status in glasshouse ornamentals like Gerbera and Chrysanthemum, where feeding injury to the flowers causes large economic losses (Smits, 1987; Smits et al., 1987a). Resistance to insecticides makes chemical control difficult. Therefore, the possibility of control with natural antagonists has been studied. A particularly promising group of potential biological control agents are the nuclear polyhedrosis viruses (NPVs).

NPVs are strictly limited in host range to insects and crustaceans. The infectious unit is the polyhedron which consists of a protein matrix with many virions, the actual virus particles, enclosed in it. Polyhedra are liberated from the dead bodies of infected caterpillars. They contaminate leaves and are taken up orally by other caterpillars. In the gut of the caterpillars, the

virions are liberated from the protein matrix, after which they infect the gut cells. The whole body can become infected, and when the caterpillar dies after a few days, up to 30% of the dry weight of the body may consist of polyhedra. Virions are sensitive to inactivation by UV-radiation (Jacques, 1975), but the protein matrix of the polyhedron and the remnants of the dead caterpillar provide some protection (Young & Yearian, 1989).

The life cycle of *S. exigua* is well known, especially from studies on artificial diets at different temperatures (e.g. Fye & McAda, 1972). The number of eggs per female ranges from 200 to 1000, the sex ratio is 1 and a multiplication factor of 50 per generation is easily attained at favourable conditions of temperatures between 20 and 35 °C, high food quality and absence of diseases and predators. The period from egg to egg varies from approximately 20 to 40 days over this temperature range. There are five, sometimes six, larval stages. On chrysanthemums, caterpillars eat approximately 70 cm^2 of leaf surface area during the entire larval period. 75% of the feeding injury is caused by the fifth larval stage, 20% by the fourth instar and 5% by the third (Smits, 1986). The first and second instar eat relatively very little, and their feeding is economically unimportant because they feed on bottom leaves. Eggs are laid in batches of 25 to 50 near the ground (Smits et al., 1986). The caterpillars migrate slowly upwards during their development and disperse from plant to plant (Smits et al., 1987b). Finally, the caterpillars originating from one egg batch occupy about 1 m^2 of crop.

We developed our model for two purposes:

1. to investigate if a biologically realistic model of the epidemiology of SeMNPV on *S. exigua* in chrysanthemums could be built with the data available in the literature and if such a model, that summarizes and integrates our knowledge about the system, would produce reasonable results.
2. second, if the model would produce realistic results, it could be used for generating and testing ideas, e.g. concerning application scenarios for the virus:
   a. dose
   b. moment of application
   c. single or split sprays
   d. scope for improvement of viral efficacy by genetic engineering of persistence or other characters.
   e. usage of SeMNPV for **inoculative** control of beet armyworm in glasshouse ornamentals, versus **inundative** control that had already been proved economically feasible by Smits et al. (1987a). Inoculative control implies that the antagonist is introduced (inoculated) only once, and propagates itself subsequently while keeping the pest below injurious levels. For inundative control, a good immediate killing effect is sufficient. Successful inoculative control requires in addition sufficient survival of virus on the right 'spots' to infect new upsurges of beet armyworm.

An earlier version of the model was described by de Moed et al. (1990). The biological parameters and structure of the present version of the model are largely the same as described in that paper. However, the description of plant growth and the deposition of sprayed virus have been modified

(see below). The model version described by de Moed et al. (1990) did not account for periodic harvests of chrysanthemums, which cause removal of virus inoculum. The present model takes this epidemiologically important phenomenon into account.

This paper gives the biologically interested reader an informal introduction to the model and the results that are obtained. A more precise account with mathematical equations will be given elsewhere.

MODEL DESCRIPTION

The model assumes that there is a large (unlimited) number of chrysanthemums in a glasshouse. The plants are grown in beds of 1 m wide with 8 plants across the bed. They are harvested at an age of ca 3 months. Plantings of 10 different ages and stages of development are present simultaneously as beds are harvested and replanted every one or two weeks. Per plant 3 new leaves appear weekly and the average leaf size is 35 cm^2.The leaf canopy grows at a rate of 0.1 LAI-unit (m^2 leaf area per m^2 ground) per day.

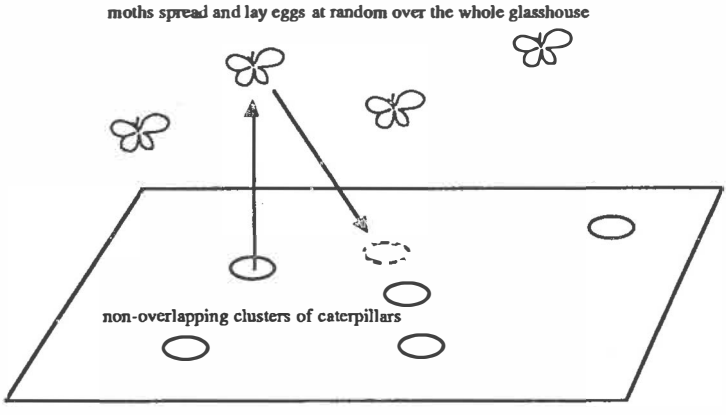

Figure 1: Schematic view of a large glasshouse with few non-overlapping randomly dispersed clusters of beet armyworm. Each cluster results from a single batch of 35 eggs, laid near the ground on one chrysanthemum plant. Caterpillars move randomly between plants, resulting in a ca 1 m^2 patch of infested crop when the caterpillars are in the fifth larval stage.

One single mated female moth flies into the glasshouse at a certain date, day 0, and lays her eggs on chrysanthemum plants of the planting that is in the preferred stage: young plants with 6 leaves. The capacity for egg laying of the female is about 500 eggs. Each egg batch comprises 35 eggs. Thus 14 egg batches are deposited in the course of a few days after the initial immigration. The batches are randomly dispersed. The caterpillar clusters that result from the egg batches will be non-overlapping because the moth is a good flyer and the crop area is large compared to the area occupied by patches of infested crop (about 14 m^2). New adults emerge from the infested patches.

They disperse over the glasshouse and start a new generation. Figure 1 illustrates these events.

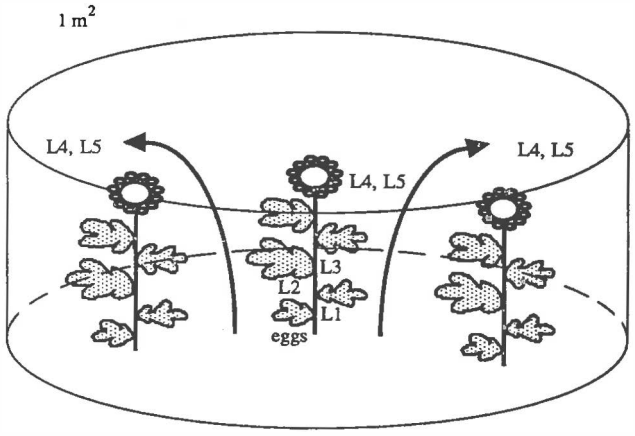

Figure 2: Schematic view of situation within a patch of infested chrysanthemum crop. Eggs are laid at the basis of the central plant. Successive stages crawl slowly upward the stems, with the fifth stage feeding in the upper leaf layers. During larval development, the initially small cluster of caterpillars spreads over more and more plants.

At a finer level of resolution, we observe the events occurring in a single patch. Eggs hatch and the first instar larvae (L1s) start feeding on the lower foliage until they moult into L2s. The age distribution changes gradually from predominantly L1s through L2s, L3s and L4s to the final fifth larval stage. While the age distribution evolves, changes occur in the vertical distribution of the caterpillars in the canopy and in the dispersion pattern in the horizontal plane (Smits et al., 1987b). These events are illustrated in Figure 2.

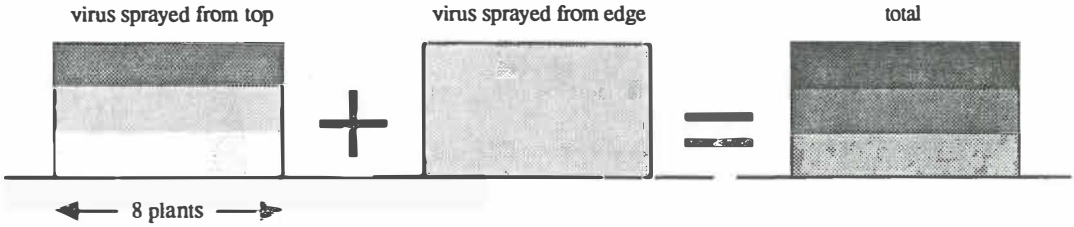

Figure 3: Cross section through chrysanthemum bed showing calculated virus concentration in different canopy layers, resulting from a 50% portion entering the canopy from above and another 50% entering the canopy from the edges. The model calculates with four canopy layers.

At the observation of feeding injury, the grower will apply a virus spray. Sprays are applied by hand. Thereby, the grower sprays not only from the top of the canopy, but also from the edges. Thus a good coverage with polyhedra is obtained on the leaves of edge plants. The model calculates an average virus concentration on the leaves for four heights in the canopy, taking the horizontal and vertical mode of applying virus into account. It is assumed that 50% of the polyhedra applied enter the canopy from above and 50% from the edges. The polyhedra entering from above are intercepted in a random fashion by leaves, resulting in an exponential virus profile. Each LAI unit of leaf area intercepts approximately 40% of the incoming polyhedra. Thus the first layer (from the top) receives a fraction of 0.4 of the amount of polyhedra applied, the second a fraction of 0.6 x 0.4, the third layer a fraction of 0.6 x 0.6 x 0.4, etc. It is assumed that the virus portion entering from the edge is distributed homogeneously in the canopy (Fig. 3).

The caterpillars take up the sprayed polyhedra with ingested leaf material. The rate at which polyhedra are ingested depends upon the feeding rate and the density of the polyhedra on the leaves:

leaf consumption rate	×	polyhedron density on leaf	=	rate of polyhedron consumption
$(cm^2 \text{ leaf } d^{-1})$		$(\text{polyhedra } cm^{-2} \text{ leaf})$		$(\text{polyhedra } d^{-1})$

Due to their greater food intake and the higher polyhedron density in the upper leaf layers, the fourth and fifth instar caterpillars ingest the highest amounts of polyhedra. The relative rate of infection is obtained by multiplying the uptake rate of polyhedra with the infection chance per single polyhedron. This infection chance decreases with stage. For the five instars, these chances are respectively 1/7, 1/4, 1/70, 1/260 and 1/18000 (Smits, 1986; de Moed et al., 1990).

rate of polyhedron consumption	×	infection chance per polyhedron	=	relative infection rate
$(\text{polyhedra } d^{-1})$		$(\text{fraction infected polyhedron}^{-1})$		$(\text{fraction infected } d^{-1})$

The portion of the model described in the foregoing is sufficient to calculate the immediate (inundative) effect of a NPV spray. For the simulation of the long term (inoculative) effect of the spray, the transmission pathways must be taken into account. There are three such pathways:

1. horizontal transmission; from caterpillar to caterpillar
2. vertical transmission; from female to eggs
3. vertical transmission; from male to eggs.

Horizontal transmission depends upon the number of encounters between healthy caterpillars and their virus-killed siblings. The model calculates the number of such encounters occurring in a patch, taking the following factors into account:

1. different instars feed at different heights in the canopy (Smits et al., 1987b). Caterpillars of different stages meet each other only on those leaves that belong to the feeding 'territory' of both stages.
2. the number of encounters increases with the number of leaves visited by a caterpillar of a certain instar in a day. The number of leaves visited per day is 1 for L1, L2 and L3, 3 for L4 and 5 for L5.
3. the size of the feeding 'territory' increases with instar (Smits et al., 1987b). L1s are found on 6 plants, including the plant on which the eggs were deposited, L2s on 15 plants, L3s on 30 plants, L4s on 47 plants and L5s on 65 plants.
4. the distribution and movement of healthy and dead caterpillars within their feeding territory is random.

Horizontal transmission is crucial for the 'survival' of the virus within a patch. When the virus is not propagated by horizontal transmission, no infected females can emerge from a patch and no epidemic will start.

Vertical transmission by females occurs when a female caterpillar is infected in the fifth stage (Smits & Vlak, 1988). Half of those caterpillars die while the other half develop into females that carry NPV with them. Approximately 15% of the eggs of these females are infected. The probability that an egg batch will contain only healthy eggs is $(1 - 0.15)^{35} = 0.0034$. Thus only 1 egg batch out of 295 produced by contaminated females will be virus-free. The average number of infected eggs in an egg batch with one or more infected eggs is 5.3.

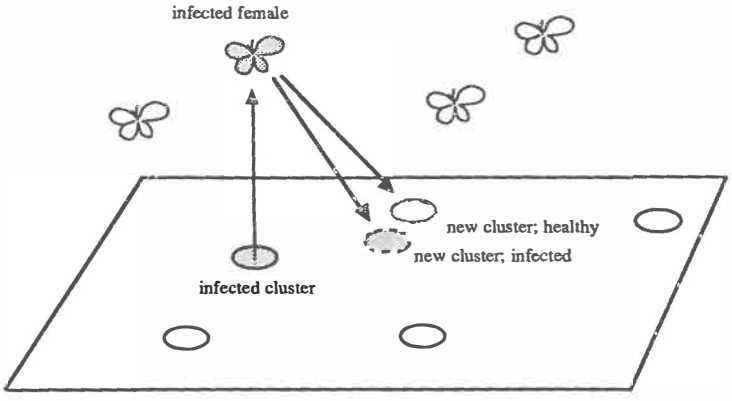

Figure 4: 50% of the infected female L5s develop into infected moths that lay infected eggs at a rate of 15%. The average number of infected eggs per batch laid by such females is 5.3. The proportion of healthy clusters produced by such females is about 0.5%.

Vertical transmission by males occurs when a male caterpillar is infected in the fifth stage. Half of those caterpillars die while the other half develop into males that carry NPV with them. At copulation, the male contaminates the female, upon which the female will lay some infected eggs (Vargas-Osuna & Santiago-Alvarez, 1988). The proportion of infected eggs due to male transmission is 7.5%, half the value taken for female vertical transmission. The probability that an egg batch will contain only healthy eggs is $(1 - 0.075)^{35} = 0.065$. Thus about 1 egg batch out of 15 produced by contaminated females will be virus-free. The average number of infected eggs in an egg batch with one or more infected eggs due to male transmission is 2.8. Females copulate only once. Under the assumption that infected males copulate as often as healthy males, the proportion of females infected at copulation equals the proportion of infected males.

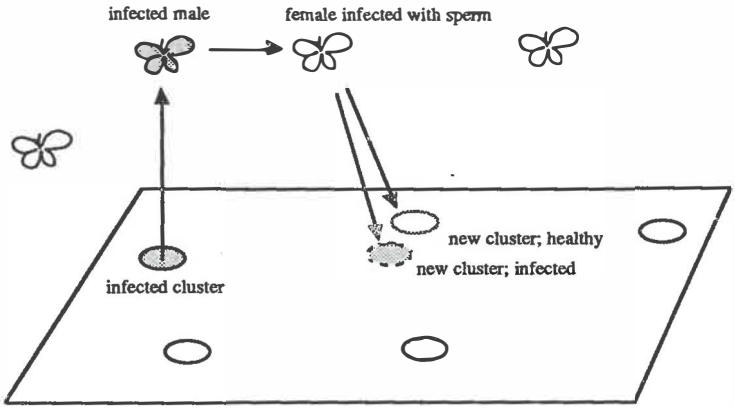

Figure 5: 50% of the infected male L5s develop into male moths that infect females at copulation. The probability of virus transmission at copulation is 1. Females infected in this way lay 7.5% infected eggs. The average number of infected eggs in an infected batch laid by such females is 2.8. The proportion of healthy clusters produced by such females is about 7.2%.

All females, whether or not infected by either pathway, deposit eggs randomly over the glasshouse. Healthy caterpillar clusters are produced by healthy females and by a fraction of the females infected by either vertical transmission mechanism. Infected caterpillar clusters are initiated by infected females. The model distinguishes between three types of patches: healthy patches, patches infected via the male transmission pathway (2.8 infected eggs initially) and patches infected via the female vertical transmission pathway (5.3 infected eggs initially). Sensitivity analyses of the model showed that a finer classification of patches according to the number of infected eggs did not significantly change the results.

Vertical transmission is crucial for the transfer of virus from one caterpillar generation to the next as patch overlap is not accounted for in the model. The simplification of neglecting patch overlap is justified at the low caterpillar densities that are relevant, taking the low damage threshold into

account. The model calculates with a time step of about one hour. Patches that are started at adjacent dates are lumped in the analysis, that is: average values are taken for all state variables. Calculations are separated for patches that are initiated at dates that are more than one week apart.

SIMULATION RESULTS

The insect model without virus produces an exponential population growth with distinct generations. As time passes the separation between generations diminishes due to individual variability in development period. Each month one generation is produced. The multiplication factor per generation is ca 100. The relative growth rate of the population is 15% per day (Fig. 6).

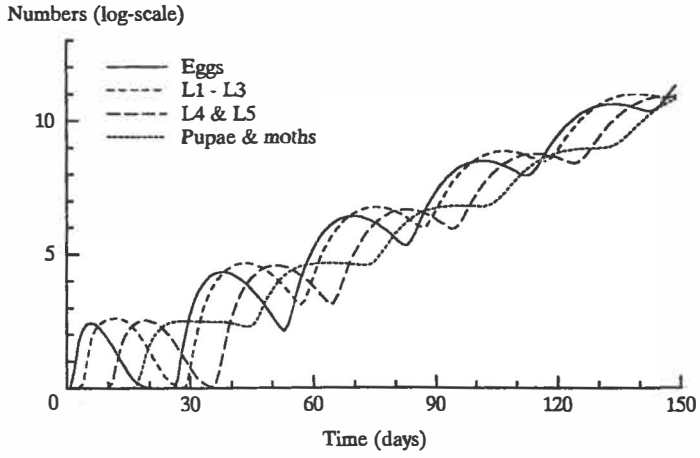

Figure 6: Simulated dynamics of healthy *Spodoptera exigua* population on glasshouse chrysanthemums at 25 °C. Time is reckoned from the day that the crop was infested by one adult female moth.

The optimal timing of the spray was the next thing that we investigated with the model. Best control during the second generation was obtained by a spray somewhat more than 40 days after the initial infestation (data not shown), that is at maximum presence of L2s and L3s. A spray at that time causes an instantaneous reduction in caterpillar numbers of 90 to 95% (Fig. 7). A good effect is obtained with an SeMNPV dose of 10^8 polyhedra/m^2. After one generation, the old pattern of population growth (15% d^{-1}) is however resumed (Fig. 7). Increasing the SeMNPV dose above 10^8 polyhedra/m^2 does not keep the population substantially longer under control. Applying the same dose of 10^8 polyhedra per m^2 in three separate sprays, each of 3 x 10^7 pol./m^2, at intervals of 7 days substantially improves control (Fig. 7).

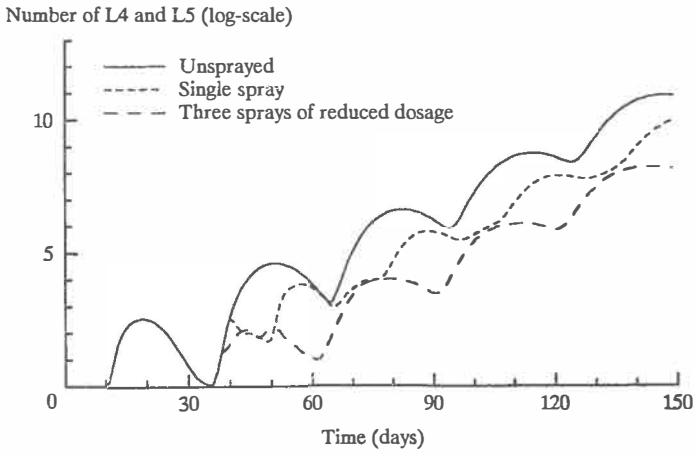

Figure 7: Population development of *Spodoptera exigua* L4 and L5 caterpillars on chrysanthemums in three situations: 1. uncontrolled; 2. single SeMNPV spray of 10^8 pol./m^2 at day 40 after initial infestation of the crop with 1 female moth; 3. three SeMNPV sprays of 3×10^7 pol./m^2 at days 38, 45 and 52 after initial infestation.

The effect of the halflife of the virus on the control achieved was also simulated and appeared to be small. Likewise, the effect of introducing a portion of 'stable' virus into the model (which is not inactivated by UV) was very small. The reason of this lack of effect is the spatial structure of the model (and of the system). Stable virus will be present on old leaves of old plants that are not anymore visited by caterpillars due to the rapid growth of the plants and frequent harvests, and is therefore ineffective. Stable virus fractions and slow virus inactivation are of more importance when the crop has not such rapid foliage growth and crop turnover rates as chrysanthemums have.

CONCLUSIONS

1. The model produces results that are logical but not self-evident. For instance the slight importance of the halflife of the virus was not anticipated. Thus model predictions serve as an eye-opener.
2. Experimental validation of model predictions is necessary. Errors may arise from an oversimplified or even incorrect representation of the structure of the system and inaccurate numerical values of the parameters. Although the model is quite comprehensive, still many processes are neglected or aggregated in a single parameter or function.
3. The best immediate control was obtained when the spray was applied at maximum presence of L2s and L3s. These caterpillar stages combine high to moderate susceptibility to the virus with an exposed position in the canopy. L1s are less exposed while L4s and L5s are less

susceptible.

4. Virus persistence in the rapidly growing chrysanthemum crop with its high turnover rate is not important but may be important in less rapidly changing crops.
5. No simulations with a long standing (inoculative) effect of SeMNPV were obtained. The model calculations suggest that the high growth and turnover rate of the chrysanthemum crop, together with the low damage threshold, will obstruct a successful implementation of inoculative control of beet armyworm with NPVs in this crop.
6. A single spray of 10^8 polyhedra/m^2 provides adequate inundative control. Model calculations suggest that three repeated sprays of 3×10^7 polyhedra/m^2 provide much better control for the same ingredient costs but higher costs for labour.

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7. Posters on entomopathogenic fungi.

The Use of Pheromones to Enhance Control of *Plutella xylostella* with *Zoophthora radicans*

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The diamondback moth, *Plutella xylostella*, is a serious pest of brassica crops, especially in areas of the Far East where it has developed resistance to all insecticides. Increased attention is therefore being paid to alternative methods of control. The aim of the current work is to develop a novel method involving a combination of pheromone trapping and the entomophthoralean fungus *Zoophthora radicans*.

One isolate of this fungus, from the homologous host in Taiwan, is highly infective for both the larvae and the adult moths and has been selected for further development. Experiments using a specially designed 'transmission box' have demonstrated that viable spores of the fungus are transmitted passively on the bodies of adults and infect larvae on cabbage plants to which the moths fly. In the control stategy being tested, adult males will be lured to a 'fast-entry/slow-exit' pheromone trap where the adults will be inoculated with spores of the fungus. The moths will then disperse in the crop and disseminate the fungus both passively and, when they die, by forming fresh foci of infection.

The fungus is cultured in a broth of yeast extract, glucose and skimmed milk and formulations for the storage of the fungus are being investigated. Field trials will be carried out in the Cameron Highlands of Malaysia where preliminary observations show that the temperature and humidity are favourable to fungal activity.

This work is being done under a grant awarded by Shell Research Ltd.

VERTICILLIUM LECANII AS A MICROBIAL INSECTICIDE AGAINST WHITEFLY

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Summary

The insect-pathogenic fungus **Verticillium lecanii** (Deuteromycetes: Moniliales) formulated as a wettable powder (MYCOTAL) can be used in cucumber, tomato and other vegetable and ornamental protected crops to control whitefly. Repeated sprayings at weekly intervals can reduce whitefly infestations by approximately 90 %, even when relative humidity is as low as 75%. In cucumber infection rates as high as 60% of western flower thrips (**Frankliniella occidentalis**) have been observed. Integration with other biological control agents and fungicide treatments is possible.

1. Introduction

V. lecanii is a well-known pathogen of arthropods: it was first described in 1861, and has been collected from numerous species of insects, spiders and mites (Rombach & Gillespie, 1988).
The mode of action is as follows: germinating spores on the cuticle of the insect produce hyphae that penetrate into the body cavity, where the fungus destroys the tissues, thus killing the insect. Later on the fungus grows out through the insect cuticle and sporulates on the outside of the body. These spores can, if spread by splashing water or other mechanical means, spread the infection to other insects.

A specific strain (KV01) of **V. lecanii** was developed as a microbial insecticide against greenhouse whitefly (**Trialeurodes vaporariorum**) in the United Kingdom between 1980 and 1985. Trials with this product in the Netherlands in 1984 were not successful. In 1988 the development of the same strain was resumed by Koppert BV in the Netherlands. Improvements in production and formulation resulted in a wettable powder with a dosage of 10^{10} viable conidiospores per gram, a 50-fold increase in comparison with the former product. Subsequent trials on cucumber and tomatoes in glasshouses proved that the product could have a very good effect on greenhouse whitefly, while the standard operating procedures of the growers could be maintained (Ravensberg et al., 1990). New efficacy trials for registration as a pesticide were successful. Registration in the UK was obtained in 1989. The registration process in the Netherlands and other countries is still under way.

The product is sprayed with standard spraying equipment at a rate of 3 kg per hectare, in two to three thousand litres of water per hectare (10^7 spores per ml). To achieve good control three treatments are recommended since mainly larvae are killed. Temperatures should range from 18 to 30 °C, relative humidity

should be above 85 %. The only restriction is that fungicides should be applied three days before or three days after **V. lecanii** applications and that the fungicides tolylfluanid and dichlofluanid are not used.

2.Effect of treatments

In cucumber five trials were done on growers premises. When cucumber is treated three times at weekly intervals the mortality of whitefly larvae usually reaches 85 % two weeks after the first treatment. Four weeks after the first treatment mortality often increases to 95 %, with a range of 70 - 98 % (see fig. 1). In the same cucumber trials **F. occidentalis** mortality reaches an average of 60 %.

In tomato (four trials on growers premises) whitefly mortality often reaches 85 % at 2 - 5 weeks after the first of three treatments at weekly intervals (see fig. 2). 80 % control can be obtained after two weeks in glasshouses with low bays (eaves 3 m high) and dense crop structure. At that point in time control is only 20 % in taller glasshouses with a more open crop structure, where, although the overall humidity can be the same, the microclimate on the leaf surface is probably quite different. Overall humidity here means the relative humidity of the air in the crop, as a contrast to the relative humidity in the boundary layer of the leaf surface.

3. Integration with other biocontrol measures

Control of **Tetranychus urticae** by the predatory mite **Phytoseiulus persimilis** was not affected by the application of **V.** lecanii. **Encarsia formosa** still parasitised larvae of **T. vaporariorum**, although the quantity of possible hosts for this parasite was of course reduced by **V. lecanii** . Negative effects of the fungus on **E. formosa** were not observed, nor on **Amblyseius** species. This means that **V. lecanii** can be safely integrated with these biocontrol agents.

4. Discussion and conclusion

Since it is well known that the relative humidity plays an important role in the infection process of **V. lecanii** and other entomopathogenic fungi (Quinlan, 1988) the average humidities at various growers were compared with the percentage mortality of the whitefly larvae. There was no relation between whitefly mortality and overall humidity. Since the variation could also not be attributed to any other factor like operator variation, fungicide treatments, etc. we assume that the microclimate on the leaf is an important cause of variation. This is substantiated by the observations on the effect of crop structure in tomatoes. The overall glasshouse humidity that is measured by the computer is not necessarily correlated with the phyllosphere humidity.

It can be concluded that **V. lecanii** can be used to control whitefly in protected cucumber and tomato crops. It is especially valuable in situations where **E. formosa** gives, temporarily or locally, insufficient control. It can also be used in the integrated control programme against **F. occidentalis**, where every added mortality factor is useful.
Trials in other crops, such as chrysanthemums and poinsettia (against **Bemisia tabaci**) are in progress.

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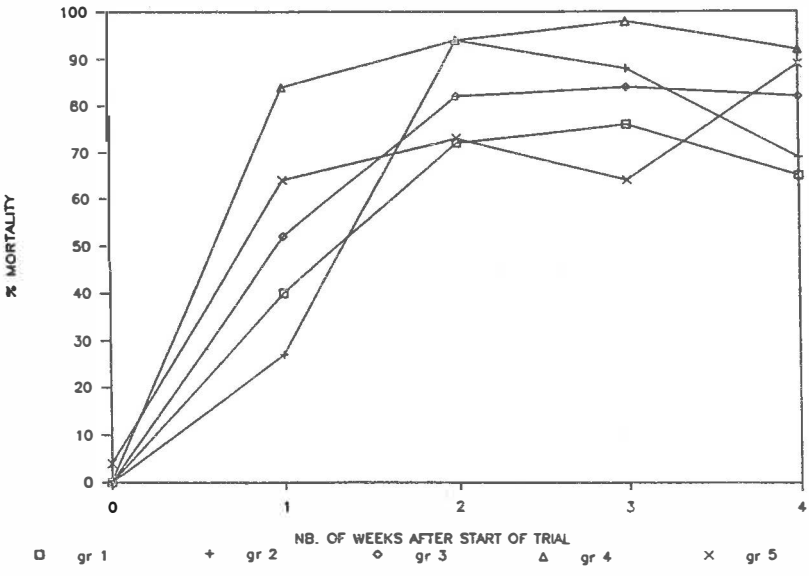

Fig. 1. Mortality of whitefly instars in cucumber greenhouses treated with Mycotal ; Mean mortality per grower at weekly intervals.
grl = grower number 1, gr 2 = grower number 2, etc.

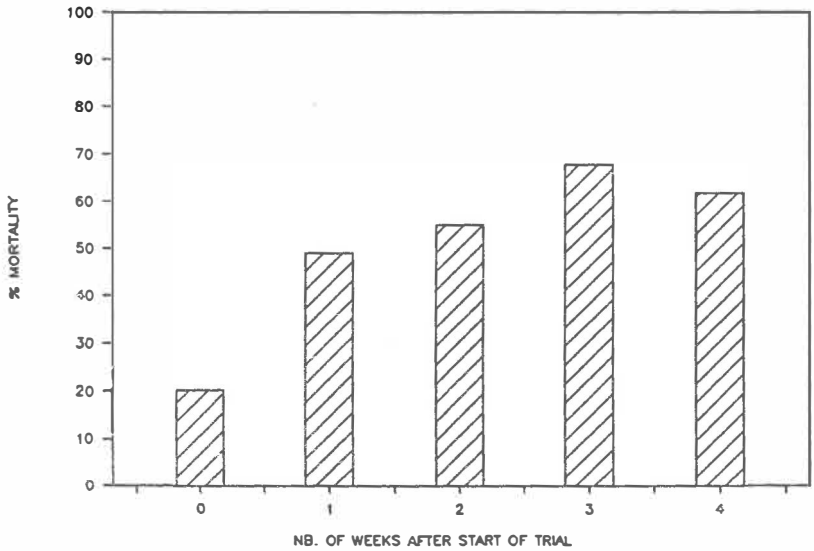

Fig. 2. Mean mortality of **Frankliniella occidentalis** larvae, pupae and adults in five cucumber crops treated with Mycotal.

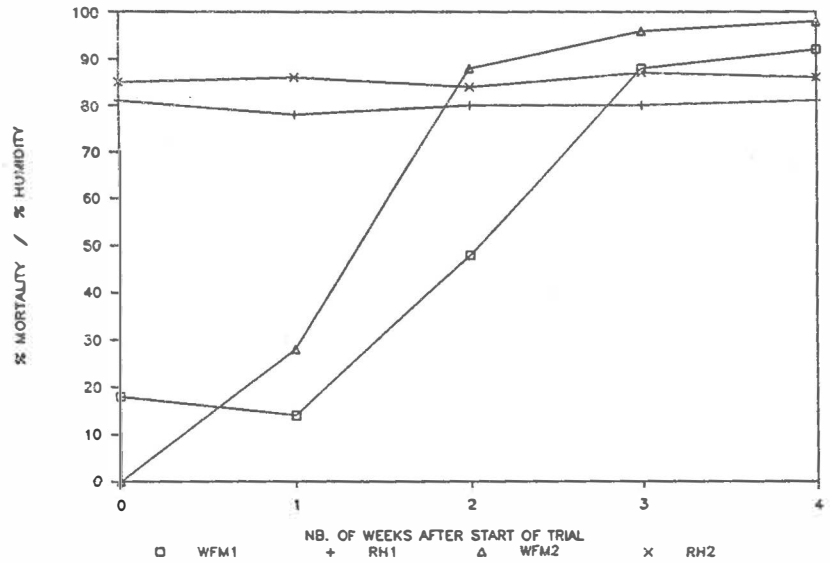

Fig. 3. Mean mortality of whitefly instars in treated areas in two tomato crops and mean relative humidity in those crops treated with Mycotal.
WFM1 = Whitefly mortality at grower 1 ; WFM2 = Whitefly mortality at grower 2 ; RH1 = Average humidity at grower 1 RH2 = Average humidity at grower 2

Entomophthorales: A key for the identification of the arthropod-pathogenic genera and their characterisation

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Introduction

During the last few years the taxonomy and systematics of the arthropod-pathogenic Entomophthorales have changed dramatically. Until 1963 only the genera Entomophthora and Massospora were recognised (MACLEOD, 1963). Two years later BATKO & WEISER (1965) described a further genus, Strongwellsea, Massospora and Strongwellsea consisted of few well-defined species, whereas Entomophthora was a collection of about 100 species, many of which were morphologically very different. BATKO (1964, 1966) laid the foundation for a new classification by splitting this large genus into a number of genera but his system included some anomalies and was not accepted. Subsequently, REMAUDIERE & HENNEBERT (1980), REMAUDIERE & KELLER (1980) and HUMBER (1981, 1984) re-worked Batko's system and defined seven genera: Entomophaga, Entomophthora, Erynia, Eryniopsis, Neozygites, Zoophthora and Tarichium. Also, some species were attributed to the genus Conidiobolus, formerly considered as including only saprophytic species. These genera have found wide acceptance.

Recently the creation of more genera has been proposed by splitting the genera Entomophaga, Erynia and Neozygites (BEN-ZE'EV & al. 1987; HUMBER, 1989). However these proposals are unsound, the definitions of the new genera failing to account for all relevant species. They are therefore considered as premature or unjustified and are not included in the following account. Also, the ordering of the genera into families (BEN-ZE'EV & al., 1987; HUMBER, 1989) is not considered here. The key presented below derives from KELLER (1987, 1991), where further literature is cited.

Key to the genera

1.	Only resting spores known	Tarichium	(10)
1.*	Conidia not forcibly discharged	Massospora	(6)
1.**	Conidia forcibly discharged		2
2.	Primary conidia uninucleate, bitunicate; elongate pyriform, fusiform or cylindrical		3
2.*	Primary conidia with more than 1 nucleus, unitunicate; campanulate, spherical, pyriform, ellipsoidal or subcylindrical		5
3.	Conidiophores branched, penetrate integument of the host.		4

3.* Conidiophores unbranched in abdominal cavity. Primary conidia subcylindrical, projected through hole on the ventral side of the abdomen of flies.. Strongwellsea (8)

4. Primary conidia ovoid pyriform or fusiform; papilla smoothly rounded, not clearly demarcated from conidial body. Secondary conidia like primary or spherical with or without small apical point ..... Erynia (4)

4.* Primary conidia fusiform or cylindrical; papilla conical, pointed or rounded, demarcated from conidial body by a collar. Secondary conidia like primary or sickle- to crescent-shaped on long, slender capillary ..... Zoophthora (9)

5. Primary conidia with apical point. Projected primary conidia sourrounded by ruptured conidial membrane ..... Entomophthora (3)

5.* Primary conidia without apical point. Projected conidia not surrounded by ruptured conidial membrane 6

6. Primary conidia elongate with about 5-15 nuclei; papilla sometimes indistinct, rounded. ..... Eryniopsis (5)

6.* Primary conidia spherical to pyriform, papilla distinct 7

7. Primary conidia relative small, spherical with flattened papilla or Montgolfière-shaped with more rounded papilla, 3-8 nuclei. Secondary conidia like primary or amygdalliform on long, slender, distally bent capillary. Resting spores ellipsoidal to spherical, darkbrown or black ..... Neozygites (7)

7.* Primary conidia relative large, subspherical, pyriform; more than 8 nuclei on average; papilla rounded or pointed. Secondary conidia like primary. Resting spores spherical 8

8. Nuclei small, not or weakly staining in lacto-phenolaceto-orcein (LPAO), on average more than 50 per conidium ..... Conidiobolus (1)

8.* Nuclei large, more or less deeply staining in LPAO, on average more than 10 per conidium ..... Entomophaga (2)

Characterisation of the genera

1. Conidiobolus BREFELD

Hyphal bodies irregular, producing single conidophore or resting spore. - Conidiophores unbranched, with or without terminal enlargement; nuclei staining weakly or not in LPAO, on average smaller than 3.0 um. - Primary conidia unitunicate, spherical with rounded or conical papilla with or without point; nuclei small, stain distinctly with the Feulgen reaction stain, very numerous, on average between 50-100 per conidium. - Secondary conidia usually like primary, produced on short lateral secondary conidiophore; sometimes microconidia around primary conidia; capilliconidia, in some species only, fusiform to elliptical. - Resting spores spherical, hyaline, surrounded by thin episporium, or villose. - Rhizoids present or absent, cystidia very rare. Most species are saprophytes, some are parasites of insects and arachnids.

Type species: Conidiobolus utriculosus BREFELD

2. Entomophaga BATKO

Hyphal bodies spherical, subspherical or irregular, each producing a single conidiophore or azygospore. - Conidiophores unbranched, with more or less distinct terminal enlargement. Nuclei staining distinctly in LPAO, usually larger then 3 um on average; diameter of nucleolus about 1/3 - 1/2 of that of the nucleus. - Primary conidia unitunicate, spherical to pyriform; papilla conical, rounded or pointed; nuclei larger than those of Conidiobolus, staining distincly in FRS and LPAO; numerous, on average between 10 and about 100. - Secondary conidia like primary, produced on short lateral secondary conidiophore. - Resting spores spherical smooth, usually hyaline. - Rhizoids present or absent. - Cystidia absent. Parasites of insects. 13 species.

Type species: Entomophaga grylli (FRES.) BATKO
Bas.: Entomophthora grylli FRESENIUS

3. Entomophthora FRESENIUS

Hyphal bodies usually homogenous and regular, spherical, subspherical or elliptical. Germinate with single germ tube. - Conidiophores unbranched, terminal portion enlarged. - Primary conidia campanulate, appearing unitunicate, bi- to multinucleate; nuclei large, deeply staining in LPAO. Projected conidia surrounded by a halo. - Secondary conidia similar to primary ones, apical point often absent or weakly developed, formed on short secondary conidiophore laterally from primary conidia. Projected secondary conidia not surrounded by a halo. - Resting spores spherical, hyaline or surrounded with dark episporium. - Rhizoids present or absent, cystidia absent. Parasites of insects. 11 species.

Type species: Entomophthora muscae (COHN) FRESENIUS
Bas.: Empusa muscae COHN

4. Erynia (NOWAKOWSKI) REMAUDIÈRE & HENNEBERT
Bas.: Erynia NOWAKOWSKI
Syn.: Erynia (NOWAKOWSKI ex BATKO) REMAUDIÈRE & HENNEBERT
Furia (BATKO) HUMBER
Pandora HUMBER

Hyphal bodies spherical, ellipsoidal, elongate, rod-shaped, filamentous, rarely branched, or hyphae-like; oligo - or multinucleate, rarely mononucleate. Nuclei large, deeply staining in LPAO. - Rhizoids monohyphal, endings rounded, branched, finger-, root- or disk-like. - Conidiophores branched, enlarged prior to the formation of conidia. - Primary conidia uninucleate, bitunicate, elongate, fusiform, conical, pyriform, ellipsoidal or ovoid, papilla rounded or conical. - Secondary conidia similar to primary or subspherical, tetraradiate in some "aquatic" species. - Resting spores spherical, hyaline or colored, smooth or ornamented. - Cystidia usually present, slender or powerful, tapering. Parasites of insects and Opiliones. 42 species.

Type species: Erynia ovispora (NOWAKOWSKI) NOWAKOWSKI
Bas.: Entomophthora ovispora NOWAKOWSKI

5. Eryniopsis HUMBER

Hyphal bodies irregular, oligonucleate, nuclei relatively large, deeply staining with LPAO. - Conidiophores usually unbranched. - Primary conidia elongate, cylindrical to fusoid, oligonucleate, unitunicate; papilla distinct, sometimes indistinct. - Secondary conidia produced laterally from primary on relatively short conidiophore, resembling the primary ones or more or less rounded, or subcylindrical on capillary germ tube. - Resting spores spherical, smooth hyaline. Rhizoids present or absent. - Cystidia unknown. Parasites of insects. 3 species.

Type species: Eryniopsis lampyridarum (THAXTER) HUMBER
Bas.: Empusa (Entomophthora) lampyridarum THAXTER.

6. Massospora PECK

Conidia globose, oboid, ellipsoid or fusiform, with 1 - 6 nuclei; not forcibly discharged. - Resting spores globose, ornamented, light or yellow brown to brown in mass. - Cystidia and rhizoids absent. - Infection limited to abdomen, conidia and resting spores liberated by sloughing off of the abdominal segments of the living host. Parasites of adult Homoptera Cicadidae. 13 species.

Type species: Massospora cicadina PECK

7. Neozygites WITLACZIL
Syn.: Thaxterosporium BEN-ZE'EV & KENNETH in BEN-ZE'EV & al.
Neozygites WITLACZIL (1885) sensu BEN-ZE'EV & KENNETH in BEN-ZE'EV & al.

Hyphal bodies regular, spherical or short rod-shaped. - Conidiophores unbranched, with more or less distinct terminal enlargement. Nuclei in hyphal bodies and conidiophores staining distinctly in LPAO. - Primary conidia unitunicate, spherical, pyriform, or in the shape of a Montgolfière (hot air balloon), hyaline or light brown, papilla cylindrical or conical, usually 4 - 8 nuclei. Nuclei not or weakly staining in LPAO. - Secondary

conidia like primary, produced on short lateral secondary conidophores, or capilliconidia amygdaliform produced on long, slender capillary, light brown with terminal drop or haptor. - Resting spores zygospores produced by conjugation of two hyphal bodies, binucleate, spherical or ellipsoidal, episporium brown or black, smooth or ornamented. Germ conidia corresponding to one of the two types of secondary conidia: spherical, hyaline on short thick germ tube or capilliconidia amygdaliform, brownish on long, slender capillary. - Cystidia absent, rhizoids usually absent. Parasites of insects and mites. 9 species.

Type species: Neozygites fresenii (NOWAKOWSKI) REMAUDIÈRE & KELLER
Bas.: Empusa fresenii NOWAKOWSKI

8. Strongwellsea BATKO & WEISER

Hyphal bodies simple, rarely branched, uni - or oligonucleate. - Conidophores unbranched, uninucleate. - Primary conidia uninucleate, bitunicate; obovoid, ellipsoidal to subcylindrical; papilla flattened to slightly rounded. - Resting spores orange, spherical to ovoid; episporium covered with broad spines. - Cystidia and rhizoids absent. - Infection restricted to abdomen, causing a nearly circular hole on the ventral side through which conidia are projected. No hole when resting spores present. - Parasites of muscoid flies. 2 species.

Type species: Strongwellsea castrans BATKO & WEISER

9. Zoophthora (BAKTO) REMAUDIÈRE & HENNEBERT
Bas.: Zoophthora BATKO

Hyphal bodies rounded, irregular or hyphae-like. - Conidiophores branched with terminal enlargement. Nuclei in hyphal bodies, conidiophores and conidia stain distinctly in LPAO. - Primary conidia bitunicate, elongate, cylindrical to slightly fusiform; papilla conical, pointed or sometimes rounded, separated from the conidial body by a raised collar. - Secondary conidia similar to primary, formed on short, thick conidiophore, or falciform to banana-like formed on long, slender capillary. - Resting spores spherical, hyaline, brown or black, smooth or ornamented. - Rhizoids monohyphal or pseudorhizomorph, with or without special holdfast, rarely absent. - Cystidia rare or absent. Parasites of insects. 16 species.

Type species: Zoophthora radicans (BREFELD) BATKO
Bas.: Empusa radicans BREFELD

10. Tarichium COHN

This genus was erected to include species known only by their resting spores. Such species can not be attributed to one of the previously described genera and must therefore be allocated to a provisional genus or considered a collection of species with uncertain taxonomic status. Parasites of insects and mites. 34 species.

Type species: Tarichium megaspermum COHN

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SOIL PERSISTENCE OF *Metarhizium anisopliae* : INFLUENCE OF FUNGAL NUTRITION ON BOTH SURVIVAL AND REMAINING INFECTIVITY OF CONIDIA

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SUMMARY

The persistence of *in vitro*- and *in vivo*-producedconidia of *Metarhizium anisopliae* in soil was investigated with an experimental biodegradation method using a trap technique. At various times of incubation, traps were collected to study changes in biomass, inoculum potential and infection potential of conidia. The biomass of culture conidia decreased rapidly whereas the biomass of host-produced conidia was preserved after 21 months. The infectivity and the viability of *in vitro*-producedconidia were affected from 2 and 6 months respectively. In contrast, host-produced conidia remained viable and infective after 2 years of incubation.

1 - INTRODUCTION

As reported by Keller and Zimmermann (1989) the persistence of mycopathogens in the soil ecosystem is important, not only with respect to their epizootology, but also in the development of strategies in biological control of soil insects. The survival of entomopathogenic fungi in the soil depends on various interacting factors, including abiotic factors, e.g. temperature, moisture, pH, inorganic and organic matters and biotic factors such as edaphic microflora and micro- and mesofauna (Ferron, 1978 ; Latteur, 1980 ; Lingg and Donaldson, 1981 ; Fargues et al., 1983 ; Studdert and Kaya, 1990 a,b ; Studdert et al., 1990). It depends also on the species, isolate and morphological stage or type of propagule of the fungus (Müller-Kögler and Stein, 1970, 1976 ; Fargues, 1981 ; Fargues and Robert, 1985 ; Perry et al., 1982). Moreover, it seems that entomopathogenic fungi persist for a long period in the soil within fungus-killed cadavers (Sprenkel and Brooks, 1977 ; Ferron, 1978 ; Coles, 1979 ; Keller, 1983). However, little is known about the effect of host-passaging on the viability of conidia produced on the surface of the mummified insect. Thus, considering the favourable effect of a single-host passage on the infection potential of two isolates of *Metarhizium anisopliae* towards scarabeid larvae (Fargues and Robert, 1983a) we investigated the effects of host-passaging on the soil persistence of *M. anisopliae* conidia.

2 - METHODOLOGY

The original strain of *M. anisopliae* was obtained from a dead larva of *Cetonia aurata*. The single-spore isolate was made 2 years before the beginning of our tests and was used in all experiments. Culture conidia were harvested from 2-week-old cultures on semisynthetic agar medium in Petri dishes incubated at 25 °C (Fargues, 1976). Host-produced conidia came from larvae killed by the fungus. They were collected by brushing the integumental surface of sporulated cadavers according to the procedure previously described (Fargues and Robert, 1983 a).

The survival of both culture and host-produced conida of *M. anisopliae* in soil was investigated under controlled conditions with an experimental biodegradation method using a trap technique (Fargues et al., 1983 ; Fargues and Robert, 1985). Conidia samples of ca. 6 g were placed in polyamid mesh bags in traps. Each trap was then buried in a 1000 g soil sample contained in an incubator. Experiments were carried out at 19 ± 0.5 °C in samples of a sandy loam, adjusted to 80 % moisture holding capacity (Fargues and Robert, 1985).

At various times of incubation in the experimental soil, traps were collected to study changes in biomass, inoculum potential and infection potential of conidia. Variations in weight of the conidial biomass were calculated by comparing the dry weight of the initial biomass with the dry weight of the incubated biomass. Inoculum potential of conidia was determined by dilution plate counts of colony forming units (C.F.U.) on semisynthetic medium. Bioassays on insect to test infection

potential of conidia were conducted on third-instar larvae of *C. aurata* (Fargues, 1976). We used dilutions of 10^{-2} obtained by mixing 1 g of fresh conidia biomass in 100 ml sterile water (Fargues and Robert, 1985). Four lots of 20 larvae were treated by spraying with each dilution of samples.

3 - RESULTS

The biomass of culture conidia decreased relatively rapidly since the percentage of dry weight loss were 32 %, 64 % and 72 % after 1, 2 and 4 months, respectively. In contrast, the dry weight of host-produced conidia was preserved well even after 21 months (29 % biomass loss).

As shown in table, the inoculum potential of culture-borne conidia remained at a high level for the first four months of incubation (3.5×10^9 living conidia per gram of dry fungal material after 4 months) but it fell down after one year. Bioassays with samples of these culture conidia showed that the remaining infectivity of living conidia was affected from 2 months of incubation (78 % mortality instead 100 % at the initial time) and that it was very low after 4 months (5 % mortality).

Conversely, host-produced conidia remained viable (2.8×10^9 living conidia per gram of fungal material) and infective (68 % mortality) after ca. 2 years of incubation.

Table : Influence of fungal nutrition on soil persistence of *Metarhizium anisopliae* propagules : Survival and infectivity of culture conidia and of host-produced conidia during incubation at 19 °C in a sandy loam.

INCUBATION[a]	CULTURE CONIDIA		HOST-PRODUCED CONIDIA	
(MONTHS)	C.F.U.[x]	LARVAL MORTALITY[y]	C.F.U.[x]	LARVAL MORTALITY[y]
0	$(12 \pm 3)\ 10^9$	90 (100%)	$(14 \pm 2)\ 10^9$	90 (100%)
1	$(16 \pm 3)\ 10^9$	90 (100%)	NC	NC
2	$(5 \pm 1)\ 10^9$	62.2 ± 7.8**	$(14 \pm 2)\ 10^9$	90 (100%)
3	$(19 \pm 5)\ 10^9$	42.1 ± 11.3**	$(10 \pm 1)\ 10^9$	90 (100%)
4	$(35 \pm 4)\ 10^8$	12.1 ± 14.8**	NC	NC
6	NC	NC	$(9 \pm 1)\ 10^9$	90 (100%)
12	$< 10^3$	0	$(15 \pm 1)\ 10^9$	90 (100%)
17	-	-	$(11 \pm 1)\ 10^9$	55.4 ± 6.0**
21	-	-	$(28 \pm 3)\ 10^8$	58.4 ± 1.8**
36	-	-	$< 10^3$	0

[a] 6 g conidia samples placed in traps buried in 1000 g soil samples at 19 ± 0.5 °C
[x] dilution plate counts (C.F.U. per gram of dry weight of conidia sample)
[y] bioassays on *Cetonia* larvae : mortality ($\overline{X}$ arc sin ± S.D.) recorded at 30 days post-treatment
NC = non controlled

4 - DISCUSSION

The results of this study showed conclusively that a single host-passage appreciably increased the persistence of free entomopathogenic conidia in soil. It was particularly interesting that the infectivity of degrading inocula was affected after 2 or 3 months in the case of *in vitro*-produced conidia instead after 17 or 21 months in that of host-produced conidia.

Previous investigations on the influence of both *in vivo* and *in vitro* passages (Fargues and Robert, 1983 a,b) suggested that changes in infectivity of conidia consisted of phenotypic responses to the nutritional sources of the fungus prior sporulation. Therefor, the authors expected that enzyme induction mechanisms could be involved in the modification of the infectivity of *M. anisopliae* conidia.

Increased infectivity following *in vivo*-passage have been reported for a lot of hyphomycetous strains (Timonin, 1974 ; Wasti and Hartmann, 1975 ; Fargues, 1981 ; Fargues and Robert, 1983 a.,b.). However, other studies showed no change in pathogenic activity after host passaging (Ferron et al., 1972 ; Hall, 1980).

Thus, the increase of soil persistence of host-produced conidia of the isolate *M. anisopliae* 32 can not be generalized, nevertheless, nutritional factors could affect the epizootic potential of inocula of numerous entomopathogenic fungal isolates in soil.

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MODELE LOGISTIQUE DE PREVISION DE L'EFFET DES RADIATIONS U.V. SUR LE POTENTIEL GERMINATIF DES SPORES DE *Paecilomyces fumosoroseus* (WIZE) BROWN et SMITH

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SUMMARY

A microclimatic approach of short term persistence of pathogens in plant canopy has been developed in order to facilitate an evaluation of individual as well as combined effects of environmental factors by means of explanatory modelling. Considering the inactivation of fungal conidia by sunlight, photic effects of radiations including UV B wavelengths were studied under controlled conditions (high-pressure metallic halogenure lamps) and natural conditions (sunlight ca. 700 $W.m^{-2}$).

A logistic model has been chosen for describing the decrease of germination as a function of UV B irradiation. Its parameters have been estimated by adjustment of this model on three different sets of data provided by these experiments. Each of these adjustments showed a good fit of predicted values to observed data.

1 - INTRODUCTION

Les perspectives offertes par la lutte microbiologique se heurtent au problème de la survie des inoculums introduits dans le milieu. La méconnaissance des conditions microclimatiques de la persistance des microorganismes est donc un des principaux facteurs limitant le développement de ces techniques. C'est pourquoi une étude analytique de la persistance des microorganismes entomopathogènes a été développée (Fargues *et al.*, 1988, 1989). Elle vise à la fois à une meilleure compréhension des mécanismes mis en jeu et à l'élaboration d'un modèle mathématique modulaire de prévision de la persistance en fonction des différents paramètres du climat.

La plupart des modèles de persistance existants ont été élaborés sur la base de données recueillies dans des conditions microclimatiques mal définies (Pinnock et al., 1971 ; Brand et al., 1975 ; Richards et Payne, 1982). Les mesures de rayonnement, en particulier, ont été très fréquemment négligées ; les effets photiques et thermiques du rayonnement n'ayant pas été séparés, il est impossible de déterminer les parts respectives de l'effet radiatif et de l'élévation de température dans la perte de pouvoir germinatif observé (Carruthers *et al.*, 1988). Les modèles ainsi établis l'ont été la plupart du temps de manière empirique, ce qui rend hasardeuse toute tentative de généralisation. Ils n'ont le plus souvent pas été testés ou validés (Onstad et Carruthers, 1990).

Le premier module, en cours d'élaboration, du modèle de persistance, concerne la perte de pouvoir germinatif de spores d'hyphomycète soumises au rayonnement.

2 - METHODOLOGIE

Ce modèle repose sur l'hypothèse que la perte de pouvoir germinatif d'un inoculum irradié à un certain niveau d'éclairement, et avec une certaine gamme de longueurs d'onde, peut être déterminée en considérant la quantité d'éclairement (en $J.m^{-2}$) reçue par celui-ci (assimilable à une dose). La grandeur que l'on cherche à quantifier est la probabilité p(x), pour une spore, de survivre (en terme de pouvoir germinatif) à une quantité d'éclairement x.

Expérimentalement, cette probabilité peut être estimée sans biais par la fréquence des spores qui germent au sein d'un échantillon ayant subi cette même dose x. Ainsi , les données utilisées pour l'élaboration de ce modèle sont les pourcentages de germination observés dans des lots de spores irradiés à différentes doses x.

Le but de cette approche est donc de représenter mathématiquement l'évolution de p(x) en fonction de x, avec, comme objectif, de pouvoir à terme prévoir la survie d'un inoculum donné en fonction de l'irradiation à laquelle il est exposé.

La construction de ce modèle passe par trois étapes :

(1) - **le choix** d'une famille de fonctions paramétriques dont les courbes représentent au mieux l'allure de la diminution de la germination en fonction de la quantité d'éclairement ;

(2) - **l'estimation**, ou **ajustement**, sur un jeu de données expérimentales correspondant à un niveau d'éclairement et à une gamme de longueurs d'onde donnée, des paramètres qui déterminent la fonction $x \rightarrow p(x)$, de telle manière que la courbe représentative de cette fonction passe le plus près possible de l'ensemble des points observés ;

(3) - **la validation** du modèle ainsi construit, qui consiste en une comparaison des prévisions faites grâce à ce modèle avec des observations réalisées dans des conditions différentes de celle son l'élaboration.

3 - PRESENTATION DU MODELE

Le choix du type de fonction paramétrique a été réalisé en tenant compte des deux critères suivants :

- la nécessité d'une bonne adéquation entre la forme des courbes représentatives de ces fonctions et l'allure des observations expérimentales;
- la possibilité d'une interprétation biologique des paramètres, permettant de juger de l'importance de chacun d'eux et du niveau de précision nécessaire.

C'est suivant ces critères que le **modèle logistique** a été choisi pour représenter la diminution du pourcentage de germination en fonction de la quantité d'éclairement reçue par l'inoculum.

Dans ce modèle, on suppose qu'en moyenne, la probabilité p(x) pour une spore de survivre (en terme d'aptitude à germer) à une quantité d'éclairement donnée x décroît en fonction de x suivant une courbe logistique :

où les **paramètres à estimer** sont :

g_0, qui représente la probabilité de germer pour une spore non irradiée,

a, qui correspond à la "vitesse de décroissance" du potentiel germinatif des spores en fonction de la dose d'irradiation, au niveau d'éclairement et pour la gamme de longueur d'onde choisis,

x_0, l'abscisse du point d'inflexion de la courbe logistique,

et où x représente la quantité d'éclairement reçue par unité de surface de l'inoculum, en $J.m^{-2}$.

La courbe logistique est continûment décroissante ; quand $x=0$, $p(0) = g_0$, et quand x devient infiniment grand, p(x) tend à s'annuler.

Une fonction p et sa courbe représentative sont entièrement déterminées par les valeurs de ces trois paramètres.

4 - ESTIMATION DES PARAMETRES DU MODELE

Cette estimation consiste à déterminer les "meilleures" valeurs pour les trois paramètres, c'est-à-dire celles qui déterminent une fonction p(x) qui soit la plus proche possible des données observées.

Les estimations ont été réalisées grâce à la procédure "NLIN" du logiciel SAS/STAT, et le critère mathématique utilisé pour déterminer les valeurs des paramètres est celui des moindres carrés. Cela consiste à retenir la fonction $x \rightarrow p(x)$ telle que la sommes des carrés des écarts entre les pourcentages de germination observés et les pourcentages lus sur la courbe soit minimale.

L'estimation des paramètres g_0, a et x_0 a été réalisée sur trois jeux de données expérimentales recueillies après irradiation des spores par trois sources lumineuses différentes : en lumière solaire directe, ou dans un banc d'irradiation (Fargues et *al.*, 1989) muni d'une source composée d'une lampe OSRAM HQI-TS à halogénures métalliques et de l'un des deux filtres WG 280 (bloquant les radiations de longueur d'onde λ<280 nm), ou WG 295 (bloquant λ<295 nm). Dans tous les cas, un système de régulation de la température de surface du support de l'inoculum a permis de ne prendre en compte que l'effet photique du rayonnement.

Ces conditions d'irradiation sont telles que la gamme de longueurs d'onde la plus active est, dans les trois cas, la portion U.V. B du rayonnement. C'est pourquoi ce sont le niveau d'éclairement en U.V. B (280 nm< λ <320 nm) et la quantité d'éclairement dans cette même gamme qui ont été pris en compte lors de ces ajustements, et sont représentés sur les graphiques correspondants.

Les résultats de ces estimations sont visualisés sur les graphes de la figure 1, et regroupés dans le tableau suivant, en fonction des conditions d'irradiation de l'inoculum:

Tableau 1 : Estimation des paramètres du modèle logistique de germination en fonction de la quantité d'éclairement reçue, dans différentes conditions expérimentales.

CONDITIONS EXPERIMENTALES Source lumineuse Filtre Niveau d'éclairement en U.V. B	Lampe HQI-TS WG 280 0,64 $W.m^{-2}$	Lampe HQI-TS WG 295 0,38 $W.m^{-2}$	Soleil aucun 1,34 $W.m^{-2}$
VALEURS DES PARAMETRES DE LA COURBE LOGISTIQUE			
g_0	101,39	101,10	100,86
a	$2,13.10^{-2}$ $J^{-1}.m^2$	$9,30.10^{-3}$ $J^{-1}.m^2$	$1,44.10^{-3}$ $J^{-1}.m^2$
x_0	376,02 $J.m^{-2}$	$1,15.10^3$ $J.m^{-2}$	$5,13.10^3$ $J.m^{-2}$
D.I. $50_{UV\ B}$ ESTIMEES PAR LE MODELE LOG-PROBIT	382 $J.m^{-2}$	1,14 10^3 $J.m^{-2}$	4,71 10^3 $J.m^{-2}$

Les D.I. $50_{UV\ B}$ sont les doses d'irradiation en UV B nécessaires pour provoquer une inhibition de la germination de 50 %. Elles ont été calculées grâce à la procédure "Probit" du logiciel SAS/STAT.

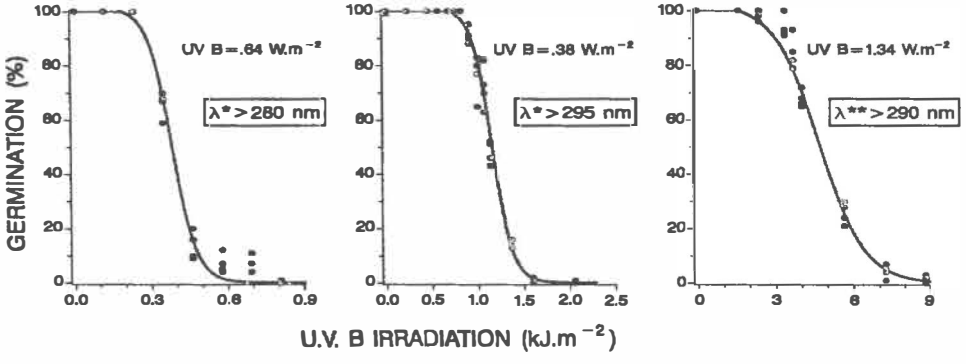

Figure 1 : Ajustement du modèle logistique sur les données de germination de l'inoculum en fonction de la quantité d'éclairement en UV B reçue, en conditions contrôlées (λ* : lampes HQI-TS) et en conditions naturelles (λ** : rayonnement solaire 700 $W.m^{-2}$).

Chacun des graphiques présente, pour un jeu de données, la courbe représentative de la fonction logistique estimée et les données expérimentales ayant servi à cette estimation. On constate, d'une manière générale, une assez bonne adéquation des estimations avec les données observées.

5 - DISCUSSION

L'élaboration d'un **modèle mathématique de persistance** de l'inoculum en fonction des conditions climatiques a été amorcée avec la construction et les premières estimations d'un module d'effet du rayonnement sur le pouvoir germinatif. On observe une **bonne concordance entre les données observées et les estimations correspondantes**. Ceci permet de considérer qu'un modèle logistique peut être retenu pour décrire ce phénomène.

La comparaison entre les abscisses x_0 des points d'inflexion des courbes estimées et les D.I. 50 calculées grâce à un modèle linéaire de type Probit (mortalité) = f(log(quantité d'éclairement)) se révèle satisfaisante. En effet, ces deux indices ont, dans les trois cas étudiés, des valeurs très proches entre elles, et représentant bien les données observées.

Cependant, les valeurs, généralement très importantes et toujours très hautement significatives, des tests de linéarité du χ^2 utilisés pour mesurer l'adéquation du modèle log-probit aux données, amènent à écarter ce modèle. De plus, le caractère essentiellement empirique de ce type de représentation ne permet pas d'accorder une signification biologique à ses paramètres (pente et ordonnée à l'origine de la droite ajustée). Il est donc impossible de juger de leur qualité, ou de se fixer des objectifs concernant, par exemple, le niveau de précision souhaité pour ceux-ci. Le modèle logistique, de type explicatif, permet de pallier cet inconvénient, puisque ses paramètres peuvent être mis en relation avec les phénomènes biologiques correspondants.

Enfin, le modèle logistique permet de prendre en compte l'ensemble des données observées, ce qui n'est pas possible avec une fonction exponentielle décroissante (Carruthers et *al.*, 1988). En effet, ce type de modèle ne représente que les points correspondants à des quantités d'éclairement supérieures à un seuil déterminé empiriquement.

La multiplication d'expérimentations dans différentes conditions devra maintenant permettre de déterminer si le niveau d'éclairement exerce ou non une influence sur la relation entre la quantité d'éclairement reçue par des spores et leur capacité à germer. Si une telle influence est démontrée, il sera nécessaire de la quantifier, afin de déterminer si, compte-tenu des objectifs, son intégration au modèle d'effet du rayonnement devra ou non être entreprise.

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Pesticide effects on *Beauveria*

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Introduction

- Our target is the control of Otiorhynchus sulcatus F. (Coleoptera Curculionidae) with the Hyphomycete Beauveria (tenella) brongniartii (Sacc.) Petch.
- We wish to check the possibilities of join use of B. brongniartii with agricultural routine products.
- We compare two "in vitro" testing methods and we observe fungal growth in both solid and broth medium.

Material and Methods

- 2 strains of B. brongniartii (isolates : 4792, 5015), 14 days old at 25°C, on 10 X diluted Sabouraud's medium, (0.2% dextrose, 0.1% neopeptone "Difco", 2% agar)
- 1 mm^3 culture is set on
- solid medium (J.J.Tuset 1985)
  potato dextrose agar
  (20% potato, 1.5% dextrose, 2% agar)
  25 ml per Petri Dish
  1 dilution/ product
  4 repetitions.
- broth medium
  Sabouraud's broth
  (2% dextrose, 1% neopetone "Difco")
  5 ml per tube
  3 dilutions/ product
  (0.1x, 1x, 10x)
  2 repetitions/dilution

Concentration 1X is the highest concentration allowed in practice.

If no growth is recorded after 14 days, new medium is provided to the fungus to test fungicidal effect.

The pesticides tested are these of the IOBC/WPRS: working group : Pesticides and Beneficial Organisms, 5th Testing programme.

Results

see tables

Conclusions

Techniques:

- Solid medium
  - easy
    - to manipulate
    - for growth observation
  - heavy
    - number of Petri dishes
    - (dilutions ?)
- Broth medium
  - easy
    - for dilutions
    - for fungicidal estimations
  - difficult
    - stuff precipitation
- Both media
  - few contaminations
  - good reading after 7 days.
  - good correlation between the 2 media

Results:

Most pesticides are harmless to B.brongniartii sometimes up to 10x concentration.
Only one : "Maneb" is fungicide against B.brongniartii

CONTROL OF APHIDS AND THRIPS WITH *VERTICILLIUM LECANII*

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At the territory of the former GDR spontaneous infections caused by Verticillium lecanii have been collected and worked on since 1979. Most of the strains were isolated from Trialeurodes vaporariorum. Only some strains were found in Thrips tabaci. For aphids virulent strains could be selected by adaption. But, however, this characteristic was lost after some subcultivations on agar. For our experiments the most promising strains were bred in submers culture and were used in form of blastospores (10^7 and 10^8 sp/ml). These special strains differ remarkably in their temperature-tolerance (in particular at temperatures below 20°C), their progress and speed of germination. For these strains also influencing measures and additional substances to spore suspension led to different reactions during the germination.

These specific characteristics of strains could be experimentally confirmed and the importance of tolerance for temperature has been emphasized.

Several promising strains were investigated in consideration of a possible multi-valency. Generally, there was a clearly visible host specifications with side-effects against at least one further pest. Only recently isolated strains show a

good efficiency against Thrips tabaci, Myzus persicae and Trialeurodes vaporariorum.

For the use of these strains in crops not only aggressiveness and appropriate climatic conditions are responsible for successes in control. Further decisive influence is given by behaviour and habit of hosts in connection with specification of different crops. With this, the neccéssity to apply the fungus in fixed intervals could be derived from. The expériments also gave information about how the characteristics of strains act under certain practical conditions.

Today these positive results justify the estimation that Verticillium lecanii can successfully enlarge biocontrol measures not only in combination with others but also allone.

8. Posters on entomopathogenic bacteria.

Laboratory and cropping chamber experiments with *Bacillus thuringiensis* for the control of the mushroom sciarid fly *Lycoriella auripila* (Diptera: Sciaridae)

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Summary

Laboratory tests illustrated the efficacy of three isolates of *Bacillus thuringiensis* against mushroom sciarid larvae (*Lycoriella auripila*). In laboratory experiments, LC_{50} values for powder formulations of IPS82 (subspecies *israelensis*), GC315 and GC327, were determined as 130, 31.4 and 12.5 mg powder/kg medium respectively. In field tests broth formulations of GC315 and GC327 were incorporated into the casing layer of a small-scale mushroom trial. A level of control equal to that achieved by the commercial chemical standard (diflubenzuron 30 mg a.i./kg casing) was attained by GC315 and GC327 at concentrations of 50 and 34 ml broth/kg casing, respectively (equivalent to 0.5 and 0.34 g powder/kg respectively).

1. Introduction

Lycoriella auripila Winn. is at present the most damaging pest of mushrooms in the UK (Fletcher et al., 1989). A similar species (*L.mali*) occupies the same niche in the USA (Cantelo 1981) and Australia (Clift et al., 1984). Unless controlled, *L.auripila* is capable of completely inhibiting mushroom production (Hussey 1973). A relationship between larval density, yield and quality loss was demonstrated by White (1986). Resistance to the chemical control agent diazinon is widespread in the UK (White et al., 1989) and control now relies on just one insecticide, diflubenzuron. With a lack of replacement insecticides attention has focused on alternative methods of control, including the use of microbial agents such as the entomopathogen, *Bacillus thuringiensis* Berliner (*B.t.*).

The *B.t.* subspecies *israelensis* (*B.t.i.*) is capable of controlling *L.mali* (Cantwell et al. 1984). Keil (1990) demonstrated that control comparable to traditional chemical insecticides was achieved, by *B.t.i.*, in laboratory and field tests. White (1989) reported several strains of *B.t.* being tested against *L.auripila* with promising results. This paper reports on the efficacy of three of these strains (IPS82, GC315 and GC327) in laboratory assays, and GC315 and GC327 in field tests.

2. Materials and methods

Laboratory experiments used larvae obtained by the method described by Binns (1973). An egg-laying chamber consisting of a polythene plastic cup holder enclosed by fly proof netting was used to hold approximately 100 gravid females. The chamber was placed on to a 7 cm Petri-dish containing moist peat and secured with an elastic band. This apparatus was lowered into a 300 ml plant propagator containing a 3 cm layer of moist peat to maintain high humidity. The lid of the propagator was replaced and secured with elastic bands. The propagator was incubated at 24°C. After two days the Petri-dish was carefully removed. Eggs which had been laid through the netting on to the moist peat were carefully removed and placed in a beaker of aerated water, horizontally divided with fine gauze. Neonate larvae emerging from the eggs passed through the fine gauze and fell to the bottom of the beaker where they were removed for experimental use.

For chamber trials eggs were obtained by the same method but could be used, once washed, after removal from the petri-dish. Eggs were then reared in a mixture of 50 g moist peat:soya medium (20:1) in 300 ml plastic pots. Emerging flies were used to infest cropping chamber experiments.

Bacterial cultures for laboratory experiments were grown in 50 ml Proflo B4 medium in 250 ml fluted flasks in an orbital shaker (250 rev/min) at 30°C. The cultures were monitored by the use of phase-contrast microscopy and harvested upon complete autolysis of the cells. Powders were made using the lactose acetone method of Dulmage et al. (1970). The activity of the IPS82 powder produced in Proflo B4 medium was 5,000 international toxicity units (ITU) per mg as determined by *Aedes aegypti* bioassay (World Health Organisation, 1981), in comparison to the international standard *B.t.i* reference powder IPS82

assigned an activity of 15,000 ITU/mg. For chamber trials GC315 and GC327 production of bacteria was as above, except that 400 ml of medium was used in 2 litre fluted flasks and the final broths were not made into powders.

2.1 Laboratory pot tests

Powders, in suspension, were serially diluted to produce a range of concentrations. 40 g of autoclaved moist peat soya medium (20:1) was treated with a prepared concentration of either IPS82, GC315 or GC327 and well mixed. The treated medium was then equally divided into a number of 170 ml plastic pots. 20 neonate *L.auripila* larvae were introduced to each pot. Four pots of untreated medium (20 g/pot), similarly infested with *L.auripila* larvae were used as controls. The pots were placed in an incubator at 24°C. Subsequent emergence of adults was recorded and used to determine the effectiveness of the treatment

2.2 Cropping chamber experiments

A standard straw-based compost (Randle, 1974) was inoculated with a commercial wheat-grain mushroom spawn of *Agaricus bisporus* Lange (Imbach) and the mycelium allowed to grow through the compost for two weeks (spawn-run) under constant temperature (24°C) and high humidity conditions. The spawn-run compost was then put into plastic plant pots (27 cm diameter) at a rate of 2.5 kg/pot. The compost was tamped down to leave a level surface about 10 cm below the rim of the plant pot. Open-ended tubes of fly-proof polyester netting (50 cm long, 30 cm diameter) were then pulled over the pots such that a 5 cm width around the circumference of the compost was covered.

A moist peat:ground chalk casing mixture (2:1 by volume) was added as an even layer (4 cm approx.) to the surface of the compost at the rate of 1.5 kg/pot, thus sandwiching the netting between it and the compost. This casing layer is required in commerce to induce fruiting of the mushroom. It is in this layer that *L.auripila* causes the most economic damage.

Prior to its application to the compost, the casing was either left untreated; treated with diflubenzuron at 30 mg a.i./kg; or treated with a broth culture of one of four doses of either GC315 or GC327. GC315 was applied at 0.4, 2.0, 10.0 or 50.0 ml/kg and GC327 at 4.0, 10.0, 25.0 or 62.5 ml/kg. Cased pots were placed in wooden mushroom trays according to a randomised plot design. The mushroom trays were then placed in a mushroom growing chamber maintained at an air temperature of 20 °C.

The mushroom chamber was infested with laboratory reared flies. After a seven day period the adult flies were killed with a non-persistent, fast-acting insecticide. A 20 x 10 cm sticky trap was placed in an inverted V (adhesive uppermost) on top of the casing. The polyester netting tube was pulled over steel wire semi-circular hoops (inserted into the casing) and secured with an elastic band forming a fly-proof enclosure over the pots. Traps were examined at regular intervals and replaced where necessary for up to 33 days post treatment. This duration was sufficient to trap first generation flies emerging from the pots.

3. Results and Discussion

3.1 Laboratory pot tests

Emergence data was assessed using probit analysis. Activities of IPS82, GC315 and GC327 are compared in Table 1.

TABLE 1 Activity of IPS82, GC315 and GC327 against *L.auripila* larvae in laboratory pot tests

Strain	LC_{50} (mg powder/kg medium)	Fiducial limits
IPS82	130.4	95.9 - 180.0
GC315	31.4	20.9 - 42.3
GC327	12.5	9.2 - 16.9

Keil (1990) in his experiments with *B.t.i.* demonstrated a 50% kill against *L.mali* with a dose equivalent to 950 µl formulated preparation/kg compost. This preparation was assumed to have an active ingredient of 1% (a typical value for commercial preparations), compared to IPS82, GC315 and GC327

which had an 'active ingredient' of approximately 4%. This indicates that in our laboratory tests IPS82, GC315 and GC327 had LC_{50}s 1.8, 7.6, and 19.1 times less (respectively) against *L.auripila* than *B.t.i* against *L.mali*.

Differences between the two *B.t.i.* strains, although small, may be explained by several reasons. The main reason could be attributed to the species differences of sciarids, even though they belong to the same genera (*Lycoriella*). Such interspecific differences have been shown in the HD-1 strain of *B.t* against two different species of *Heliothis* (*H.virescens* and *H.armigera)* (Jarrett et al., 1986). The LC_{50} values against *H.virescens* and *H.armigera* varied by a factor of 4.88, with another, transciplent, strain exhibiting a nine fold difference. In both cases *H.virescens* was the most susceptible. Bioassay procedures and formulation techniques may also contribute to the differences observed in *B.t.i.* strains. The increase in activity demonstrated by GC315 and GC327 compared to both *B.t.i* formulations was sufficiently large to demonstrate the superiority of GC315 and GC327 in controlling *L.auripila* larvae.

3.2 Cropping chamber experiments

Incorporating GC315 and GC327 into the casing material caused a reduction in the emergence of first generation *L.auripila*. Levels of control comparable to diflubenzuron were obtained with GC315 and GC327 at 50 and 33 ml broth/kg casing, respectively. Fig. 1 illustrates the greater level of control exhibited by GC327. Estimates of LC_{50} values were obtained from the regression lines; these were 3.8 and 1.1 ml broth/kg casing for GC315 and GC327 respectively, demonstrating the superiority of GC327 over GC315 in the control of first generation *L.auripila*. Equivalent LC_{50} values of 38 and 11 mg powder/kg casing for GC315 and GC327, respectively, compare directly with the results of the laboratory pot tests.

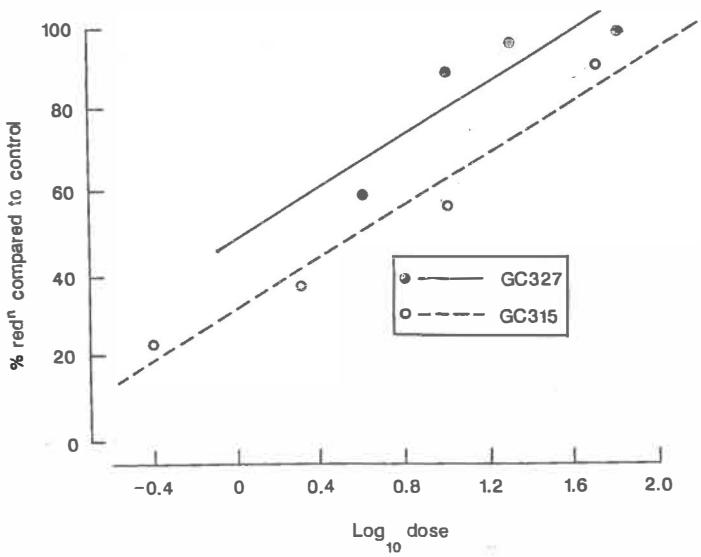

FIGURE 1 Percentage reduction in fly emergence compared to the control with increasing dose of GC315 and GC327

In a series of mushroom farm experiments Keil (1990) applied *B.t.i.* at various concentrations to mushroom crops at two stages during the cropping cycle: 177.6 ml product/m^2 (top rate) to the surface of the compost about a week after spawning; and 37.7 ml product/m^2 (only dose) as a surface drench to the casing immediately after its application - a total of 215.3 ml product/m^2 bed area. At this combined dose, a level of control of *L.mali* was achieved which was equal to, or better than, registered larvicides (including diflubenzuron) and adulticides.

The rates at which GC315 and GC327 gave comparable levels of control to diflubenzuron were equivalent to 14.63 and 9.65 g powder/m^2 bed area respectively. If it is assumed that the same

concentration of 'active ingredient' ratio is maintained for each formulation (4%:1%, see above), this represents doses 3.7 and 5.5 times less for GC315 and GC327 respectively compared to that used by Keil (1990). This appears to indicate a loss in the field efficacy of both GC315 and GC327 compared to the *B.t.i.* strain used by Keil (1990) - a phenomenon identified by Beegle et al. (1982) when comparing laboratory and field efficacies of various *B.t.* isolates against *Tricoplusia ni*. Another factor to be considered is that Keil (1990) applied a double dose of *B.t.i*, one of which was to the compost surface. This will have had an advantageous effect on the overall control of *L.mali* as, in commerce, both *L.auripila* and *L.mali* first infest the compost at or soon after spawning with subsequent generations developing in the casing layer (Fletcher et al. 1989; Macdonald, 1972). This indicates that a compost treatment, by controlling a proportion of the very first generation of larvae, may have greater success than a casing treatment alone.

Although the results from this field test are only applicable to the first casing generation of larvae, it is clear from these experiments that GC315 and GC327, in both laboratory and field tests, are capable of controlling *L.auripila* larvae at lower concentrations than that achieved by *B.t.i.*. GC327 exerts a greater level of control than GC315. In an effort to achieve maximum control of *L.auripila* however, future trials should give attention to compost as well as casing treatments.

Acknowledgements

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INVESTIGATION ON NATURAL OCCURRENCE OF *BACILLUS THURINGIENSIS* IN DIFFERENT SOILS

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Strains of *B.t.* are used to control pest insects of the order of Lepidoptera, Diptera and Coleoptera. A main source of isolation has been dead insects.
Several authors suggested on the other hand that the usual habitat of *B.t.* is the soil. For this reason we have investigated soil samples from the area of Darmstadt.

Materials and methods: 30 Soil samples were collected in a depth of 2 cm, by scraping off the surface material with a spatula. These samples were stored in plastic boxes at a temperature of 4°C.
To isolate *B.t.* from soil samples, 1g of soil was added to 20ml sterile, destilled water in a 200ml fourfold baffled flask.
This mixture was shaken for 15 minutes at 200rpm.
After shaking, the sample was treated with heat in a waterbath for 10 min at 75°C.
One drop (0,05ml) of a 1:10 dilution was plated on a T3-medium (TRAVERS et al., Appl. Environ. Microbiol. 53, 1263-1266, (1987)).
The colonies were allowed to grow overnight. Suspected colonies were chequed for the presence of crystals, which was the criterion to confirm isolates as *B.t.*.
Some *B.t.*-isolates were characterized by biochemical tests and SDS-PAGE.
Biochemical tests used, were the fermentation of mannose, sucrose, cellobiose, esculine, salicine and the production of acetylmethylcarbinol, arginine-dihydrolase, amylase, chitinase, tween-esterase and urease.

Results: 90 % of the soil samples contained suspected colonies. In 8 soil samples 155 *B.t.*-isolates were established. The quantitative distribution was very variable among the samples examined.
By light microscopy of the crystal morphology *B.t.*-isolates were examined. We were able to distinguish flat plate-like, bipyramidal and spherical crystals.
One of the isolates with flat plate-like crystals was tested in two bioassays. The protein crystals exhibited toxicity against larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*), but not against the larvae of the cabbage moth (*Plutella xylostella*). SDS-PAGE with this isolate and *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*). showed a very similar protein pattern.

Discussion: We were able to show that *B.t.* occurs ubiquitous in soil.
The isolate with the flat plate-like crystals, seems to be similar to *B.t.t.*, because the results of the bioassays and the pattern of SDS-PAGE are analogous to *B.t.t.* The question of identity can only be answered by further investigations e.g. by serological tests.

PATHOTYPIC BOUNDARIES FOR *SERRATIA* SPP. CAUSING AMBER DISEASE IN THE NEW ZEALAND GRASS GRUB, *COSTELYTRA ZEALANDICA*.

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Summary
A number of strains of the bacteria *Serratia entomophila* and *S. proteamaculans* have been found to consistently cause amber disease when fed to larvae of the grass grub, *Costelytra zealandica*. Despite considerable variation between isolates, no consistent biochemical, physical or ecological similarities have been found common to the pathogenic strains. The disease-causing strains adhere to the larval foregut wall and cause cessation of feeding, gut clearance and death. These strains constitute a pathotype and cause symptoms of amber disease only in larvae of *C. zealandica*, but not in closely related species, indicating a specific host factor involved in the disease.

Introduction

The grass grub, *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae) is the target of a biological control programme utilizing the amber disease-causing bacterium, *Serratia entomophila* (Enterobacteriaceae), in New Zealand (Jackson et al., 1991). In order to utilize the bacterium successfully it is important to understand the pathology of the disease and ecology of the bacterium.

Disease can be defined as a state deleterious to an organism which can be produced by microorganism. The development of a disease, however, depends on the coincident occurrence of pathogenic organisms and susceptible hosts (see Lysenko, 1981). Insect pathogens vary in their host range from highly specific to general pathogens capable of attacking hosts belonging to different orders. Some conditions, recognised as diseases, can be caused by a range of different organisms. For example, bacterial septicaemia, caused by the proliferation of bacteria in the haemocoel of an insect, can be caused by a number of species. Among the Scarabaeidae, bacterial septicaemia is often attributed to bacteria in the genera, *Serratia*, *Pseudomonas*, *Micrococcus*, *Bacillus* and others (Klein and Jackson, 1991). Several of these species can also cause septicaemia among other insect hosts. They possess in common a means of invading the haemocoel and overwhelming the hosts defence system. Most bacteria causing general septicaemia are potential or facultative pathogens and are able to survive and multiply outside the host. Other diseases are highly specific, with a distinct pathology caused by a single agent. Milky disease occurs uniquely in the Scarabaeidae and is caused only by strains of *Bacillus popilliae*. Some variation in strains exists, but this is accompanied by a clear selectivity for a particular host. The milky disease-causing bacteria are obligate pathogens and are generally unable to grow outside their host.

Pathogens capable of causing the same disease symptoms can be grouped together as a pathotype. For example, *Bacillus thuringiensis* serotype 8a8b includes three pathotypes showing lepidopteran, dipteran and coleopteran activity respectively (Krieg et al., 1987). A pathotype will include one or a number of strains or species with particular characteristics and have a defined host range. Amber disease in grass grub is caused by a number of strains of

Serratia. In this paper we report on studies to define the bacterial pathotype capable of causing amber disease and efforts to determine the host range showing expression of disease.

Bacterial pathotype causing amber disease in grass grub.

Amber disease is primarily characterised by a cessation of feeding and gut clearance in larvae of *Costelytra zealandica*. The disease was first recognised as a bacterial infection in 1981, when the bacterium was incorrectly identified as *Hafnia alvei* (Trought et al., 1982). A subsequent screening of bacteria isolated from the guts of healthy and diseased larvae showed that amber disease in grass grub could be caused by two species of *Serratia* (Stucki et al., 1984). These were later identified as strains of *Serratia proteamaculans* and a new species, *S. entomophila* (Grimont et al., 1988). Subsequently, grass grub larvae have been tested with a large number of *Serratia* isolates and other Enterobacteriaceae.

Bacteria for testing are generally isolated from the soil or larvae onto the selective CTA agar and further characterised by biochemical tests (O'Callaghan and Pearson, 1989). Isolates are then tested in a simple bioassay. Six to ten, 2nd or early 3rd instar larvae are fed with bacterial impregnated carrot (approx 10^6 bacteria per dose) in individual chambers of trays held at high humidity at 15°C. After 2-3 days the larvae are refed fresh carrot and examined after 7 days. Amber disease-causing strains produce a cessation of feeding and gut clearance in this time. Reference strains have also been tested from the culture collections of the Pasteur Institute and the University of California, Berkeley. The range of strains tested, isolated from insects where possible or the grass grub soil environment, is shown in Table 1.

Table 1. Bacterial isolates tested for producing amber disease in grass grub.

Serratia species	Pathogenic isolates (total no. tested)	Reassociation of DNA with *S.entomophila* DNA[1] (total no. strains)
S. entomophila	46 (82)	93 (13)
S. ficaria	0 (2)	58 (13)
S. marcescens	0 (26)	50 (12)
S. liquefaciens	0 (1)	38 (1)
S. plymuthica	0 (2)	34 (1)
S. proteamaculans	18 (45)	32 (1)
S. rubidaea	0 (6)	32 (1)
S. grimesi	0 (2)	28 (2)
S. odorifera	0 (2)	24 (1)

Other genera and species tested without effect[2];
Acinobacter calco, *Acinetobacter calcoaceticus*, *Bacillus* sp., *Bacillus thuringiensis* San Diego[3], *Citrobacter freundi*, *Citrobacter diversus*, *Enterobacter aglomerans*, *E. cloacae*, *Escherichia coli*[3], *Flavobacterium* sp., *Klebsiella oxytoca*, *Lactobacillus* sp, *Pseudomonas putida*, *Pseudomonas* sp, *Sporolactobacillus* sp, *Xenorhabdus nematophilus*[3], *X. luminescens*[3], *Yersinia enterocolytica*.

[1] From Grimont et al. (1988). [2] Bacteria isolated from the grass grub gut or soil environment (Stucki et al., 1984 or Jackson unpublished) or [3] reference strains.

Amber disease has only been produced in grass grub by isolates of *S. entomophila* or *S. proteamaculans*. Pathogenicity is a stable trait and approximately 50% of the isolates of each species have caused the disease.

The unique nature of the pathogenesis suggests a close relationship between the two species, however DNA relatedness studies show considerable divergence between *S. entomophila* and *S. proteamaculans* (Table 1). Variation exists within both species, however pathogenic and nonpathogenic strains cannot be distinguished by differences in biochemical derived biotype (Grimont et al., 1988), serotype (Allardyce et al., 1991), or sensitivity to a range of specific bacteriophages (Wilson, 1988; Turnbull, 1991).

The primary stage of infection is adhesion of the bacteria to the tissues of the foregut. Pathogenic strains of *S. entomophila* adhere to the foregut membrane while nonpathogenic do not (Wilson, 1988). This adhesion is mediated by fimbriae (Glare and Jackosn, 1990), but pathogenicity is not solely determined by the presence of fimbriae as both pathogenic and non-pathogenic strains of fimbriated bacteria have been encountered (Glare unpublished; Sadler, 1991). A genetic region encoding fimbrial production, and linked to pathogenicity, has been located in *S. entomophila* (Upadhyaya et al., 1990) but this is found to be widely homologous throughout *Serratia* spp. (Sadler, unpublished).

Following ingestion and adhesion of even small numbers of bacteria to the foregut of the larvae, the insect rapidly ceases feeding and clears the gut, taking on the characteristic disease symptoms. We have suggested that this is due to the production of an antifeedant toxin by the bacterium, but no direct evidence of such a toxin has yet been found. In the final stages of the disease (one or two months after dosing), the bacteria will penetrate the weakened gut lining and enter the haemocoel where a general septicaemia ensues.

Thus, it appears that amber disease in *C. zealandica* larvae is only produced by strains of *S. entomophila* or *S. proteamaculans*. These cells must be fimbriated and adhesive to the foregut. Pathogenic cells must cause the antifeedant reaction. Current research centres on differentiation of adhesion and "toxin".

Host range

The host range of the virulent bacteria has been investigated in feeding tests with a range of insects, including closely related scarabaeids (Table 2). The tests have all been by direct feeding of the bacteria or by environmental saturation in a manner that would cause disease in *C. zealandica*. These tests are continuing, but to date only *C. zealandica* has developed amber disease as a result of ingestion of pathogenic strains of either bacterium. It is not clear whether the failure of disease expression is due to lack of adhesion or some other aspect of disease. Bacteria have been noted in large numbers in the crops of some apparently nonsusceptible species.

Similar conditions, cessation of feeding and gut clearance, have been noted in other species but the causative agents have not been isolated. It is possible, as amber disease is chronic rather than acutely invasive, that similar diseases in other insects have been overlooked. The disease remained unrecognised during more than 50 years of intensive research on grass grub although it is now recognised as a major factor in population collapse in some areas.

Table 2. Insects tested for susceptibility to *Serratia entomophila*

Coleoptera	Scarabaeidae		
		Melolonthinae	*Costelytra zealandica* (White), *Pyronota festiva* (F.), *Odontria striata* White, *Odontria* sp. *Chlorochiton* sp.
		Dynastinae	*Adoryphorus couloni* (Burm.), *Heteronychus arator* Burm., *Pericoptus truncatus* (F.)
		Aphodinae	*Aphodius tasmaniae* Hope
		Rutelinae	*Popillia japonica* Newman
	Lucanidae		*Lissotes* sp.
Lepidoptera	Galleriidae		*Galleria mellonella* L.
	Tortricidae		*Epiphyas postvittana* Walker
	Hepialidae		*Wiseana* sp.
Hymenoptera	Apidae		*Aphis mellifera* L.

Amber disease appears to be unusual in that it is a highly specific disease affecting a single known host species, but it is one that is caused by facultative bacteria with the ability to survive and multiply outside the host. Therefore the "host factor" determining susceptibility is of interest. The site of bacterial adhesion, the chitinous foregut membrane is the subject of further study.

S. entomophila and *S. proteamaculans* - Parallel evolution or genetic transfer?

Two species within a genus causing the same disease is perhaps not unexpected, especially considering that bacterial taxonomy is largely based on biochemical reactions, and species definition is somewhat arbitrary. However, it is unlikely that amber disease is a case of divergent evolution. Studies on DNA homology among *Serratia* (Grimont et al., 1988) demonstrate that other species, present in New Zealand, are more closely related to *S. entomophila* than is *S. proteamaculans*. As the expression and characteristics of amber disease are indistinguishable when caused by strains of either species, questions are raised about how pathogenicity developed in only these bacteria. Nonpathogenic strains of both species are still common in the pasture environment, and the very specific nature of the disease suggests that this bacterium/insect interaction is unlikely to have independently developed in two *Serratia* species in the same country. More likely, some genetic interchange has occurred between strains, possibly by conjugal transfer of plasmids or transduction via infecting bacteriophage.

Investigation into the basic genetics of both species is a promising area of current research, and capable of resolving these questions.

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RECHERCHE DE SOUCHES SAUVAGES DE *Bacillus thuringiensis*. SENSIBILITE DE *Leptinotarsa decemlineata* (COLEOPT. *Chrysomelidae*) A LA SOUCHE LM 63

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La Station de Recherches de Lutte Biologique de La Minière a créé, en 1988, une chaîne de recherche et de criblage de souches de *Bacillus thuringiensis* ayant de nouvelles propriétés insecticides.

Un réseau de correspondants à travers le monde, a permis de récolter plus de 4000 échantillons divers provenant de 75 pays différents. Nous avons récolté également des échantillons dans 90 départements français.

Après analyse des échantillons, les souches isolées sont cultivées pour permettre leur caractérisation biochimique par électrophorèse et leur caractérisation biologique par essais sur différentes espèces d'insectes.

A partir de 3000 échantillons analysés, un peu plus de 900 souches ont été isolées. Parmi elles, deux se sont révélées être actives contre les larves du doryphore, *Leptinotarsa decemlineata*, les souches LM 63 et LM 79.

L'analyse électrophorétique de ces deux souches a mis en évidence la présence de 3 bandes de protéines à 70-73 Kd.

Nous présentons l'activité de la souche LM 63, sur différents stades larvaires de *L. decemlineata*. A la dose utilisée, 100.10^{-5} mg de protéine par cm^2, la mortalité est très importante sur les deux premiers stades, elle diminue lorsque les insectes atteignent les troisième et quatrième stades larvaires. On notera qu'à la dose utilisée, les adultes ne sont pas sensibles.
Afin de mieux situer le degré d'efficacité de la souche LM 63, nous avons comparé sa dose létale 50 sur *L. decemlineata* aux premier et deuxième stades larvaires, à celle de la souche Aizawa 7-29 utilisée sur *Spodoptera littoralis* (la noctuelle du cotonnier) connue pour être active sur cette espèce. Au premier stade larvaire les DL 50 sont sensiblement égales (2.10^{-5} mg de protéine par cm^2 pour *S. littoralis*). Au deuxième stade larvaire, la DL 50 est trois fois plus élevée sur *Leptinotarsa decemlineata* (34.10^{-5} mg par cm^2) que sur *Spodoptera littoralis* (10.10^{-5} mg par cm^2).

9. Posters on entomopathogenic viruses.

ACCUMULATION AND PERSISTENCE OF A NUCLEAR POLYHEDROSIS VIRUS OF AUTOGRAPHA CALIFORNICA IN FIELD SOIL

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Summary
In risk assessment studies, the field persistence of nuclear polyhedrosis virus was determined by estimation of the virus quantity in soil over several weeks. In 1989 and 1990, plots in sugar beet fields were sprayed with the nuclear polyhedrosis virus of *Autographa californica* (AcNPV) at a concentration of $5x10^8 p/m^2$, and seeded with larvae of the beet armyworm, *Spodoptera exigua*. Soil was sampled weekly from the plots and bioassayed with different methods against two host insects. The results in the first year indicated a tenfold increase of the amount of virus during a period of four weeks. The concentration of virus from the soil in the 1990 field trial showed a decline over the whole observation period of ten weeks. The virus amount decreased by a factor 5,5 as compared to the virus quantity directly after the treatment. This inconsistency from one year to the other is probably related to the different weather conditions in the two years.

1. Introduction

The role of soil as a reservoir for the initiation of viral epizootics in field populations of insects has been discussed previously (Jaques,1974). The aim of this study was to evaluate possible risks associated with the release of genetically engineered baculoviruses for use in plant protection. Since field trials with genetically modified viruses are not welcome in Germany, a natural virus, which usually is not present in the given crop system, was used as a substitute.

2. Methods

In summer 1989 AcNPV was applied by a hand sprayer to a plot of $6,3m^2$ in a sugar beet field at a dose of $3,1x10^9$ polyhedra (corresponding to $5x10^{12}$ polyhedra per hectar). A second application of the same virus concentration was carried out in summer 1990 in eight separated plots of $1,4m^2$ in another sugar beet field. Since natural attack was not expected, second instar larvae of *S.exigua*, (80 larvae per m^2) were released in each plot after the spray deposits had dried. During one month in summer 1989 soil samples were taken weekly from the surface of different parts of the field plot. In 1990, in order to be able to estimate the virus amount over a longer period, each soil sample was collected from a different plot.
The viral activity of the field samples was determined in bioassays. Aliquots of soil were incorporated in a semi-synthetic diet and fed to first instar larvae of S. *exigua*. For this, polyhedra were recovered from soil samples by a simplified extraction procedure with SDS (Evans et al.,1980), or aliquots of the soil were mixed directly into the medium. When virus mortality in bioassays was low, a more susceptible host insect, *Autographa gamma*, was used. In standard bioassays the virulence of AcNPV against *A.gamma* was about ten times higher than against *S. exigua*.
The weather data recorded during the experimental periods (July/August 89 and July-September 90) are shown in figure 1.

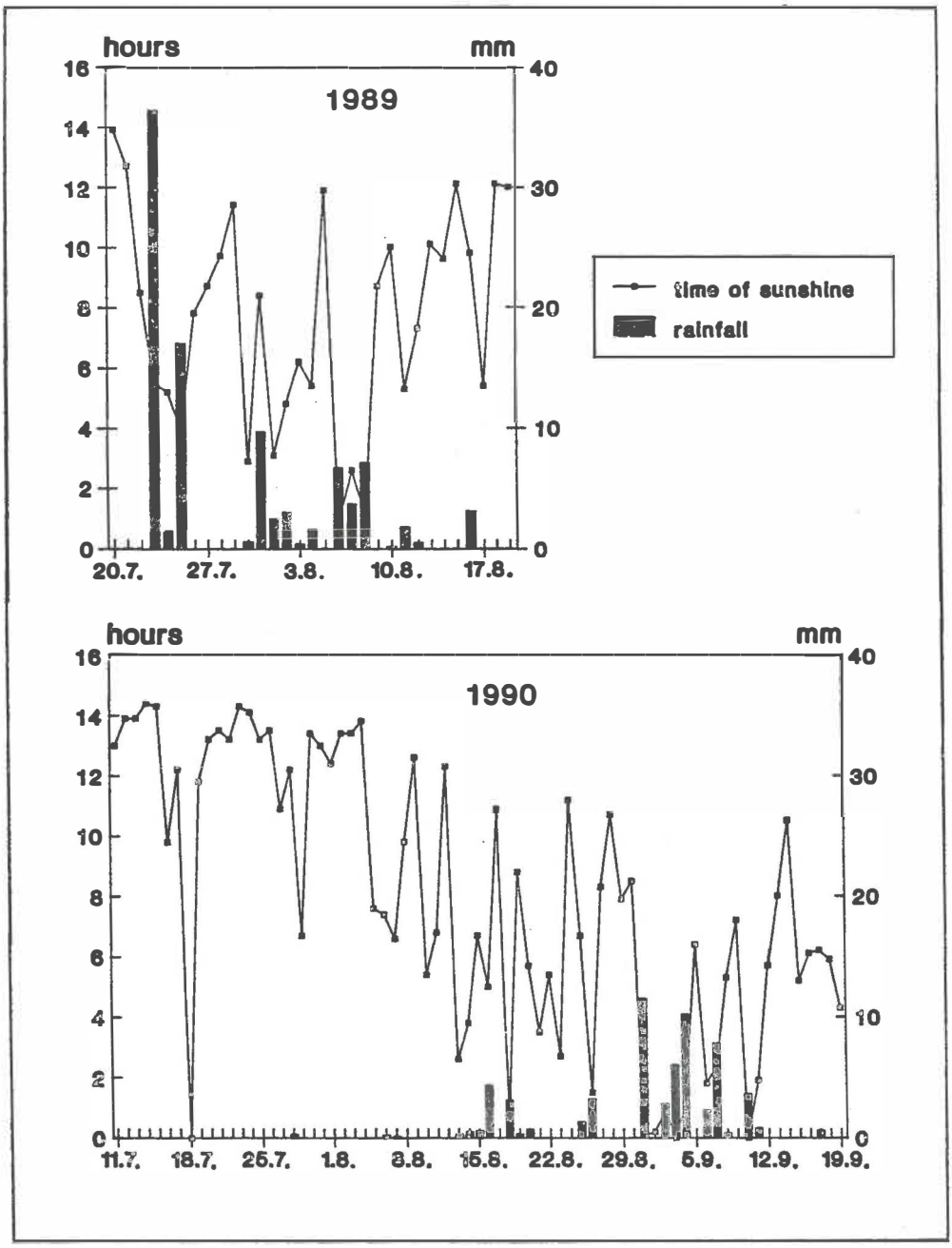

Fig.1: Rainfall and sunshine-hours in 1989 and 1990

3. Results

Field trial 1989: The results obtained from soil samples bioassayed against *S. exigua* are presented in figure 2. The data of the virus quantification show an increase of the virus amount over the whole observation period of four weeks. In the first sample, one week after spraying, $2{,}4 \times 10^3$ polyhedra were detected per g soil. In the second and third week the virus amount increased by a factor 2 ($4{,}9 \times 10^3$p/g soil) and 2,4 ($5{,}8 \times 10^3$p/g soil), respectively, as compared to the virus residues found in the first week. At the end of the experiment the virus amount was ten times higher than after the first week.

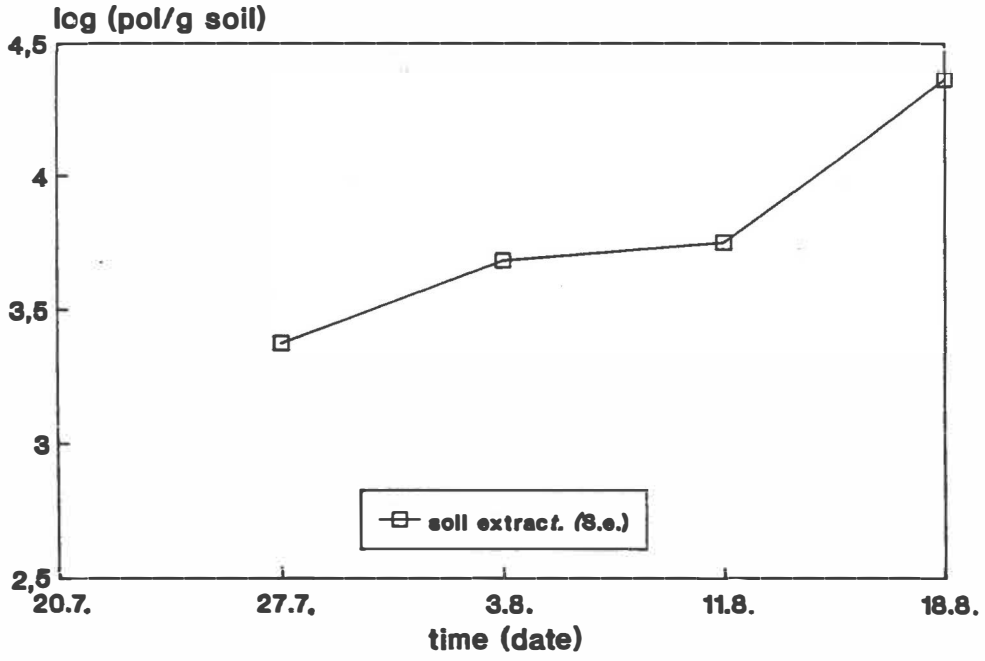

Fig.2: Change of virus concentration in soil in 1989. Data were obtained by a bioassay method with extracted soil fed to larvae of *S. exigua*

Field trial 1990: The results obtained from soil samples bioassayed against *S. exigua* and *A. gamma* are compared in figure 3. Generally all three lines obtained by different bioassay methods showed the same tendency of virus decline in soil. Directly after spraying, an average of 1×10^4 polyhedra were detected per g soil. In the first and second week, the virus amount dropped by a factor 2,5 ($4{,}1 \times 10^3$ p/g soil) and 3,6 ($2{,}8 \times 10^3$p/g soil), respectively, as compared to the virus determined directly after the treatment. The following four weeks gave a fairly constant concentration of $2{,}5 \times 10^3$p/g soil. From the sixth week after spraying, the virus concentration in soil was too low to be detected in bioassays with *S. exigua*. At the end of the experiment the virus amount was five times lower than at the beginning.

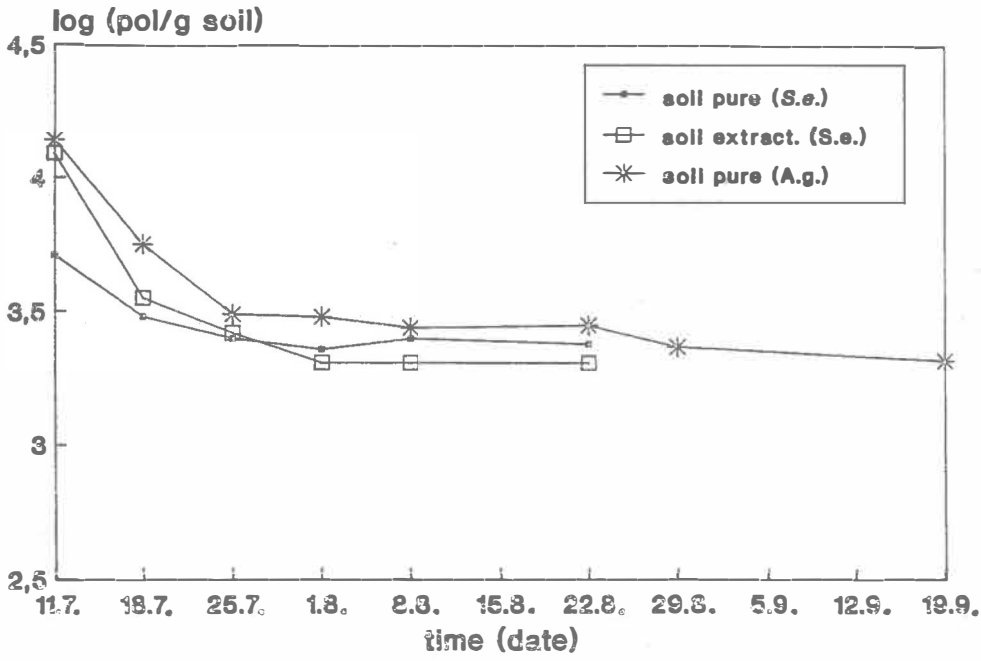

Fig.3: Change of virus concentration in soil in 1990. Data were obtained by three bioassay methods.

4. Discussion

The results of the field trials were rather different from one year to the other. This discrepancy may be attributed to differences in the weather conditions recorded for both years. The increase of virus in the field is due to the virus propagation in infected host insects. From larval cadaver a large number of viruses are released representing new sources of infection for healthy host larvae. Eventually the virus is washed into the soil by rain. The virus increase in soil found in the 1989 field trial corresponds very well to the rainfall pattern during the experiment. In 1990, the amount of virus in the field soil decreased, particularly at the beginning. This can be explained by a period of intensive sunshine during the first three weeks of the experiment. It is known, that the sun's UV-radiation inactivates the viruses within short time. The absence of rain on the other hand prevented that the virus was washed from the foliage. The results of the trials demonstrated that the fate of viruses released in the field depends very much upon environmental conditions. They emphasized the necessity of further studies.

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Persistence of insect viruses in tropical and temperate zones

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Introduction

Research on formulation of insect viruses at NRI has used *Spodoptera littoralis* NPV as a model system. As part of this research extensive studies on environmental persistence have been undertaken. These persistence studies have been carried out mainly in Egypt. It was concluded that two factors determine the persistence of the virus in the field, these were inactivation by sunlight and physical loss of polyhedra from the crop. Based on this study a wettable powder formulation of the virus has been developed and the persistence of this formulation predicted (Jones 1988, McKinley *et al* 1989). It is important, however, to determine whether the results obtained in Egypt can be applied to other areas so that, if necessary, the formulation can be adapted.

This paper describes the first steps to the assessment of persistence in different regions of the world and will concentrate on the effects of sunlight on the virus.

Relationship between solar radiation and virus inactivation.

The wavelengths present in sunlight and reaching the Earth's surface that are responsible for inactivating the virus were determined by exposing virus deposits to sunlight filtered through Schott colour glass filters. Results confirmed that most inactivation results from exposure to wavelengths below 320 nm; although there was some inactivation due to exposure to wavelengths above

665 nm this represented less than 10% of the total inactivation observed. Experimental design eliminated the possibility that this latter inactivation was a result of heating of the samples. It was concluded that for practical purposes measurements of the intensity of solar UV radiation at wavelengths below 320 nm could be used to determine the relationship between exposure to sunlight and virus inactivation.

Experiments to relate solar UV dose to virus inactivation were carried out over a four year period. Virus was exposed to direct sunlight in Egypt between 10.00 and 14.00 hrs, throughout the exposures the UV dose below 320 nm was measured using polysulphone film (Davis *et al* 1979). From the results a calibration curve relating UV dose to virus inactivation was constructed.

Inactivation of NPV in different regions of the world.

The actual degree of inactivation (for Scotland, England and Egypt) and degree of inactivation estimated from UV dose (India and Thailand) of *S. littoralis NPV* at five different regions in the northern hemisphere was estimated. Exposures/estimates were made for times of the year when pest control operations would be undertaken (Egypt, England and Scotland = June, Thailand = September, India = November). The degree of inactivation increased toward the equator, as did the UV dose received. After 8 hrs exposure the degree of inactivation of virus exposed in Thailand was estimated to be one hundred times that observed with virus exposed in Scotland.

Rates of inactivation of virus at each region were estimated from the slopes of the inactivation curves and these were plotted against latitude. The resulting relationship can be used to estimate the degree of inactivation at different sites.

Conclusions

The results of this study can be used to aid the prediction of virus persistence in different countries. The relationships obtained are as one would expect i.e. in tropical regions the rate of inactivation is significantly faster than in temperate regions.

These results, however, do need to be put into the context of practical pest control. Virus sprays are rarely exposed to direct sunlight, rather they are deposited on crops which provide shade. Thus in Egypt UV doses received on the undersurface of leaves on the cotton plant (where the target insects are located) have been measured to be between 1 and 11% of the dose received in direct sunlight (Jones 1988). Similar figures have been obtained for Thailand. This means that the rate of inactivation of the virus on parts of a plant where it matters i.e. where the target insects will ingest the virus, is slower than results obtained in direct sunlight suggest. However, the persistence in different regions of the plant could be estimated from UV readings by use of the calibration curve relating dose to degree of inactivation; this has been done in Egypt (Jones 1988, McKinley *et al* 1989).

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SUSCEPTIBILITY OF THE OLIVE FRUIT FLY TO VIRAL INFECTIONS

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The olive fruit fly, *Dacus oleae* (Gmelin), is the key pest of olive cultures in Mediterranean countries. Electron microscope examination of homogenates from insects collected from 22 areas of Greece showed three types of parasphaerical virus particles approximately 60, 40 and 30 nm diameter.

Of these 3 viruses, the largest one received more attention. Examination of purified suspensions showed that the virions consist of a double capsid. Positive reaction to orcinol and polyacrylamide gel electro-phoresis revealed that the viral genome consists of 10 segments of double stranded RNA. These data along with the morphology of the virion strongly suggest its relationship to the Reovirus group.

The other two types of virus particles were tentatively assigned to other groups of viruses on the basis of their morphological characteristics. Thus the virus particles of 40 nm in diameter are similar in size and shape to the β Nudaurella group, while the virus particles of 30 nm diameter are morphologically similar to the Picornavirus group.

Experimental infections of virus-free flies both *per* os and by inoculation using purified suspensions of the Reovirus type and of 8 other entomopathogenic viruses proved that olive fruit fly is sensitive to some of them.

In the case of experimental infections with viral particles of the Reovirus type, 95% mortality occured within 15 days whereas the average lifespan of healthy adult is 27 days in laboratory conditions (26±1°C and 65% RH). Electron microscope examination of ultrathin sections showed an intense multiplication of the virus in the midgut epithelial cells. Large viroplasms developed in the cytoplasm of infected cells as well as accumulation of virions in paracrystalline arrays. These are typical features of cells infected with Reo-viruses.

Among the other types of viruses used the Picornavirus CrPV and the Iridovirus CIV proved to be the most infectious by killing 85% of the insects within 3 and 7 days, respectively, when administrated by injection. E.M. examination of thin sections of inoculated insects showed that the main organs of the adult insects, are infected.

These experimental infections showed that the olive fruit fly is sensitive to several viruses and indicate the possibility of using these viruses as regulating factors of natural populations of this pest.

VIRAL PATHOGENS OF THE ARCTIIDAE Ocnogyna baetica RAMB.

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During a four year period (1987-1990) from November to April, Ocnogyna baetica larvae were collected weekly from infested fields at different localities situated in a circle of 20 km around Córdoba. In the laboratory the collected larvae were cheked every twenty four hours to determine the presence of pathogens and parasites.

Two viral pathogens, a granulosis virus (GV) and an entomopoxvirus (EPV), were identified by phase-contrast and electron microscopy. The GV was the most common and widespread entomopathogen. These are the first two reports of viral pathogens on this species.

Third instar larvae of four noctuid species, Agrotis segetum, Spodoptera littoralis, Spodoptera exigua and Mythimna loreyi, were used in cross infectivity tests. The EPV gave infection to A. segetum and S. littoralis larvae.

Dose-mortality and time-mortality relations were obtained by bioassays at 26 C using newly molted third-instar larvae of O. baetica. LD_{50} values were 44 and 6663 IBs/larva for GV and EPV respectively. The mortality periods were ranging between 5 and 25 days in both viruses.

After these results, the possible use of these viruses for biological control of O. baetica populations is being investigated.

EPIZOOTICS CAUSED BY A NUCLEAR POLYHEDROSIS VIRUS (NPV) IN POPULATIONS OF Spodoptera exigua IN SOUTHERN SPAIN

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In the South of Spain a Nuclear Polyhedrosis Virus (NPV) has been observed as a mortality factor in populations of Spodoptera exigua, the beet armyworm. The incidence of this NPV and other control agents have been determined from samples of larvae collected from sunflower fields in Azuaga (Badajoz), during an unusual high infestation in July of 1988, and from vegetable greenhouses in El Ejido (Almería), during experiments conducted without application of chemicals insecticides in late May and June of 1990.

The NPV was the only disease agent identified in both sites. Beet armyworm mortality in Azuaga was also caused by three parasitoid species, the braconid Meteorus pulchricornis, the ichneumonid Hyposoter dydimator and the tachinid Gonia bimaculata, and an unidentified fungus.

A epizootic was evident in each site at the first sampling date with 4.3 and 22.5% of S. exigua larvae infected by a NPV in Azuaga and El Ejido respectively. Since the sunflower fields at Azuaga were sprayed with chemical insecticides against S. exigua, it is not known how effectively the epizootic has controlled the insect population. Through the period surveyed the pecentage of infected larvae increased from 4.3 to 23%. Larval mortality due to parasitoids also increased from 12.1 to 29.9% for M. pulchricornis and from 6.2 to 12.6% for H. dydimator, but G. bimaculata contributed relatively little to larval mortality. Simultaneous infections due to the NPV and M. pulchricornis were found in six larvae on July 15. Parasitoid survival was possible in all of these.
During the course of the study at El Ejido, a S. exigua population density peak occurred around June 6, followed by a quick decline the week thereafter. The percentage of NPV-infected larvae detected at the first sampling date increased, coincidently with the increase of the beet armyworm population, and at the peak population time reached a 81%. After this time the percentage of the NPV infection remained above 81% and ultimaly reached 100%. The S. exigua population decline was attributed to the NPV.

No mortality attributed to the NPV was found in samples from Azuaga of Heliothis armigera, Peridroma saucia and Plusia gamma or in collection from El Ejido of S. littoralis and P. gamma.

This is the first report of epizootics caused by a NPV in S. exigua populations in field and greenhouse crops in Europe.

DIVERSITE GENETIQUE ET SPECTRE D'HOTES DE POPULATIONS NATURELLES DE BACULOVIRUS DE POLYEDROSE NUCLEAIRE D' ANTICARSIA GEMMATALIS

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Summary

The analysis of the genetic variability and of the evolution of natural and experimental populations of baculoviruses are an efficient means to assess the capacity of change in the viruses. Possible introduction of foreign genes in the genetic pool of natural population through engineered virus released in nature should be exhaustively considered. Two Anticarsia gemmatalis MNPV natural populations from Brazil have been studied. A tremendous genetic variability has been observed in both viral populations by DNA restriction analysis. Modifications of the composition of the two natural populations have been observed when AgMNPVs are multiplied on Spodoptera frugiperda or Spodoptera littoralis cells. The implications of such a variability on recently proposed strategies of biological controle are adressed.

1. Introduction

Il y a une différence radicale entre la situation d'un germe manipulé confiné dans des installations industrielles qui doit impérativement disparaître au plus tôt de l'environnement en cas de dissémination accidentelle et le cas des germes manipulés qu'on introduit volontairement d'une manière massive et répétitive dans l'environnement dans le but de détruire des populations d'insectes. Dans la première situation les mutations destinées à réduire les probabilités de survie du germe dans la nature sont des précautions utiles, car elles diminuent les risques associés aux gènes potentiellement dommagables lorsqu'ils sont déconnectés de leur genome d'origine. Dans le cas des insecticides viraux cette précaution n'a pas beaucoup de sens et risque pour le moins d'être illusoire. En effet, toutes les conditions sont remplies lors des traitements par insecticides viraux pour implanter dans les populations d'insectes les séquences issues des manipulations génétiques. Si de telles séquences sont des séquences de virus d'insecte, les virus manipulés ne sont guère différents des recombinants naturels qui jalonnent l'évolution des baculovirus.

Si on considère que des gènes ne doivent pas s'implanter dans des populations naturelles parce qu'ils présentent potentiellement un danger, il est illusoire de croire que la présence de mutations destinées a réduire la persistance des virus manipulés soit efficace pour interdire les immigrations de gènes et ceci à cause de l'importance considérable exercée par les recombinaisons génétiques légitimes et illégitimes chez les baculovirus.

Dans le cadre d'une Réunion qui traite d'Ecologie et d'Epizootiolgie d'Entomopathogènes naturels et relâchés notre propos est de montrer que l'examen de la diversité des populations naturelles de baculovirus, telle qu'elle ressort de l'exemple du baculovirus de la polyédrose nucléaire d'Anticarsia gemmatelis (AgMNPV), ainsi que l'étude de l'évolution de ces populations chez des hôtes de remplacement (culture cellulaire de Spodoptera frugiperda ,lignée Sf9 et de Spodopera littoralis, lignée SL) montrent une placticité de ces virus qui doit être prise en compte dans les stratégies de fabrication des insecticides viraux.

2. Matériel et Méthode

- Virus. Deux préparations d'AgMNPV originaires du Brésil sous forme de suspension de polyèdres destinées pour la lutte biologique désignées par souche Ag et souche F3 ont été multipliées sur cultures cellulaires.

- Cultures cellulaires. Les cellules de la lignée Sf9 sont entretenues en milieu TCl00, 10% SFV. Les cellules de la lignée SL sont entretenues sur milieu de Grace, 15% SFV. Les cellules sont en premier lieu transfectées à l'aide des ADN issus des polyédres par la méthode de transfection au DOTMA. Quatre séries de 10 passages des surnageants infectieux(notés Pl à Pl0) avec une multiplicité d'infection toujours supérieure à 100 (m.o.i.>100) ont été réalisées. Deux purifications par la technique de dilution limite (trois dilutions limites successives) ont été réalisées avec la souche virale F3 sur Sf9 passage P0 et sur la lignée SL passage Pl0.

- Analyse des populations virales. Les ADN des deux préparations initiales ainsi que l'ADN issus des virus purifiés par dilution limite ont été analysés par les profils de restriction pour les endonucléases BamHl, EcoRl, Bgl2, BstE2, HinD3 et Pstl. Le virus purifié de référence AgMNPV-2 nous a été aimablement fourni par J.Maruniak ; la carte physique du génome correspondante est celle établie par D.Johnson et J.Maruniak (1989). La diversité des populations et leur évolution sont appréciées par les variations des fragments inframoléculaires et par le typage des virus clonés établis sur plusieurs enzymes. L'ADN nécessaire au typage d'un virus est obtenu par purification des polyédres issus de 5 à 10 ml de culture.

3. Résultats

Spectre d'hôtes . Les souches F3 et Ag d'A.gemmatalis se répliquent sur les larves de Spodoptera littoralis, Prodenia litura, Mamestra brassicae et Galleria mellonella pour des doses massives de virus injectées en provenance de surnageant de cultures cellulaires ou de polyédres solubilisés par des solutions de carbonate de sodium. Ces infections croisées ont toutes été authentifiées par identification des profils de restriction.

La transfection in vitro a été réalisée pour les souches F3 et Ag sur les cultures Sf9 et SL. Les surnageants de transfection sont hautement infectieux . Au cours des passages successifs le pourcentage de cellules Sf9 présentant des polyédres est passé de 20 à 80% , les premiers passages se sont effectués tous les 6 jours pour finir par des passages tous les trois jours. La multiplication des populations virales sur les cellules SL est beaucoup plus faible et 10% des cellules au maximun présentent des polyèdres; les passages s'effectuent tous les sept jours.

Tableau

TYPAGE DES PLAGES : POPULATION AGMNPV-F3								
ENDO NUCLEASES	PLAQUES							
	REF	1A	7A	8A	9A	2B	3B	1G
BamHI	1	1	1	1	1	1	1	1
BstEII	1	2	2	2	2	2	2	2
BglII	1	1	2	1	2	1	1	2
PstI	1	2	3	2	3	3	2	3
EcoRI	1	3	2	3	3	3	2	2
HinDIII	1	3	4	2	4	2	3	4
General Restriction Types	A	B	C	D	E	F	G	C

Diversité génétique . L'importance des fragments inframoléculaires observés dans les profils de restriction des ADN des populations Ag et F3 indique une hétérogénéité génétique majeure dans ces populations naturelles. Ainsi le clone AgMNPV-2 posséde 10 fragments EcoRl et on dénombre dans les deux populations 8 fragments EcoRl infra-moléculaires supplémentaires . Les 7 premiers clones de la population parentale F3 (purifiés par dilution limite sur cellules Sf9) se répartissent par typage à l'aide de 6 endonucléases dans 6 types différents comme indiqué dans le tableau 1.

Influence des hôtes secondaires sur l'évolution des populations virales. L'examen comparatif des profils de restriction obtenu à partir de l'ADN des populations parentales obtenues sur larve d'A.gemmatalis et après 10 passages des populations Ag et F3 sur les cellules Sf9 et SL montre une évolution quantitative des principaux fragments inframoléculaires.

4 .Discussion

Le clonage de 6 génomes différents pour les 7 premiers clones tirés d'une population virale laisse non seulement présager une multitude de types viraux mais signe un brassage important des marqueurs de restriction dans la population. Un tel exemple qui demande toutefois un supplément d'examen n'a rien pour surprendre quand on considére l'ampleur de la recombinaison génétique chez ces virus. Ces résultats recoupent ceux de Maruniak(1989) qui obtient 13 types de restriction différents pour 34 clones issus d'une population d'AgMNPV après 20 passages sur Diatrea saccharalis. La recombinaison de gènes chez les baculovirus est et sera de plus en plus exploitée à des fins biotechnologiques. Une évaluation réaliste de l'impact de cette recombinaison dans les opérations de lutte biologique est nécessaire en vue d'homologuer de futurs procédés d'emploi d'insecticides viraux.

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SURVIVAL OF GENETICALLY MODIFIED NPVs IN A MODEL-ECOSYSTEM

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Summary

To assess the potential risk of the release of genetically modified baculovirus, a contained model-ecosystem has been developed, in which the field release of a virus can be simulated. Microcosms components included soil, tomato plants and *Spodoptera exigua* larvae, simulated wind, rain and sunlight. The study concentrated on budgeting of NPV production and dispersal and involved foliage, soil and infiltrating water. When the recombinant virus of Autographa californica (AcMNPV/p10Z), containing a p10/β-galactosidase fusion gen, and the wild type AcMNPV were applied as a mixture (in equal quantity) at a single dose of $2x10^8$ PIBs, the total virus amount in the ecosystem increased by a factor of 13 as compared to the original inoculum. About 74 % of the virus was found in the soil, 26 % on the leaves and none in the drainage. Examination of the progeny virus showed predomonation (93 %) of the wild-type.

1. Introduction

Some wild type baculoviruses are already established as environmentally safe biopesticides. Efforts are being made to improve there competitiveness with chemical insecticides by genetic engineering. The objective is to enhance their viral activity, speed of action, and host range in the field. The use of these recombinant viruses in plant protection requires basic ecological studies for assessing possible risks associated with their release into the environment.

2. Methods

The studies were carried out in a contained model-ecosystem (microcosm) in which the release of the virus in a 0,6 x 1,20 m part of a cultivated field could be simulated. The facility incorporated controlled filtered air flow, light (including UV) and simulated rainfall. Water percolating through the soil could be collected for analysis.

In the present experiment, two different virus strains - the wild-type multiple embedded nuclear polyhedrosis virus of *Autographa californica* (AcMNPV), strain E2 (Oxford, GB) and the genetically manipulated AcMNPV/p10Z strain AK2 (VLAK et al., 1988) - were used. The latter, a recombinant virus expressing a p10-β-glactosidase fusion gene can be detected in smear preparations of virus killed host insects by the appearance of blue colour in the presence of X-Gal (substrate for β-galactosidase).

The investigations were concentrated on the environmental behaviour of the genetically modified virus, in particular their movememt and dispersal as compared to the unmodified virus.
The microcosm components included soil as a substrate, tomato plants and first to third instar larvae of *Spodoptera exigua*. To study interactions with naturally occuring virus, a mixture of both viruses (modified and unmodified in equal quantity) was introduced at a single dose of 2 x 10^8 polyhedra, corresponding to $5x10^{12}$ polyhedra per hectar. One month later, the concentrations of polyhedra on the foliage, in the soil and in the infiltration water were measured. In order to do this, virus particles were first washed from plants and extracted from soil samples by a procedure modified from EVANS et al. (1980). Aliquots of the plant and soil extracts were then mixed into semisyntetic diet and bioassayed against first instar larvae of *Spodoptera exigua*. Bioassays were incubated 12 days at 26 ^{0}C and 16 h daylight. To determine the virus concentrations, mortality data in the test assays were calibrated with standard virus suspensions of known concentration.

3. Results

The results of the virus quantification showed that compared to the original inoculum the total virus amount in the ecosystem had increased by a factor of 13. Analysis of the general distribution of the virus among the system components showed that approximately 74 % of the total virus amount was found in the soil, 26 % on the leaves and none in the drainage (figure 1).

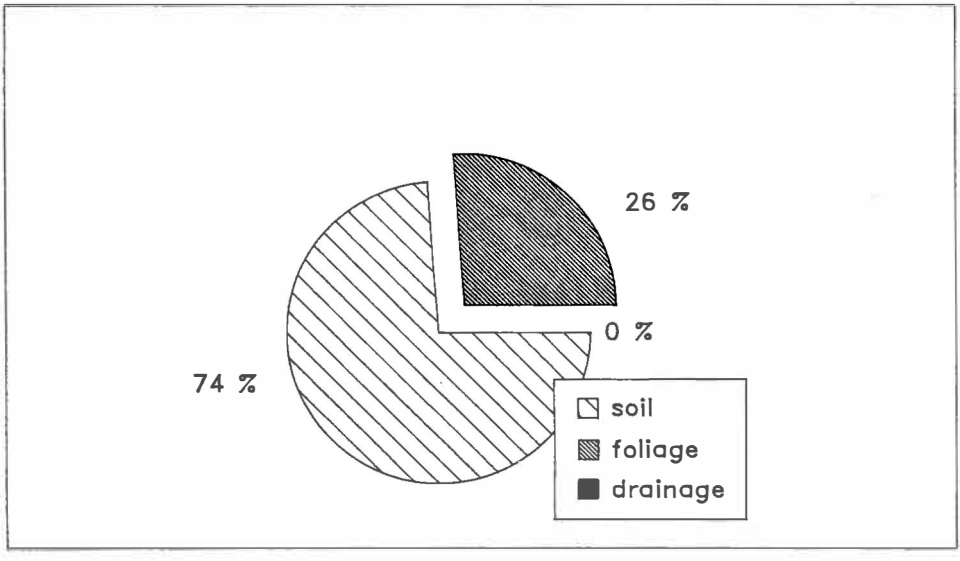

Fig. 1 General distribution of virus among the components of the ecosystem.

To estimate the ratio of both virus types (recombinant and unmodified virus) the larvae from the bioassays were analysed for the presence of the engineered virus, using the β-galactosidase reaction as an indicator. The following figure 2 represents the amount of recombinant and wild type virus determined on the foliage and in the soil in comparison to the inoculum sprayed.

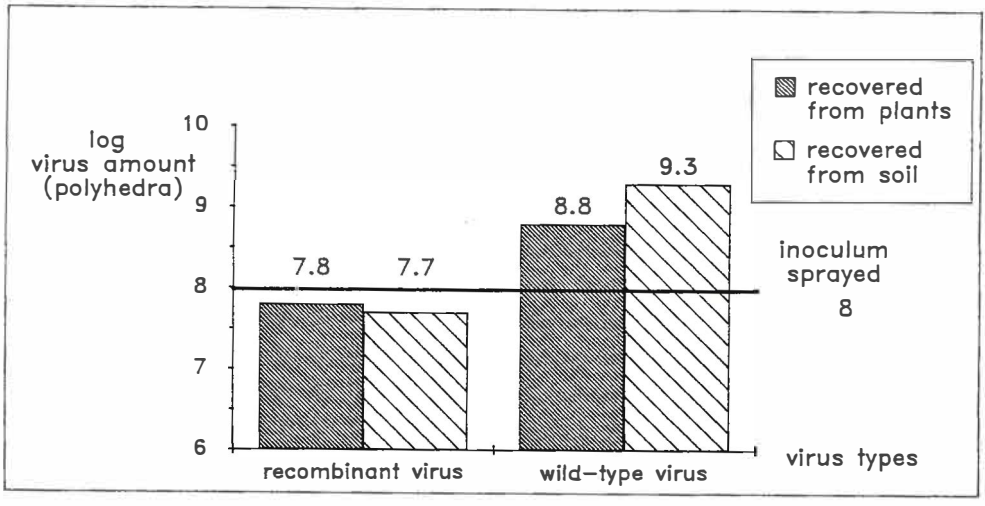

Fig. 2 Total virus amount in the components of the ecosystem one month after application, in comparison with the initial inoculum.

The results showed that the recombinant virus yield was less than for the wild type virus. In comparison with the inoculum (1 x 10^8 Pol), the wild type virus increased by a factor of approximately 25, whereas the amount of the recombinant virus remained nearly the same (1,12 x 10^8 Pol). Only 8-9 % of the polyhedra recovered from the plants and approximately 3 % of the virus found in the soil was identified to be of the recombinant type.

4. Discussion

The budgetary studies in a contained microcosm have demonstrated that a spray release can result in an increase of the total virus amount as compared to the initial inoculum. Simulated rain dispersed viruses on the leaves, washed them off and led to an accumulation in the soil. The finding, that no virus was found in the percolation water, emphasize that soil is very effectiv in virus adsorption to prevent contamination of groundwater.

Despite similar levels of infectivity of recombinant and wild viruses, less recombinant virus was produced in the microcosm when a mixture of both was applied to the same larval population. This result was also confirmed in further experiments, where each of the two viruses was applied individually in a separate containment of the microcosm. The lower persistence of the recombinant virus is probably due to the deletion of the p10 gene, causing a failed or reduced cell lysis (WILLIAM et al., 1989). Therefore, recombinant viruses were less dispersed from larval cadavers than the wild type.

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GENETIC ENGINEERING OF BACULOVIRUSES: RISK ASSESSMENT ON GRANULOSIS VIRUSES

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Summary

The likelihood of recombination between the granulosis viruses of the codling moth (CpGV) and the false codling moth (ClGV) was examined *in vivo* by coinfecting *Cryptophlebia leucotreta* larvae *per os* with the two viruses. The mixed infections were carried out at different ratios of CpGV and UV-exposed ClGV-CV3. The characterization of the virus progeny resulted in 4 new virus clones (MCp4, MCp5, MCp10 and MCl3) differing substantially in their DNA pofiles from the parent viruses. By REN double digestions the variations in the genomes were localized and identified as changes of restriction sites and DNA insertions. Southern hybridization proved that neither of the clones was a recombinant of CpGV and ClGV-CV3 inoculum. Bioassays with neonate larvae indicated no change in the virulence of the virus clones, except for the MCp10 which was significantly less infectious for *C. pomonella* than the native virus.

Introduction

Genetic engineering offers exiting opportunities to improve traits of baculoviruses with regard to their use as biological insecticides. Before engineered baculoviruses are considered for a deliberate release their safety has to be assessed. Particular attention must be paid to the possible risk of an uncontrolled spread of modified characteristics by genetic interactions. Recombinations are potential mechanisms for the transfer of genetic modifications to naturally occurring virus populations in the environment. There are evidences that baculoviruses form recombinants, *in vitro* as well as *in vivo* (SUMMERS et al., 1980; CROIZIER et al., 1988). In this study the likelihood of recombination of granulosis viruses (GVs) is examined *in vivo* in larvae of the false codling moth.

Material and Methods

Since the natural way of infection is the virus ingestion *per os*, larvae of the false codling moth *Cryptophlebia leucotreta* were simultanously fed two granulosis viruses: a genotype of the specific ClGV (ClGV-CV3) and the codling moth granulosis virus (CpGV). Both GVs can replicate in *C. leucotreta*, but the infectivity of the ClGV is is much higher than that of the CpGV. In order to reduce the virulence of the ClGV-CV3, this granulosis virus had been exposed to UV light before using it for the mixed infections.

The concentration of the UV-exposed ClGV-CV3 was 6×10^4 G/ml (= LC_{50}) in a first coinfection experiment and 3×10^3 G/ml (= LC_{10}) in a second one. In both, the concentration of the CpGV was 6×10^4 G/ml (= LC_{50}).
In order to screen for recombinants the virus progeny of the mixed infections was cloned *in vivo* in larvae of *Cydia pomonella* and *Cryptophlebia leucotreta* (SMITH and CROOK, 1988). Virus clones from single infected larvae were identified by restriction enzyme analysis (REN). Those virus clones which differed from the CpGV and ClGV-CV3 inoculum were characterized by Southern hybridization and their virulence was determined in bioassays with neonate larvae of *C.pomonella* and *C.leucotreta*.

Results

The analysis of virus clones obtained after the coinfections and *in vivo* cloning showed that all of the clones from *C.leucotreta* were of the ClGV type and those from *C.pomonella* of the CpGV type. But 4 virus clones differed substantially in their DNA restriction profiles from the parent viruses. One of these clones (MCp10) derived from mixed infection at LC_{50} : LC_{50} ratio of CpGV and ClGV-CV3 and was cloned in *C.pomonella*. The three others resulted from coinfection with LC_{50} of CpGV and LC_{10} of ClGV-CV3 and cloning in *C.leucotreta* (MCl3) and *C.pomonella* (MCp4 and MCp5).
In REN double digestions it was found that all of these clones carry insertions of DNA fragments. The MCp4 showed an insertion of 3.0 kbp generating a new HindIII site. The MCp5 carried an insertion of 4.5 kbp flanked by two HindIII sites. The MCp10 contained a DNA insertion of 1.0 kbp within the BamHI fragment C and an additional HindIII restriction site outside of this insertion (**Figure 1**). The MCl3 shows an extension of the EcoRI fragment H of about 1 kbp and a loss of an EcoRI site resulting in a fusion of the fragments C and S (**Figure 2**).
Analysis by Southern hybridization indicated that the virus clones were not due to recombination of the inoculated viruses. In particular the 4.5 kbp insertion of the MCp5 was neither homologous to the CpGV, nor to the ClGV-CV3 inoculum.
In bioassays the LC_{50} values of the virus clones were determined and compared to those of CpGV and ClGV-CV3. The data presented in **Table 1** show that the virulence of one MCp clone has changed. The MCp10 proved to be about 30 times less infectious for *C.pomonella* than the specific CpGV. The larvae of *C.leucotreta* were as susceptible to MCl3 than to ClGV-CV3.

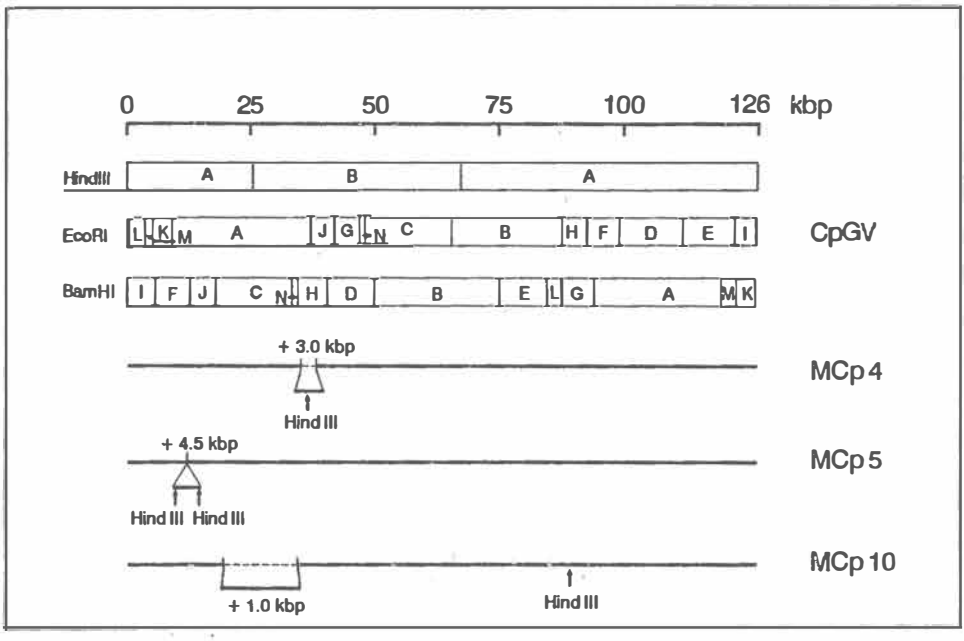

Figure 1 Linearized physical map of HindIII, EcoRI and BamHI restriction sites on CpGV (CROOK et al., 1985) compared with the isolated clones MCp4, MCp5 and MCp10. The dashed lines indicate the areas of insertions and the arrows new restriction sites.

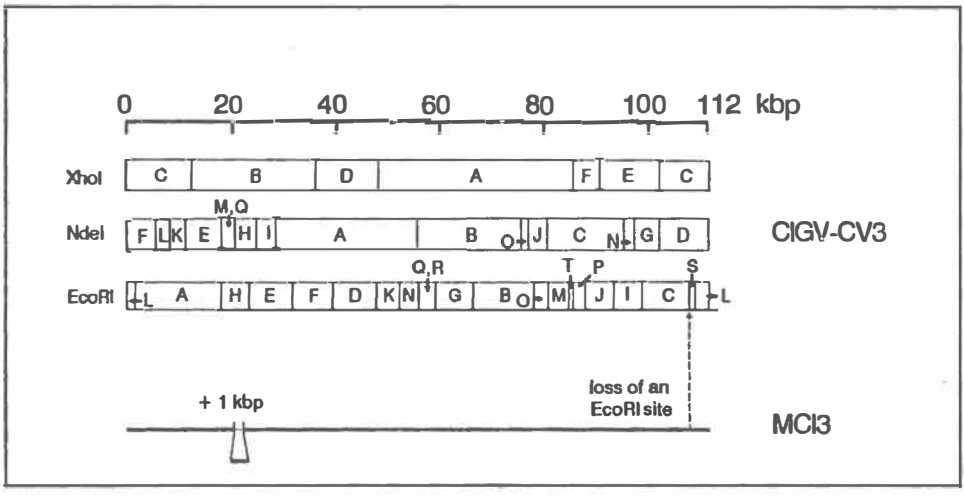

Figure 2 Linearized physical map of XhoI, NdeI and EcoRI restriction sites on ClGV-CV3 compared with the clone MCl3. The dashed line indicates the area of insertion.

virus	LC_{50} (G/ml)	test insect
CpGV	2.6×10^3	Cp
MCp10	8.4×10^4	Cp
MCp4	4.1×10^3	Cp
MCp5	4.0×10^3	Cp
MCl3	2.8×10^3	Cl
ClGV-CV3	2.7×10^3	Cl

Table 1 Biological activity of the clones MCp4, MCp5, MCp10 and MCl3 determined in bioassays with neonate larvae of *Cydia pomonella* (Cp) and *Cryptophlebia leucotreta* (Cl)

Discussion

None of the new virus clones obtained after the coinfections of *C.leuocireta* larvae with CpGV and ClGV-CV3 were recombinants. There seems to be only a low possibility for a genetic exchange *in vivo* between these granulosis viruses showing slight DNA homologies. The MCp clones as well as the MCl3 acquired additional DNA fragments. It is not supposed that the MCp clones represent spontaneous mutants, since nearly all CpGVs isolated in various laboratories all over the world had been identical. The only two distinct CpGV variants are described by CROOK et al. (1985).
The 4.5 kbp insertion fragment of clone MCp5 proved to be neither homologous to the CpGV nor to the ClGV-CV3 inoculum. The origin of this insertion is not known, but it can be assumed that it derived from the genome of the hosts during the coinfection experiments. There is evidence for acquisition of host DNA by baculoviruses (FRASER et al., 1983). So far these DNA insertions have only been observed in cell culture systems of nuclear polyhedrosis viruses, but never with *in vivo* replication of granulosis viruses.
The data of the bioassays showed that neither the MCl3 was more infectious for *C.leucotreta* nor the MCp clones for *C.pomonella*. Whether the decreased virulence of MCp10 for *C.pomonella* is really due to the genetic variations observed in the REN has to be examined in further studies.

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NUCLEOTIDE SEQUENCE OF THE POLYHEDRIN GENE OF *SPODOPTERA EXIGUA* NUCLEAR POLYHEDROSIS VIRUS.

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Summary

Spodoptera exigua nuclear polyhedrosis virus (*Se*NPV) is a baculovirus specific for the beet armyworm, *Spodoptera exigua*. As part of an investigation on the genomic organization and replication of *Se*MNPV, the gene encoding the viral occlusion body protein (polyhedrin) was detected and its nucleotide sequence determined. The gene encompasses 738 nucleotides encoding 246 amino acids. Comparison of the *Se*NPV polyhedrin gene with polyhedrin genes of other NPVs indicated a high degree of nucleotide and amino acid sequence homology. *Se*NPV and *S. frugiperda* NPV have the most closely related polyhedrin genes sharing 86% and 98% of their nucleotides and amino acids, respectively. Most differences in the nucleotide sequence are point mutations without affecting the encoded amino acids. The elucidation of the SeNPV polyhedrin gene sequence may aid in the genetic engineering of *Se*NPV to improve its efficacy in insect pest control.

1. Introduction

The beet armyworm, *Spodoptera exigua* (Lepidoptera, Noctuidae), is a polyphagous pest insect of economically important crops in the Northern hemisphere. The multiple-nucleocapsid form of *S. exigua* nuclear polyhedrosis virus (*Se*NPV) is a baculovirus (Baculoviridae) specific for beet armyworm and is attractive as a biological insecticide (Gelernter & Federici, 1986; Smits, 1987). However, as with all baculoviruses the speed of action is rather slow, as it may take several days to weeks before virus infection renders effect. Attempts are being made to improve the pathogenic properties of baculoviruses by genetic engineering (Vlak, 1991). Detailed analysis of the genome structure and replication strategy of baculoviruses are therefore required.

Baculoviruses represent a group of insect viruses, whose virions are usually found occluded in polyhedron-shaped capsules, also called polyhedra or occlusion bodies. These bodies are thought to protect the virus from environmental decay and play an important role in the epizootiology of the disease. Polyhedrin, a protein of approximately 30 kDa, is the priciple component of these polyhedra. We report here the sequence of the SeNPV polyhedrin gene.

2. Materials and methods

A *S. exigua* NPV isolate (*Se*NPV/US) was obtained from dr. B.A. Federici and described elsewhere (Gelernter & Federici, 1986a). The virus was propagated in 4th instar larvae of *S. exigua*. Hemolymph from infected insects was used to infect cultured *S. exigua* cells (Gelernter and Federici, 1986b). Viral DNA was isolated from the extracellulair, non-occluded virus of a plaque-purified isolate of *Se*NPV according to Summers & Smith, (1987).

To locate the DNA containing polyhedrin gene sequences, the viral DNA was digested with various restriction enzymes, blotted onto nitrocellulose filters and hybridized with the [^{32}P]-labelled *Hin*dIII-V fragment of *Autographa californica* (*Ac*) NPV DNA according to procedures described by Sambrook et al. (1989). This fragment contains polyhedrin gene sequences (Smith et al., 1983). *Se*NPV DNA fragments which

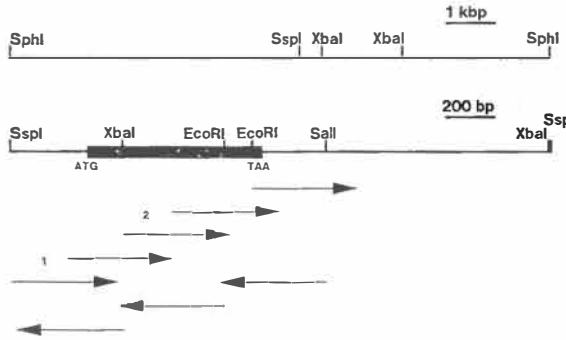

Figure 1. Location of restriction sites on the *Se*NPV DNA *Sph*I fragment of 11.4 kbp (A) and its *Ssp*I subfragment of 2.2 kbp containing the polyhedrin gene sequence. The start (ATG) and stop (TAA) codons of the polyhedrin gene are indicated. The arrows point in the direction the clones were sequenced.

promoter region

```
                                                                M  Y  T  R  Y  S
AATTGTAAGTAATTTTTTCCTTTCGTAAAACATTGTGAAAAAATAAATATAATGTATACTCGCTACAGCT
-50          .           .           .           .          1           .

Y  N  P  A  L  G  R  T  Y  V  Y  D  N  K  F  Y  K  N  L  G  S  V  I  K
ATAACCCAGCCTTGGGTCGCACTTACGTGTACGACAACAAATTCTACAAGAATCTTGGTTCCGTCATCAA
.           .           .          50           .           .           .

N  A  K  R  K  E  H  L  L  Q  H  E  I  E  E  R  T  L  D  P  L  E  R
AAACGCCAAACGCAAGGAGCATCTATTGCAACATGAAATTGAAGAGAGAACTCTCGATCCTCTAGAAAGG
.          100          .           .           .           .          150

Y  V  V  A  E  D  P  F  L  G  P  G  I  N  Q  K  L  T  L  F  K  E  I
TATGTCGTCGCCGAAGATCCCTTCTTGGGACCCGGCAAAAACCAAAAGTTGACTCTCTTCAACGAAATTC
.           .           .           .          200          .           .

R  I  V  K  P  D  T  M  K  L  V  V  N  W  S  G  K  E  F  L  R  E  T  W
GTATCGTCAAACCGGACACGATGAAACTGGTCGTCAACTGGAGCGGCAAAGAGTTTCTCCGCGAAACTTG
.           .          250          .           .           .           .

T  R  F  M  E  D  S  F  P  I  V  N  D  Q  E  I  M  D  V  F  L  V  I
GACGCGTTTCATGGAAGACAGCTTTCCCATCGTCAACGATCAAGAAATCATGGACGTTTTCCTCGTAATC
300          .           .           .           .          350          .

N  M  R  P  T  R  P  N  R  C  F  R  F  L  A  Q  H  A  L  R  C  D  P
AACATGAGACCAACGAGACCTAACCGTTGCTTCCGATTTTTGGCTCAGCACGCTCTCCGTTGCGATCCCG
.           .           .          400          .           .           .

D  Y  V  P  H  E  V  I  R  I  V  E  P  V  Y  V  G  T  N  N  E  Y  R  I
ACTACGTTCCCCACGAAGTCATCCGCATCGTCGAGCCCGTGTACGTCGGCACCAACAACGAATACCGCAT
.          450          .           .           .           .          500

S  L  A  K  K  G  G  G  C  P  V  M  N  L  H  S  E  Y  T  N  S  F  E
CAGTTTAGCCAAAAAGGGCGGCGGTTGCCCCGTCATGAATCTCCACTCCGAGTACACCAACTCGTTCGAG
.           .           .           .          550          .           .

E  F  I  N  R  V  I  W  E  N  F  Y  K  P  I  V  Y  V  G  T  D  S  G
GAATTCATCAACCGCGTCATTTGGGAGAACTTTTACAAACCCATCGTTTACGTAGGAACCGACTCGGGTG
.           .          600          .           .           .           .

E  E  E  E  I  L  L  E  L  S  L  V  F  K  I  K  E  F  A  P  E  A  P  L
AGGAAGAGGAAATCCTCCTCGAACTGTCGCTCGTGTTCAAGATCAAGGAATTCGCACCCGATGCGCCTCT
650          .           .           .           .          700          .

Y  N  G  P  A  Y  *
TTACAACGGACCCGCCTATTAAAAACGTAATTAAAAACACAAAAGGTAAGTATTACAATTTTATTAATCT
.           .           .          750          .           .           .
```

Figure 2. Nucleotide sequence of the SeNPV polyhedrin gene and predicted amino acids (one letter code designations). The putative transcription initiation and termination signals are underlined.

hybridized with the *Ac*NPV *Hin*dIII probe were isolated from agarose gels and (sub)cloned into the plasmid pTZ18R. The *Se*NPV DNA inserts were sequenced using T7 DNA polymerase and [^{35}S]-dATP according to Sanger et al. (1977).

3. Results and discussion

The location of the *Se*NPV polyhedrin gene was determined by hybridization using the *Ac*NPV *Hin*dIII-V fragment as a probe. Despite the limited overall DNA homology beteen *Se*NPV and *Ac*NPV (Smith & Summers, 1982), the conservation of polyhedrin genes among baculoviruses (Rohrmann, 1986) allowed the detection of homologous polyhedrin sequences. A *Sph*I fragment of 11.4 kilobase pairs (kbp) and a *Xba*I fragment of 1.7 kbp of *Se*NPV DNA hybridized to the *Ac*NPV probe and were further analyzed. The location of various restriction sites on these *Se*NPV DNA fragments were determined and subclones were prepared for DNA sequence analysis (Fig. 1).

The coding sequence of the *Se*NPV polyhedrin gene contained 738 nucleotides coding for 246 amino acids (Fig. 2). The molecular weight of *Se*NPV polyhedrin as determined by SDS-polyacrylamide gel electrophoresis (31 kilodalton (kDa), data not shown) is close to the predicted molecular weight of 29 kDa from the amino acid sequence. Comparison of the *Se*NPV polyhedrin gene and polyhedrin genes of other NPVs, *Autographa californica* NPV (Hooft van Iddekinge et al., 1983), *Bombyx mori* NPV (Iatrou et al., 1985), two *Orgyia pseudotsugata* NPVs (Leisy et al., 1986a,b), *Panolis flammea* NPV (Oakey et al., 1989), *Mamestra brassicae* NPV (Cameron & Possee, 1989), *Spodoptera frugiperda* NPV (Gonzales et al., 1989) and *Lymantria dispar* NPV (Smith et al., 1988; Chang et al., 1989) showed them to be closely related (Fig. 3; Table 1). The polyhedrins of *Se*NPV and *Ld*NPV are most distantly related with only 73% nucleotide and 81% amino acid sequence homology. The highly conserved

```
SeMNPV MYTRYSYNPA LGRTYVYDNK FYKNLGSVIK NAKRKEHLLQ HEIEERTLDP LERYVVAEDP
SfMNPV .........S .......... .......... ........AL .......... ..........
MbMNPV .........S .......... Y......... ..N..R.YIE ..L..K.... .D..L.....
PfMNPV .........S FP........ Y.....A... ..N..NDFIE .QL..K.... .D..L.....
OpSNPV .........S .......... Y.....A... .....K.QIE ..A..H..... .DK.L.....
BmMNPV .-PN.....T I......... Y.....GL.. .....K..IE ..K..KQW.L .DN.M.....
AcMNPV .-PD...R.T I......... Y.....A... .....K.FAE .....A.... .DN.L.....
OpMNPV .-PD...R.T I......... Y......... .....K...E ..ED.KH... .DH.M.....
LdMNPV .HNF.N.S.. ..K....E.. Y.....T... Q...QK..E. ......S..H .D..L.....
                                                                        60

SeMNPV FLGPGJNQKL TLFKEIRIVR PDTMKLVVNW SGKEFLRETW TRFMEDSFPI VNDQEIMDVF
SfMNPV .......... .......... .......... .......... .......... ..........
MbMNPV .......... .......N.. .......... .......... .......... .....V....
PfMNPV .......... .......N.. .......... .......... .......... .....V....
OpSNPV .......... .......N.. .......... .......... .......... ..........
BmMNPV .......... .....V.N.. ......I... .......... ...V...... .....V...Y
AcMNPV .......... .......N.. ........G. K....Y.... .......... .....V....
OpMNPV .......... .......N.. ......I... .......... ...V...... .....V....
LdMNPV .Y........ .......N.. .......... .......... .......... .....V..IY
                                                                       120

SeMNPV LVINMRPTRP NRCFRFLAQH ALRCDPDYVP HEVIRIVEPV YVGTNNEYRI SLAKKGGGCP
SfMNPV .......... .......... .......... .......... ...N...... ..........
MbMNPV .......... ....K..... .......... .........S ...S.....V ....R.....
PfMNPV .......... ...YK..... ......E... .........S ...S.....V ....R.....
OpSNPV .......... .......... ......E... .........S ...S...... ....R.....
BmMNPV ..A.LK.... ...YK..... ...W.E.... ......M..S ...M...... ..........
AcMNPV ..V....... ...YK..... .......... .D........S W..S...... ..........
OpMNPV ..V....... ...YK..... ...W.C.... .........S ...M...... ..........
LdMNPV .T..V..... ...YK.V... .....EG... .........S T.E-...... ....R.....
                                                                       180

SeMNPV VMNLHSEYTN SFEEFINRVI WENFYKPIVY VGTDSGEEEE ILLELSLVFK IKEFAPDAPL YNGPAY
SfMNPV .........H .......... .......... .......... .......... .......... ......
MbMNPV .......... .......... .......... .....A.... ....V..... .......... ......
PfMNPV .......... .......... .......... .....A.... ....V..... .......... ......
OpSNPV .....A.... .........H .......... .....A.... ....V..... .......... .S.K..
BmMNPV I..I...... ...S.V.... .......... I....A.... ..I.V..... .......... FT....
AcMNPV I.......... ...Q..D... .......... I....A.... ....V..... V......... FT....
CpMNPV I..I.A.... ...S.V.... .......... I....S.... ..I.V..... V......... FT....
LdMNPV IR....A..T ...H.L.S.. .D........ ...T.A.... ....V..... .......... FQ....
                                                                       246
```

Figure 3. Amino acid sequence comparison of nine polyhedrins. Dots indicate identical amino acids.

nature polyhedrins suggests that baculoviruses stem from a common ancester. The divergence of polyhedrins may be the result of co-evolution during speciation of the various insect species. The highly conserved nature of polyhedrins may also refelect the importance of occlusion bodies in the life cycle of NPVs.

Most notable is the high degree of homology between *Se*NPV and *Sf*NPV with only a five amino acids difference (98% amino acid sequence homology). These amino acid substitutions occurred in areas that were also variable in other polyhedrins. Most nucleotide differences (86% nucleotide sequence homology) are silent substitutions resulting in synonymus codons for the same amino acid. The close relationship between the polyhedrin genes of *Se*NPV and *Sf*NPV provide support to the co-evolution hypothesis, as *S. exigua* and *S. frugiperda* are closely related species. It is interesting to note that *Se*NPV is species-specific.

The promoter region of the *Se*NPV polyhedrin gene, located upstream from the ATG translational start signal (Fig. 2), appeared to be identical to that of *Sf*NPV and is highly conserved as compared to other baculoviruses (data not shown). A putative transcriptional start signal (TAAG), preceding all baculovirus polyhedrin genes (Rohrmann, 1986), was located at position -47. The 3'-end of the coding sequence contained two putative transcriptional termination signals (ATTAAA), the most likely candidate situated twelve nucleotides downstream from the translational stop signal TAA. The 3'-end is considerably diverged in length and sequence among baculoviruses.

In this contribution the location and the sequence of the *Se*NPV polyhedrin gene was determined. The *Se*NPV polyhedrin gene sequence was compared to known polyhedrin sequences of other NPVs. The polyhedrin gene promoter can be exploited to drive the expression of insecticidal genes and hence improve the efficacy of *Se*NPV.

	*Sf*MNPV	*Ha*MNPV	*Pf*MNPV	*Op*SNPV	*Bm*MNPV	*Ac*MNPV	*Op*MNPV	*Ld*MNPV
Nucleotide	86	82	81	79	74	76	77	73
Amino acid	98	92	89	91	82	85	84	81

Table 1. Homology (in parcentages) of polyhedrin nucleotide and amino acid sequence of *Se*NPV and other NPVs.

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INSECTICIDAL ACTIVITY OF A *BACILLUS THURINGIENSIS* TOXIN EXPRESSED BY BACULOVIRUS RECOMBINANTS

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Summary

Baculoviruses are insect pathogens with a relatively slow speed of action. This property has limited their commercial success as control agents of insect pests. Introduction into these viruses of genes coding for proteins interfering with insect metabolism or metamorphosis, such as toxins, hormones or enzymes, may enhance the pathogenicity of these viral insecticides. In this paper we report on the potential of *Autographa californica* (*Ac*) nuclear polyhedrosis virus (NPV) recombinants expressing a toxin gene of the entomopathogenic bacterium, *Bacillus thuringiensis* (*Bt*) subsp. *aizawai*. In recombinant *Ac*NPV-infected *Spodoptera frugiperda* (*Sf*) cells both the complete and a truncated *Bt* protoxin gene were expressed at high level. The biological activity (toxicity) of these insect-cell derived proteins against the large cabbage white, *Pieris brassicae*, was comparable with that of authentic *Bt* toxin. The perspectives of baculovirus-*Bt* recombinants in the control of insect pests are discussed.

1. Introduction

Baculoviruses are insect pathogens that can be used for the control of insect pests in agriculture and forestry (Martignoni, 1984; Granados & Federici, 1986). In contrast to chemical insecticides, baculoviruses are species-specific and safe for the environment. However, there are limitations to the successful use of these viruses as they act slowly. Hence, chemical insecticides are often preferred when immediate insect control is required. Improving the speed of action of baculoviruses is therefore an important focus of research. In addition to strain selection, genetic engineering offers new opportunities to achieve this goal.

Introduction of genes into baculoviruses whose products interfere with larval metabolism (Vlak, 1990), such as hormones (Maeda, 1990), enzymes (Hammock et al., 1990) or toxins (Carbonell et al., 1988), may be suitable for this aim. Research efforts are mainly focused to achieve a reduction of feeding of larvae soon after infection. In this report the introduction of a toxin gene of the entomopathogenic bacterium *Bacillus thuringiensis* (*Bt*) is documented and the effects on insects are discussed.

B. thuringiensis is a Gram-positive soil bacterium that produces one or more crystals during sporulation (Krieg, 1985). These crystals contain the 130 kDa protoxin. Upon ingestion by insect larvae these crystals are digested by alkaline gut proteases releasing proteins of 65 kDa that are toxic to insect larvae (Höfte & Whiteley, 1989). The *Lepidopteran*-specific crystals are bipyramidal in shape and contain one or several toxic proteins with a molecular weight of approximately 130 kDa (Bulla et al., 1977). Each of these proteins appears to have its own specific toxicity spectrum with respect to insect hosts (Höfte et al., 1988; Visser et al., 1988).

Efficient expression of foreign genes in baculoviruses is achieved by using the promoters of polyhedrin (PH) or the p10 genes (Luckow & Summers, 1988; Miller, 1989; Vlak & Keus., 1990). These genes are dispensable for virus replication in cell culture and the loci of these genes can be used for insertion of toxin genes. We already introduced the complete *Bt* toxin gene as shown in figure 1 into the polyhedrin locus (Martens et al., 1990) of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) and determined the biological activity of the toxin produced against *Pieris brassicae*. Here we compare the properties of the complete and a truncated version of the *Bt* gene produced by the polyhedrin promotor.

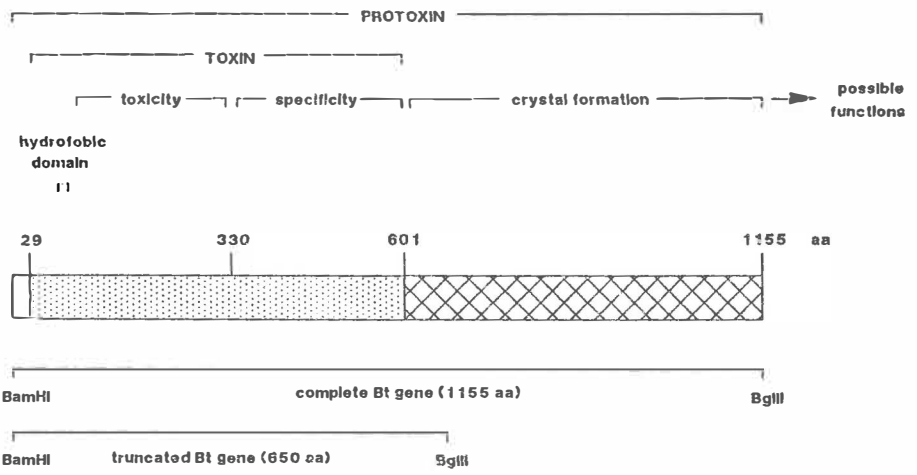

Figure 1. Organization of the cryIA(b) toxin gene (Höfte and Whiteley, 1989) of *Bt* subsp. *aizawai* strain 7.21A. The various putative functional domains are indicated and their size is approximated by amino acid numbers. The constructs and restriction sites used in this experiment are shown at the bottom in single lines. BglII and BamHI are restriction enzymes from *Bacillus globigii* and *B. amyloliquefaciens*, respectively.

2. Materials and methods

The *Bacillus thuringiensis* subsp. *aizawai* strain 7.21 was supplied by Dr. H. de Barjac, Institut Pasteur, Paris. A recombinant *Escherichia coli* (7.21A), containing the cryIA(b) protoxin gene from *Bt* subsp. *aizawai* strain 7.21 on plasmid p7.21A (Honée et al. 1990), was used. The structure of the *Bt* gene is shown in figure 1. The *Spodoptera frugiperda* cell line IPLB-SF-21 was maintained in plastic tissue culture flasks in TNM-FH medium supplemented with 10% foetal calf serum (Vaughn et al., 1977).

The multiple nucleocapsid form of the nuclear polyhedrosis virus of *Autographa californica* (*Ac*NPV), strain E2 (Summers & Smith, 1978), was used as wild-type (wt) virus. Recombinant plasmid p*Ac*DZ1 contained a deletion of the polyhedrin gene and an insertion of a Lac2 gene cassette to facilitate screening of recombinants by using 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) as ß-galactosidase activity indicator (Zuidema et al. 1990).

The *Bt* protoxin gene (3.4 kbp) and the truncated *Bt* gene (1.9 kbp) were inserted into the BamHI side of the transfer plasmid p*Ac*DZ1 and recombinant baculoviruses were isolated according to Martens et al. (1990). The recombinant containing the entire protoxin gene was named *Ac*NPV/JM3 (Martens et al., 1990) and the one containing the truncated protoxin gene *Ac*NPV/JM4.

To determine the biological activity of recombinant insect cell-derived *Bt* toxin protein, *Ac*NPV/JM3- and *Ac*NPVJM4-infected *S. frugiperda* cells and *E. coli* 7.21A cells were tested on second instar larvae of *P. brassicae*.

Table 1. Toxicity of *Bt* toxin protein produced by *Ac*NPV/JM3- and *Ac*NPV/JM4-infected *S. frugiperda* cells against second instar larvae of *P. brassicae*. Mortality was scored two days after application of cell samples and is expressed as percentage of the total number of larvae used per assay (n = 32)

Source	Mortality
E. coli	0
E. coli (7.21A)	100
S. frugiperda cells	3
S. frugiperda cells + *Ac*NPV	0
S. frugiperda cells + AcNPV/DZ1	3
S. frugiperda cells + *Ac*NPV/JM3	97
S. frugiperda cells + *Ac*NPV/JM4	100

Table 2. LC_{50} of *E. coli* (7.21A) cells and *Ac*NPV/JM3- and JM4-infected *S. frugiperda* cells. LC_{50} is presented in cells/cm^2 leaf disk.

Source	LC_{50}	95% fiducial limits		Slope	Intercept	X^2/DF
		lower	upper	+ SD		
E. coli (7.21A)	2.7×10^4	1.7×10^4	4.6×10^4	0.8 (±0.3)	0.8	2.7/5
S. frugiperda cells + *Ac*NPV/JM3	3.4×10^2	1.9×10^2	5.9×10^2	1.0 (±0.2)	-0.4	3.3/5
S. frugiperda cells + *Ac*NPV/JM4	2.1×10^2	1.2×10^2	3.5×10^2	1.4 (±0.4)	-0.2	3.6/5

3. Results and discussion

S. frugiperda cells were infected with the recombinant *Ac*NPVs expressing the Bt protoxin gene (*Ac*NPV/JM3) or the truncated protoxin gene (*Ac*NPV/JM4) (Figure 1). These recombinants produced a protein of 130 kiloDalton (kDa) and a truncated 70 kDa protein, respectively, at high level. At 48 h post infection the amount of *Bt* protein approached 5% of the total cell protein mass. The identity of these proteins was confirmed by immunoblot analyses with antiserum, raised against dissolved *Bt* subsp. *aizawai* crystals.

In the cytoplasm of *S. frugiperda* cells infected with recombinant (*Ac*NPV/JM3) bipyramidal crystals were often seen, comparable in size and shape with those present in *Bt* subsp. *aizawai* itself (Martens et al., 1990). This observation was confirmed by immunofluorescence and electron microscopy. Cells infected with the recombinant (*Ac*NPV/JM4) containing the truncated *Bt* gene did not show these crystals, suggesting that the 3'-end of the protoxin might be involved in crystal formation. This 3' end is very conserved among *Bt* genes and might therefore allow crystallisation of different toxins in one crystal.

Both the complete and the truncated *Bt* protein produced by these infected *S. frugiperda* cells were fed to *Pieris brassicae* larvae and appeared to be highly toxic. *P. brassicae* is not susceptible to wt *Ac*NPV and this allows the analysis of only the toxic properties of the recombinant *Bt* proteins in the absence of virus infection. The toxicity of *S. frugiperda* cell-derived *Bt* toxins was comparable with that of the *Bt* gene products expressed by *E. coli* cells (Table 1 and 2). Infection of *Ac*NPV-susceptible insects, such as *Heliothis virescens*, with recombinants *Ac*NPV/JM3 and *Ac*NPV/JM4 is difficult to achieve. By oral ingestion the non-occluded form of baculoviruses is much less infectious to insect larvae than the occluded form (Volkman & Summers, 1976). Despite this lower infectivity, comparative bioassays with the polyhedron-negative mutant *Ac*NPV/D21 as control indicated that

an increased speed of kill using these *Bt* recombinants was not achieved.

Polyhedron-minus recombinants would also be impractical from the application point of view. They will only shortly survive and persist in the environment to cause an infection (Bishop et al., 1988). In order to engineer baculovirus/*Bt* recombinants that are active under more natural conditions, recombinants are now being prepared, which produce normal polyhedra. The *Bt* protein genes will then be expressed under the control of the p10 promoter replacing the *Ac*NPV p10 gene.

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10. Posters on entomopathogenic nematodes.

Commercialisation of Entomopathogenic Nematodes

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Summary

Steinernematid and heterorhabditid entompathogenic nematode-based products are commercially available for insect control. Recent developments in mass rearing by liquid fermentation have enabled nematodes to become economically competititve in certain crops. There has been a substantial improvement in the stability of formulations by partial desiccation of large numbers of nematodes on specific carriers such as clay, polyacrylamide and alginate gels. Standardised experimental protocols and multiple tests are needed to evaluate the environmental impact on insect-nematode relationships to optimise nematode efficacy.

Introduction

Steinernema and *Heterorhabditis* and their associated bacteria *Xenorhabdus* spp parasitise a wide range of insect hosts and actively seek out their hosts. They have been successfully developed for commercial use against a range of insect pests in soil and in cryptic environments (Table 1). These nematodes have been shown to be effective alternatives to soil insecticides, many of which have environmental residue problems or have been banned.

The most successful and widespread applications of these nematodes have been against various weevil species, eg the control of the black vine weevil, *Otiorhynchus sulcatus* on potted plants in Europe, Australia and USA, and the control of weevils in citrus orchards and in cranberry bogs in the USA (Klein, 1990).

Production and Formulation

The liquid fermentation process (Friedman, 1990) and the solid media culture (Bedding, 1984) are the current methods used by industry to mass produce these nematodes.

The Bedding (1984) monoxenic solid media culture process provides a large surface area to volume ratio by rearing the nematode-bacterium complex on shredded plastic foam soaked with a medium (preferably chicken offal) and placed in sacks. This process includes a semi-automated harvesting process capable of handling large quantities of foam. In a scale-up model, Friedman (1990) reported that the solid culture method is economically feasible up to a production level of approximately 10×10^{12} per month. Labour costs increase significantly for nematode production beyond this level, making a less expensive method of large-scale production such as liquid fermentation necessary for countries where cost of labour is high.

Table 1 Current and potential crops for steinernematid and heterorhabditid-based products.

Crops	Target insects	Countries
Artichoke	Artichoke plume moth (*Platyptilia cardiuidactyla*)	USA
Citrus	Sugarcane rootstalk borer (*Diaprepes abbreviatus*)	USA, West Indies
	Blue green weevil (*Pachneus litus*)	USA
Cranberry & Berries	Black vine weevil (*Otiorhynchus sulcatus*)	USA, W. Europe, Canada, Australia
	Strawberry root weevil (*O. ovatus*)	USA, W. Europe, Canada
	Cranberry girdler (*Chrysoteuchia topiaria*)	USA, Canada
	White grubs (various species)[a,b]	USA, Canada
Turf	Black cutworm (*Agrotis ipsilon*)	USA, Japan[a], Canada
	Japanese lawn cutworm (*Spodeptera depravata*)[a]	Japan
	Armyworm (*Pseudaletia unipuncta*)	USA
	Bluegrass webworm (*Parapediasia teterrella*)	USA, Japan[a]
	Bluegrass billbug (*Sphenophorus parvulus*)	USA
	White grubs (various species)[a,c]	USA, Japan, Canada
	Mole crickets (*Scapteriscus* spp)[a]	USA
Greenhouse/ Nursery Plants	Black vine weevil (*O. sulcatus*)	USA, W. Europe, Australia, Canada
	Strawberry root weevil (*O. ovatus*)	USA, W. Europe, Canada
	Fungus gnats (*Bradysia* spp)	USA, W. Europe, Canada
	White grubs (various species)[d]	USA

a Expected markets in 1991-1993
b Major genera are *phyllophaga* and *Anomala*
c Major insects are japanese beetle (*Popillia japonica*), european chafer (*Rhizotrogus majalis*), masked chafers (*Cyclocephala* spp), June beetles (*Phyllophaga* spp) and soybean beetle (*Anomala rufocuprea*)
d Major insects are japanese beetle, european chafer and oriental beetle (*Anomala orientalis*)

Currently Steinernematid nematodes can be produced consistently and effectively in a 15,000 litre fermenter (with projections to 80,000 litres) using an ox kidney homogenate-yeast extract or a medium containing soya flour, yeast extract, corn oil and egg yolk (Friedman, 1990): yields as high as 100,000 nematodes per ml of extract have been obtained, as long as optimum aeration is maintained and the shear sensitivity of the nematodes is taken into account.

For commercial use the ability of the nematodes to be stored (have a satisfactory "shelf-life") is critical if they are to compete with chemical insecticides. Prior to formulation nematodes are stored under refrigeration in aerated aqueous suspension. The oxygen and moisture parameters for the two genera/species must be ascertained and once the requirements of the individual species have been defined then selection of formulation, packaging size and storage conditions for product development and distribution can be undertaken.

Improvement of formulation stability has been obtained by partially desiccating or immobilizing the nematodes on specific carriers such as clay, polyacrylamide and alginate gels which reduce nematode metabolism thus improving their tolerance to temperature extremes. The storage period is related to the oxygen and moisture requirements of the nematode.

For Steinernematids 10 million nematodes placed on 12 x 60 cm alginate sheet in a 0.5 litre container will store for 5 months at room temperature and for at least 12 months in a refrigerator but heterorhabditids are more difficult to store, 6 million nematodes on a clay formulation only lasting 3 months kept at 10° - 15°C.

Efficacy

Entomopathogenic nematodes are not generalist in terms of their effectivenese for pest control, each species having a number of preferred insect hosts, and therefore screening of a range of entomopathogenic nematodes against a particular target host is esssential in any control programme (Gaugler, 1988; Georgis, 1990). It has become evident that the ability of the nematode to penetrate the host insect is only one component of infectivity: consideration should also be given to the environment in which the nematode will be used, eg field soil, potting soil, foliage or galleries in a tree.

Entomopathogenic nematodes are mobile and it has been assumed that they seek out their insect host (as demonstrated in the control of borers such as *Synanthedon* and *Holcocerus*, Curran, 1990) but there is little evidence that they do this in field soils. If the ability of entomopathogenic nematodes to disperse through soil is limited then the correct placement of the nematodes is crucial for efficient control. Based on results obtained in various parts of the world, it seems that a dose of $1 \times 10^5 - 1 \times 10^6$ nematodes per squre metre, equivalent to about 5 billion nematodes per hectare, is required to give effective control compared to insecticides. In some conditions these numbers may be not great enough to overcome the limitations on dispersal and persistence imposed by the environment (Georgis, 1990).

Conclusions

The "efficacy gap" between pesticides and entomopathogenic nematodes has been narrowed in various crops and there have been significant developments in quality control of nematode products, the selection of suitable target environments and the host targets themselves. It is essential to identify effective nematode strains for a particular host target, to time applications properly, as well as choosing the most appropriate means of application or delivery to increase the probability of successful contact between the nematode and the target insect (Georgis, 1990).

Entomopathogenic nematodes have emerged as effective "biological" soil insecticides, especially in markets where safety and restricted use of insecticides are an issue. Future developments may include immobilizing infective juvenile nematodes in a gel polymer to give formulations which are easier to handle and store than at present, incorporating certain desirable traits, eg host seeking enhancement, UV tolerance and toleration to desiccation, and the possibility of finding low temperature pathogenic strains of *S. feltiae* (= *bibionis*) or *Heterorhabditis*. These efforts should go alongside investigations into better understanding of the insect-nematode ecology with particular reference to the biology of the insect host where the potential target is at present considered too difficult or an impractical host for using entomopathogenic nematodes.

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Identification of *Heterorhabditis* spp. by morphometric characters and RFLP and of their symbiotic bacteria *Xenorhabdus* spp. by species specific DNA probes

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Introduction

During the second meeting of the IOBC/WPRS working group on Microbial Control in Rome, 1989 a cooperative approach to identification of heterorhaditid nematodes and their symbiotic bacteria *Xenorhabdus* was decided on in order to clarify the taxonomic position of European isolates. This paper is the result of the combined activity of the subgroup "Entomopathogenic Nematodes".

Methods

Heterorhabditis isolates were sent to the authors by a large number of researchers (see table 1). They were propagated on *Galleria mellonella* larvae. *X. luminescens* was isolated by streaking haemolymph of infected larvae on agar media (Akhurst, 1980).

Morphometric characters (total length, distance head to excretory pore and head to pharynx base, tail length) of 25 dauer juveniles were measured and compared with data published for *H. bacteriophora* (Poinar, 1975), *H. heliothidis* (Khan et al., 1976) and *H. megidis* (Poinar et al., 1987).

The total DNA of the nematodes was analysed by comparing restriction fragment length patterns (RLFP) according to Curran et al. (1985). For detailled information refer to Smits et al. (1991).

To distinguish between the bacterial symbionts DNA probes complementary to a variable domaine of the 16S rRNA of two reference strains (ATCC 29304 and HL-81) were used in dot blot hybridisation experiments according to Pütz et al. (1990). The sequences of the probes are given in table 2.

Table 1. Origin of the *Heterorhabditis* isolates

Isolate code or name	Original place of isolation	Obtained from the collection of
1. HL-81	Limburg,The Netherlands	Groene Vlieg
2. HF-85	Flevoland, The Netherlands	Westerman/Simons
3. HFR-86	Friesland, The Netherlands	Westerman/Simons
4. HNH1-87	N-Holland, The Netherlands	Westerman/Simons
5. HW-79	Wageningen,The Netherlands	Westerman/Simons
6. HNB-87	N-Brabant, The Netherlands	Westerman/Simons
7. HB-87.1	Bergeyk, The Netherlands	Smits
8. HB-87.2	Bergeyk, The Netherlands	Smits
9. HE-87.3	Eindhoven, The Netherlands	Smits
10. HK-3	Darmstadt, Germany	Bathon
11. HK-6	Darmstadt, Germany	Bathon
12. HD-01	Darmstadt, Germany	Ehlers (1)
13. HSH-1	Kiel, Germany	Ehlers
14. HSH-2	Kiel, Germany	Ehlers
15. HI-23	Italy	Ehlers (2)
16. HI-127	Italy	Ehlers (2)
17. HI-82	Italy	Westerman/Simons(2)
18. HSie	Siedlce, Poland	Bednarek
19. HPB	Puszcza Blata, Poland	Bednarek
20. H. bacteriophora W	Warsaw, Poland	Bednarek
21. HMo!	Moldavia, USSR	Bednarek
22. HIR-K122	Ireland	Griffin
23. HIR-M145	Ireland	Griffin
24. HIR-M170	Ireland	Griffin
25. HSP-1	Barcelona, Spain	Del Pino
26. HSP-2	Barcelona, Spain	Del Pino
27. HNZ	New Zealand	Ehlers (3)
28. HT-327	Australia	Ehlers (4)
29. *H. bacteriophora* K	Brecon, S-Australia	Kaya
30. *H. bacteriophora* NJ	Brecon, S-Australia	Bednarek (5)
31. *H. heliothidis*	N-Carolina, USA	Kaya
32. HPTI	California, USA	Kaya
33. HP-88	Utah, USA	Kaya
34. *H. megidis*	Ohio, USA	Georgis/Westerman
35. HI-?	Italy	Deseo
36. HI-483	Italy	Deseo
37. HI-493	Italy	Deseo
38. HI-191	Italy	Deseo
39. HI-100	Italy	Deseo
40. HI-174	Italy	Deseo
41. HI-775	Italy	Deseo
42. HCH-H2	Switzerland	Grunder
43. HCH-HW79	Switzerland	Grunder
44. BS	Israel	Glazer
45. CDC 3106-77*	-	Grimont
46. CDC 3265-86*	-	Grimont

Groene Vlieg Company, Nieuwe Tonge, The Netherlands; P. Westerman, Friesland Agricultural College, Leeuwarden, The Netherlands; R.-U. Ehlers, Christian-Albrechts-University, Kiel, W-Germany; H.K. Kaya, University of California, Davis, USA; A. Bednarek, University of Warsaw, Poland; C.T. Griffin, St. Patricks College, Dublin, Ireland; F.G. del Pino, University of Barcelona, Spain; H. Bathon, Institute for Biological Control, Darmstadt, W-Germany; ( ) Isolates originally from: (1) H. Bathon, W-Germany. (2) K.V. Deseo, Italy. (3) W. Wouts, New Zealand. (4) D.H. Molyneux, Australia. (5) R. Gaugler, USA.
* - only *X. luminescens* isolates

Table 2. Sequence of the probes used for the hybridisation experiments.

probe lumi 1	CCGGACTTCTCCCGC	
probe lumi 2	ATCGGACTTGTCCCAACTTAA	
	458	478

Results and Discussion

Morphometric data are given in table 3. According to these results two groups can be discriminated and assigned to *H. megidis* or *H. bacteriophora* on the basis of all 4 characters listed. The exceptional high values for the tail length published by some authors probably concern measurements including the L2-cuticle.

Table 3. Morphometric characteristics of infective juveniles of *Heterorhabditis* isolates.

isolate	total length	distance head to pore	distance head to pharynx	tail length
1. HL-81	804 (759-840)	134	152	80
3. HFR-86	764 (684-828)	127	148	79
13. HSH-1	806 (749-859)	135	152	82
36. *H. megidis*	768 (736-800)	131	155	119*
22. HIR-K122	785 (744-835)	-	-	-
12. HD-01	590 (538-625)	117	132	62
25. HSP-1	567 (560-622)	112	130	62
28. HT-327	578 (521-620)	108	130	58
42. HI-174	552 (445-606)	107	128	56
27. HNZ	685 (570-740)	112	140	102*
29. *H. bacteriophora*	570 (520-600)	104	125	91*
31. *H. heliothidis*	644 (618-671)	112	133	102*

* probably including L2-cuticle.

The results obtained from the comparison of the digestion patterns of the total nematode DNA of the isolates studied (table 4) are published by Smits et al. (1991). Three groups could be distinguished on the basis of the digestion patterns using the restriction enzymes EcoR1 and Mspl. The line drawing of the patterns are given in figure 1. With this method three groups were identified: The *H. bacteriophora*, the NW European and the Irish group. The RFLP confirmed the separation on the basis of morphometric characters and could even separate the *H. megidis* group of table 3 into the NW European and the

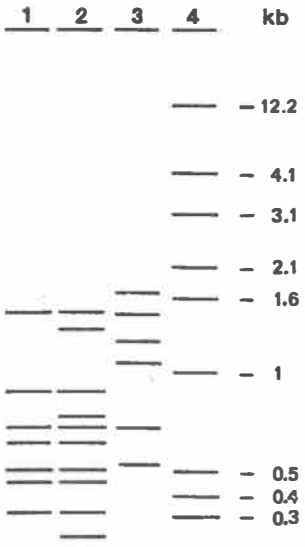

Figure 1. Line drawing of typical MspI digestion patterns of total DNA showing repetitive DNA fragments of three groups of *Heterorhabditis* isolates. 1. NW-European group; 2. Irish group; 3. *H. bacteriophora* group; 4. 1 kb DNA marker.

Table 4. Grouping of nematodes based on identical RFLP patterns with EcoR1 and MspI digestion.

H. megidis group	Irish group	*H.bacteriophora* group
1. HL-81	22. HIR-K122	10. HK-3
2. HF-85	23. HIR-M145	11. HK-6
3. HFr-86	24. HIR-M170	12. HD-01
4. HNH1-87		15. HI-23
5. HW-79		16. HI-127
6. HNB-87		17. HI-82
7. HB-87.1		21. HMol
8. HB-87.2		25. HSP-1
9. HE-87.3		26. HSP-2
13. HSH-1		27. HNZ
14. HSH-2		28. HT-327
18. HSie		29. *H. bacteriophora* K
19. HPB		30. *H. bacteriophora* N
20. (*H. bacteriophora* W)*		31. *H. heliothidis*
36. *H. megidis*		32. HPTI
		33. HP-88

* wrong name given obviously

Table 5. Grouping of *Heterorhabditis* isolates based on hybridisation of the DNA of their *Xenorhabdus* symbionts with Lumi 1 & 2 probes.

Lumi-1 group	Lumi-2 group	Negative group
10. HK-3	1. HL-81	9. HE-87.3
11. HK-6	2. HF-85	12. HD-01
25. HSP-1	4. HNH1-87	15. HI-23
27. HNZ	6. HNB-87	16. HI-127
28. HT-327	7. HB-87.1	21. HMol
31. *H. heliothidis*	8. HB-87.2	22. HIR-K122
32. HPTI	14. HSH-2	23. HIR-M145
36. HI-483	18. HSie	24. HIR-M170
44. BS	42. HCH-H2	33. HP-88
	43. HCH-HW79	35. HI-?
		37. HI-493
		38. HI-191
		39. HI-100
		40. HI-174
		41. HI-775

Irish group. Table 4 lists the isolates which were assigned to the different groups. According to the large number of the common bands in the digestion patterns obtained with EcoR1 and Msp1 the NW European and the Irish group are closely related to each other. *H. megidis* has an identical pattern as the NW-European group.

The results obtained by the hybridisation experiments and additional data of the sequences of the variable domaine of further reference strains indicate no group-specific correlation of the nematode-bacterium complex *Heterorhabditis-X. luminescens* as has been proven for the steinernematid nematodes with their species-specific symbionts (Pütz et al., 1990 and Ehlers et al. unpublished data). The *X. luminescens* isolates hybridising with the lumi 1 probe are closely related to the ATCC strains 29999 and 29304. Strains hybridising with the lumi 2 probe are associated with the NW European nematode group with the exception of the strain HFR-86 which has the same sequence within the variable domaine as the Irish M-145 and the CDC strain 3106-77. The CDC strain 3265-86 differs from the other CDC strain in one base. The hybridisation results are listed in table 5. The sequences within the variable domaine of the strains HMol and HD-01 are different from all other strain sequences. The strains hybridising with the probes probably are two different species and the remaining isolates consist of at least 5 subgroups with a possible separation on the species level.

Conclusions

1. There are 3 groups of nematode isolates occuring in Europe representing two described and a new undescribed species:
   1. *H. bacteriophora*
   2. *H. megidis* (NW European group)
   3. Irish group

2. *H. bacteriophora* seems to occurr in warmer regions such as the Mediterranean and Central Europe whereas *H. megidis* and the Irish nematodes are found in more temperate zones.

3. Each of the *Heterorhabditis* species seem to be associated with more than one *Xenorhabdus luminescens* subgroup. The subgroups probably represent different species.

4. The New Zealand strain HNZ did not have a distict RLFP profile which would allow a taxonomic seperation of a *H. zealandica* (Poinar, 1990) from *H. bacteriophora*.

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DISCUSSION OF FACTORS INFLUENCING A SURFACE-APPLICATION OF ENTOMOPATHOGENIC NEMATODES WITH SPECIAL REFERENCE TO *SYNANTHEDON MYOPAEFORMIS* (SESIIDAE)

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SUMMARY

In the last decade numerous investigations on the use of entomopathogenic nematodes for control of different cryptic living wood and bark boring noxious insects were published. The results of the treatments varied substantially. This paper exhibits a number of factors influencing an application of nematodes to foliage, branches or trunks. A special note is taken on humidity conditions.

INTRODUCTION

The use of entomopathogenic nematodes against soil-dwelling insects has been improved to a commercial extent in Europe. Above all the control of the black wine weevil, *Otiorrhynchus sulcatus* (Curculionidae), is focussed (Klingler, 1988).

Besides, the parasitic potential of nematodes was demonstrated in small scale applications against bark- or wood inhabiting insects with variable results (e.g. Miller & Bedding, 1982; Deseö & Miller, 1985; Kaya & Brown 1986; Lindegren et al. 1987).

Fig. A: Main factors influencing the application of entomopathogenic nematodes

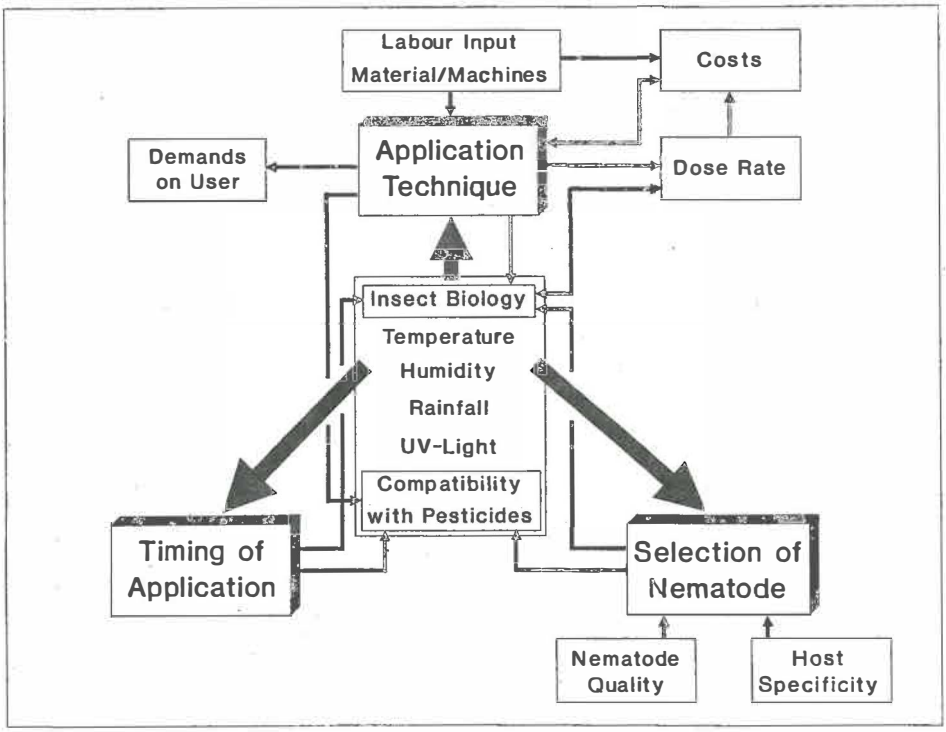


Figure A gives a general overview of biotic, abiotic and economic factors having an influence on the procedure of a nematode application. A carefully balanced consideration of all factors is required for the selection of the most favourable procedure.

Apple clearwing moth, *Syanthedon myopaeformis* (Sesiidae) is choosen as an example in order to discuss the influence of some factors on a surface application in more detail:
Changes in cultivation techniques in apple growing systems - especially the widespread grafting on M9 rootstocks - led to a gradation of the insect in many orchards in Germany since the early 70ties. The larvae of this noxious insect feed in galleries made in the cambial tissue of trunks or branches of apple-trees thus favouring secondary infections with cancer (*Nectria galligena*). Due to distinct reduction of yield and lifespan of infested trees *S. myopaeformis* became a pest of economic importance (Dickler, 1986).

SELECTION OF NEMATODE

HOST SPECIFICITY

In all investigations only J3 of the genera *Steinernema* proved to be successful for control of *S. myopaeformis* larvae. Treatments with *Heterorhabditis* spec. (Deseö & Miller, 1985: Stamm IH; Nachtigall & Dickler, in press: Stamm HW79, HD 01) did not result in any parasitation of the host larvae or pupae. Bedding and Miller (1981) also found a reduced effectiveness of *H. heliothidis* compared to *S. feltiae* or *carpocapsae* after treatment of blackcurrant cuttings infested with *S. tipuliformis*.

NEMATODE QUALITY

The infectious potential of the antagonist is influenced for example by age of population, fat reserves, J2-cuticle, number and age of symbiontic bacteria (Westermann, 1990; Ehlers, in press). For field applications a constant quality should be assured by the producer.

APPLICATION TECHNIQUES

At present several techniques are available for application of entomopathogenic nematodes to control *S. myopaeformis*:

I. Spraying a nematode-water-suspension manually with a knapsack-sprayer on the trunks of infested trees (mostly 1 - 2 x 10E6 J3/trunk). In order to prevent damage of the nematodes the diameter of the nozzle should exceed 0,8mm, the pressure should be less than 5 bar.

II. Spraying the trunks with a herbicide-sprayer provided with a pump keeping the nematode-water-suspension in constant rotation to ensure a uniform distribution of the parasites.

III. On young trees more than 80% of the larvae can be found in frass galleries in the nonlignified area of the grafting knot. Therefore a 'collar' made of sponge-rubber material (thickness: 0,5-1cm) soaked in a nematode suspension (1 x 10E5 J3/tree) was fixed around that area together with a thin plastic foil preventing rapid desiccation (Nachtigall & Dickler, in press).

IV. Injection of nematode-suspension in each frass gallery by means of a syringe (e.g. Kaya & Brown, 1986: 1,8 - 3,6 x 10E4 J3/gallery against *S. culiciformis*) or inserting nematode soaked cotton-sticks (Q-tips).

The selection of the application technique mainly depends on its entire costs. These are determined by labour input, material and mashine costs as well as the required dose rate . At present the labour input is the most important factor. Due to the fact that technique II requires the least labour input only this method can be considered as practically relevant for large scale application.

The time consuming techniques I, III and IV are suitable only for small scale areas such as housing gardens or single treatments of ornamental plants.

Techniques III and IV are independant of the impact of abiotic conditions at the time of application resulting in reproducible high mortality rates of the target insect. Surface sprayings, in contrast, gave variable results stressing the importance of the influence of the prevailing abiotic conditions.

TIMING OF APPLICATION

As the developmental cycle of the insect is characterized by a flight period lasting from May to August/September as well as an irregular 1- or 2-year larval feeding stage the following application periods are possible:

a. before or at beginning of pupation in April or May
b. in September or October at the end of the flight period

COMPATIBILITY WITH OTHER PESTICIDES

Intensive laboratory investigations during the last years indicate that most of the commercially used chemicals had no or little adverse effect of viability, infectivity and motility on the infective juveniles of *S. carpocapsae* Weiser and *S. feltiae* Filip. (Syn. *S. bibionis* Bovien (Rovesti & Deseö, in press; Rovesti et al, 1990). Their use in Integrated Pest Management (IPM) strategies seems feasible.

TEMPERATURE AND UV-LIGHT

Kaya (1977) found an optimum range for *S. carpocapsae* within 15-27°C where the J3 easily penetrate its host, kill an multiply in it.

Short waved UV-light (254nm) as well as natural sunlight causes high mortality of the entomoparasites (Gaugler & Boush, 1978). Consequently, nematodes should not be applied before sunset.

HUMIDITY AND RAINFALL

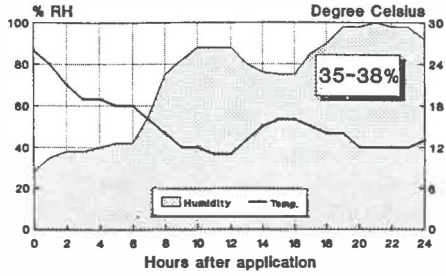
It is generally agreed that the influence of the humidity conditions must be taken into account when applying the nematodes on foliage, branches or trunks. High rel. humidity (RH) near 100% is required to enable the J3 to move, a prerequisit to actively search out and parasitize their hosts. Low RH causes rapid desiccation of the spraying deposit, so the J3 dry out and die.

Considering the relevance of surface spraying application in fruit growing practice own investigations were focussed on the influence of weather conditions during and shortly after application on the efficiency of the treatment. Aim of the study was to determine suitable temperature and humidity conditions at the time of application and for how long these conditons must continue to ensure sufficient parasitation. Nematode sprayings were carried out under variable weather conditions and data of temperature, humidity and rainfall are recorded for 10 different treatments for the first 24 hours after application against *S. myopaeformis* (fig. 1-4) or 5th instar larvae of codling moth, *Cydia pomonella* (Tortricidae) (fig. 5-10). The resulting parasitation rates are also given in each figure. All experiments were conducted in the late afternoon between 5 and 7pm. Only experiment 4 was carried out at 2pm under cloudy conditions.

RH above 80% or little rainfall during application had a fostering effect on parasitation rates of the target pest (fig. 2, 3, 6 - 9). Under humid conditions desiccation of the spraying deposit is delayed and the period for the nematodes to actively search and penetrate their host prolonged.

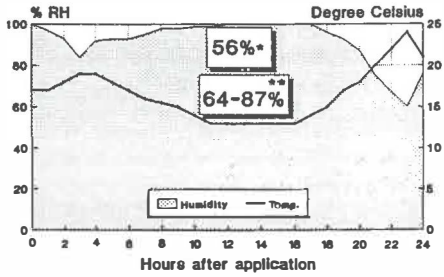
Fig. 1-4: Spraying application of *S. feltiae* on trunks of apple-trees against larvae of *Synanthedon myopaeformis* (0,5 - 1,0 x 10E6 J3/trunk):
Sight B: Encaged trunks of apple-trees at sight A
Sight C: Commercial orchard at Winden (South Palatinate)

FIGURE 1:
Sight B, Knapsack-sprayer, April 19th
Dosage: 1x10E6 J3/trunk



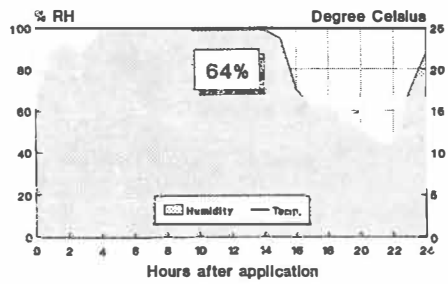
Rainfall: hour 18-20 (3,9 mm)

FIGURE 2:
Sight B, Knapsack-sprayer, May 16th
• = 1x10E6 J3, •• = 2x10E6 J3/trunk



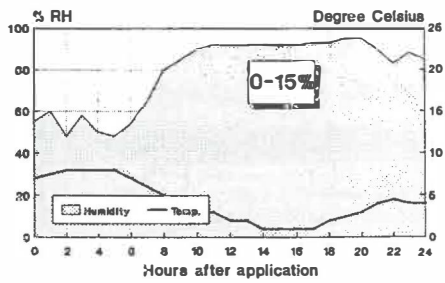
Rainfall just before appl. (5,8 mm)

FIGURE 3:
Sight B, Knapsack-sprayer, June 3rd



Rainfall:
hour 0-1 (2 mm), 4-14 (20,5 mm)

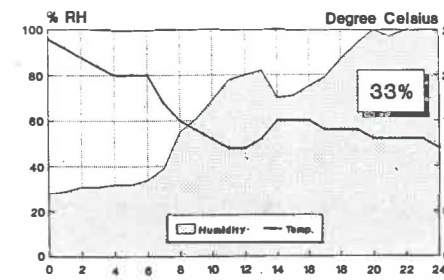
FIGURE 4:
Sight C, Herbicide-sprayer, April 27th
Spindle-bush double row system



Little rainfall hour 12-18

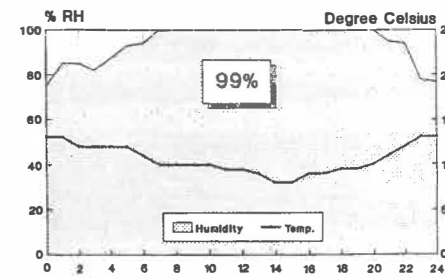
Fig. 5-10: Knapsack spraying application of *S. feltiae* against laboratory reared larvae of *C. pomonella* (0,5 - 1,0 x 10E6 J3/trunk):
Sight A: Experimental field at Institute, Dossenheim

FIGURE 5:
Sight A, April 19th



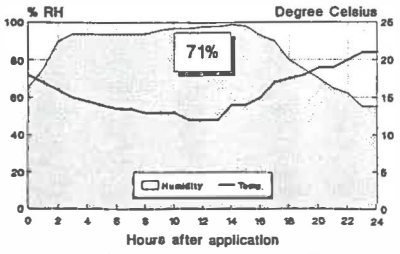
Rainfall: hour 18-20 (3,9 mm)

FIGURE 6:
Sight A, April 27th



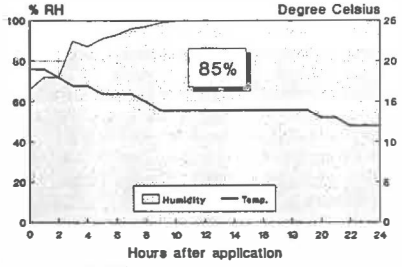
Rainfall: hour 3-7 (1,8mm), 15-18 (2mm)

FIGURE 7:
Sight A, May 16th
Nematode: S. carpocapsae



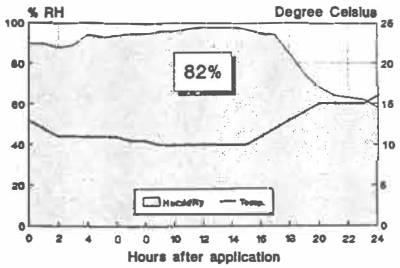
Rainfall: hour 1-3 (1,5 mm)

FIGURE 8:
Sight A, May 17th



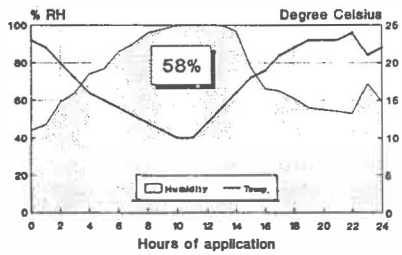
Rainfall: hour 17-22 (7 mm)

FIGURE 9:
Sight A, June 11th



Rainfall: hour 0 (2 mm)

FIGURE 10:
Sight A, June 15th
Dosage: 1.2x10E5 J3/trunk



No rainfall

When RH were below 60% at the moment of application and for 7 hours afterwards low mortality rates could be observed (fig. 1, 4, 5). The negative effect of these conditions could not be compensated by rising RH after that period. Screening and field-testing of various antidesiccants could lengthen the period of convenient moisture resulting in an increasing mortality of the target insect (review: Kaya, 1986)

In all experiments temperatures remained within the optimum range for entomoparasites during the first 24 hours after application (see above), so that the influence of temperature conditions can be expected not to be responsible for the variability of the results. An exception is given in fig. 4 where temperature did not exeed 8°C. A comparison of fig. 1 and 4 shows equally unfavourable RH conditions for parasitation of *S. myopaeformis* in both experiments. It can be assumed that the higher parasitation rate in fig. 1 in comparison to the results in fig. 4 is due to the higher temperature conditions.

CONCLUSIONS

1. Field application of entomopathogenic nematodes of the genera *Steinernema* proved to be principally suitable for use in biological and/or integrated pest management systems as a method for control of different cryptic living noxious insects like *S. myopaeformis*.

2. Due to its relatively low labour input only surface spraying application is justified for large scale treatments. As the success of the spraying technique largely depends on the weather conditions during and in the first hours after treatment, the moment of application must be carefully determined.

3. Inspite of an optimization of the application technique the use of entomoparasites in commercial practice is still limitated by the high costs of the nematode material. Therefore more efficient rearing techniques are required to reduce the costs of nematode production and hence render nematode application with an economically promising alternative.

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ON THE OCCURRENCE OF A MERMITHID ON SPANISH ACRIDIDS

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SUMMARY

A mermithid of the genus *Hexamermis*, probably *Hexamermis albicans* (v. Sieb.) emerging from the second to the fifth instars of *Dociostaurus maroccanus* (Thunb.), *Dociostaurus genei* (Ocsk.) *Calliptamus italicus* (L.), *Sphingonotus azurescens* (Rb.) and *Oedipoda charpentieri* Fieb. has been found in the permanent breeding areas of the Spanish locust. This is a new species for Spain and the combinations of the mermithid with the hosts *C. italicus, S. azurescens* and *O. charpentieri* are unrecorded elsewhere.

INTRODUCTION

Two species of the Acrididae, *Dociostaurus maroccanus* (Th.) and *Calliptamus italicus* (L.), are the most important locust pest in Spain. The former, the Moroccan locust, has been responsible for most of the historicaly reported outbreaks (VAZQUEZ-LESMES and SANTIAGO-ALVAREZ, 1991).

Uncultivated hilly lands in the meridional part of Spain (Extremadura and La Mancha) are the permanent breeding areas (CAÑIZO and MORENO, 1940) where appropriate control measures must be taken to avoid undesired locust outbreaks.

When the phase transformation theory of migrant locust (UVAROV, 1921) was assumed for *D. maroccanus* (CAÑIZO and MORENO, 1940; MORENO, 1942) the antiquated methods for locust control were discarded, assuring the succees of outbreak prevention with a reduction in human efforts and costs. Since then the risk of locust plague could be eliminated by means of chemical insecticides applied oportunely; nowaday control continues to rely on these products (MORAL, 1986).

As is common in many other pest control situations, there is an increasing interest on new alternative methods, with emphasis on enviromental preservation. Given the importance of the manipulation of natural enemies for biological control we have begun the study of the entomopathogens that affect locust populations at the permanet breeding areas. The work is a colaborative project between the S.I.A. of Badajoz and the Cátedra de Entomología Agrícola (E.T.S.I.A.) of Córdoba.

This paper is a preliminary report dealing with an entomophilic nematode encountered on many acridid species.

MATERIAL AND METHODS

Acridids at different instars were collected at the permanent breeding areas in Badajoz (La Serena and Llerena) from March to August 1990. Samples of acridid fauna were collected weekly by means of a sweep net. The individuals brought to the laboratory were introduced in plastic cups (6.5 cm.h.x 6.5 cm. O), one per cup, supplied with pieces of a semidefined diet (SANTIAGO-ALVAREZ, 1977) and maintained at room temperature, until death. Every twenty four hours the cups were checked to determine the presence of diseased or parasitized individuals. When a diseased insect was observed we determined the etilogical agent.

RESULTS AND DISCUSSION

During the survey period, 2012 acridid larvae of seven species were collected (TABLE 1). Our field observations indicate greater abundance of *D. maroccanus* than of *D. genei*. The reason that the number of *D. maroccanus* larvae collected was lower than that of *D. genei* may be due to differences in the species' development, so that the larval instars of the two species do not overlap.

TABLE 1. Acridid species collected in Badajoz (Spain).

SPECIES	Number of collected larvae	Percentage
Dociostaurus maroccanus (Thunb.)	661	32.9
Calliptamus italicus (L.)	466	23.2
Dociostaurus genei (Ocsk.)	707	35.1
Sphingonotus azurescens (Rb.)	105	5.2
Oedipoda charpentieri Fieb.	6	0.3
Other	67	3.3
TOTAL	2012	

Acridids showing reduced mobility, abdominal enlargement and weakly transparent integument were killed by a long nematode lodged in the hemocoele that emerged through the natural openings or in some cases through the integument. The emerged worms were juveniles whose characteristics corresponded to those of the family Mermithidae. This mermithid appears from mid April to the end of June.

The post-parasitic juveniles were transferred to cups containing clean wet sand, maintained in laboratory at 23±2 ℃, to facilitate adult transformation. This takes between 42 and 57 days (n = 21; $\bar{x}$ = 51) and the molting process takes 13 to 30 days (n = 16; $\bar{x}$ = 17,6). The length of males was between 7 and 14,5 cm (n = 27; $\bar{x}$ = 10,2) and the length of females between 18,5 and 29 cm. (n = 7; $\bar{x}$ = 23,6).

All postparasitic juveniles preserved short finger-like tail appendages. Adults had 4-6 rows in a typical pattern; males had two short spicules and the females vagina was S-shaped whith a well developed vulval cone . These characteristics correspond to those of the genus *Hexamermis* Stainer and probably to *Hexamermis albicans* (v.Sieb.) which

has a wide range of host terrestial insects and world wide distribution (Nickle, 1972). H. *albicans* (=*Mermis albicans*) was cited on *D. maroccanus* (Uvarov 1928) and the combinations of this mermithid with the hosts *Calliptamus italicus*, *Oedipoda charpentieri* and *Sphingonotus azurescens* found during this survey are unrecorded elsewhere. This is a new species for Spain and with the former *Mermis nigrescens* encountered on *D.maroccanus* adults (Benlloch, 1947) is the second mermithid species atacking the Morrocan locust in Spain.

Larvae of all the acridid species were killed by the nematode (TABLE 2) with a mean percentage mortality of 10 %, although nematode incidence reached 45 %, in a site sampled (near Campanario, Badajoz) with well drained gravelly loamy sand from granite. A remarkable spacial variability in mermithid parasitation percentages was recorded by Poinar & Gyrysco (1962) and the higest incidence of parasitism occurreed in a soil type with similar structure. Probably, good drainage permits mobility and high survival rate of mermithids which is reflected in parasitatism. The most parasitized especies were *D. maroccanus* and *C. italicus*, with 12 and 13 % respectively. In general, more females than males parasitized by the nematode were observed which agrees other reported data (GLASER and WILCOX, 1918). It has been suggested that the development period of the nematode is slower in females and thus parasitized females die after parasitized males (NICKLE , 1982). This could explain the difference in gender ratio in some cases. However, our results could not be explained by an imbalance in the gender ratios in parasitized field samples. In our *C.italicus* samples, for instance, a larger number of males than females was collected and the higher percentage of parasitized females prevailed (TABLE 2). Thorough researh on mermithid-host systems is needed to confirm the differences between males and females susceptibility.

The emergence of the nematode occured from the second to the fifth instars, but mostly at the host third-instar; nematode were never observed emerging from adults or in adult disections. One to four worms normally emerged from each parasitized host; sometimes more worms emerged, as was the exceptional case of one 5-instar *D. maroccanus* specimen that harboured 35 nematodes. Infective stages of the nematode probably enter the host larvae at the earliest instars; nevertheless, the small size nematodes found in the host nymphs may have entered at an advanced instar which would impede their normal development. This observation is agrees with those reported on *Hypera postica* parasitized by *H. arvalis* (POINAR & GYRYSCO, 1962).

TABLE 2. Percentages of parasitization by Hexamermis on acridids according to their sex.

SPECIES	Males		Females		Unknown sex		Total	
	N	%	N	%	N	%	N	%
D.maroccanus	283	9.8	281	14.5	97	10.0	661	11.1
C.italicus	239	9.6	198	17.6	29	6.8	466	12.8
D.genei	295	6.4	368	6.7	44	9.0	707	6.7
S.azurescens	34	5.8	40	10.0	31	3.2	105	6.6
O.charpentieri	-	-	-	-	6	33.3	6	33.3
Other	-	-	-	-	67	7.4	67	7.4
TOTAL	851	8.4	887	11.8	274	8.7	2012	9.9

N = Number of collected larvae
% = Percentage of parasitized larvae

The possible use of this nematod for the biological control of the Moroccan locust in Spain is now under research in our laboratory.

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FORM VARIANTS OF *XENORHABDUS LUMINESCENS*

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Introduction

Xenorhabdus spp. are insect pathogenic bacteria symbiotically associated with nematodes of the genera *Steinernema* and *Heterorhabditis* (Thomas & Pionar, 1979). Each species of nematode is associated with a single bacterial species; all *Heterorhabditis* spp. have *X. luminescens* as the symbiont but each species of *Steinernema* is associated with a different species of bacterium (Akhurst, 1980; Boemare & Akhurst, 1988).
Xenorhabdus isolates tend to produce two colony forms, a primary and a secundary form. The primary form is preferentially transmitted by the infective dauerlarvae ; it is unstable in culture where it converts into the secundary form. The primary form has the ability to produce antibiotics and a pigment, can absorp dye from agar media and, in case of *X. luminescens*, is luminescent. The secondary form has lost these abilities (Akhurst, 1980; Akhurst & Boemare, 1990; Boemare & Akhurst, 1988).
In this study form variants of *X. luminescens* XE-87.3, symbiont of *Heterorhabditis* strain HE-87.3, are described.

Methods

Bacteria were isolated directly from infective stage nematodes. The nematodes were surface-sterilized in 0.1% merthiolate, washed in sterile Ringer's solution (Oxoid) and macerated with a glass rod (Akhurst, 1980). Samples of the macerate were spread on nutrient agar (Lab Lemco, Oxoid) and inoculated at 25°C. The bacterium of *Heterorhabditis* strain HE-87.3 was also obtained by sampling the heamocoel of waxmoth larvae (*Galleria mellonella*) infected by nematodes. Red or orange luminescent colonies were selected. The strains were individually subcultured every 3 or 4 days on nutrient agar. Luminescence was determined by observation of cultures in a dark room. The production of antimicrobial subtances by *Xenorhabdus* strains was determined as described by Akhurst (1982). Uptake of neutral red and bromothymol blue (BTB) was tested on MacConkey agar and NBTA (Akhurst, 1982) respectively. Restriction endonuclease analysis, purification of DNA, agarose gel electroforese and sodium dodecyl sulfate polyacrylamide gel electroforese (SDS-PAGE) were performed as described by Sambrook et al. (1989). RFLP was performed with total DNA of the bacterium digested with the restriction enzyme MspI. The fragments were separated on an 0.8% agarose gel. Protein patterns of whole cell extracts of *X. luminescens* were made on an 12% SDS-poly-acryl-amide gel.

Results

A red *Xenorhabdus* variant (XE-red) was isolated, both from the nematode and from infected waxmoth larvae. XE-red has all the abilities of a primary form (Table 1). Next to the red variant

always a small, round, white colony (XE-white) appeared. It was not possible to separate the two forms but selection for more red than white colonies and vice versa was possible. After 2 to 3 months of subculturing XE-red, two other forms were found: a yellow and a pink variant. The red and pink variant have large cells, 4 to 8 μm. The white and yellow variant have small cells, 1 to 2 μm. Table 1 summarises the main characteristics of the four XE-variants.
The white and yellow variant are very unstable. After 3 to 5 days red sectors appear in the colony. These sectors contain large cells, like XE-red. Young colonies were composed of almost only small cells but after 48 hours the amount of large cells increased.
RFLP patterns of total bacterial-DNA, where identical, suggesting that all four variants are genetically similar and are therefore derivatives of the same parent.
The SDS-PAGE protein patterns showed some differences between the four variants. Characteristic is a large protein band of approximately 10 kDalton. This band is absent in XE-white and less intense in XE-yellow.
The small white variant is also found in all other *X. luminescens* strains we worked with, namely in nine strains of the North-West-European *Heterorhabditis* group (Smits & Ehlers, 1991), in the Irish *Heterorhabditis* strain M145 and in a *H. bacteriophora* strain.

Table 1. Characteristics of the four form variants of *X. luminescens* XE-87.3.

Variant	XE-RED	XE-PINK	XE-YELLOW	XE-WHITE
pigmentation on NA*	red	pink	yellow	none
colony form	large, uneven margin	large,uneven margin or round	medium,round, smooth margin	small,round, smooth margin
cell size	large,4-8um	large	small	small,1-2um
inclusion granules	+**	+	+-	-
luminescence	++	++	+	+-
antibiotic production	++	++	+	-
uptake neutral red	++	-		
uptake BTB	+-	-	-	-
stability	stable	stable	unstable, after 4 days shift to XE-red	unstable, after 4 days shift to XE-red

* NA= Nutrient agar (Lab Lemco agar, Oxoid)
** ++ positive respons, + moderate respons, +- weak respons, - negative respons

Conclusions and discussion

The red variant should be considered as the primary form of *Xenorhabdus* XE-87.3.
A secondary form, as described in the introduction, was not found for XE-87.3. The small white variant is not a secondary form because of its small cells and instability (Akhurst & Boemare, 1990). The pink and yellow variant still have the ability to produce antibiotics and are luminescent. This suggests that the terms primary and secondary are not applicable to the variants of this *Xenorhabdus* strain.
The small white variant is typical for several *X. luminescens* strains. It is always associated with the red variant of these strains. Hurlbert *et al.* (1989) described a colony form of *X. luminescens* strain HP88 that is similar to the white variant described here. They also found this small variant associated with the secondary form of HP88. This supports the conclusion that the white variant is not the secondary form. XE-pink and XE-yellow appeared only a few times. They could be the result of an incomplete switch from the primary form to XE-white or vice versa.
The significance of the polymorphism of *Xenorhabdus* spp. for the bacterium itself is unknown at the present. The small variant could have a survival advantage. Small cells with very little secundary metabolitic activity reproduce more easily. The mechanism by which these variants arise is still a question.

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Use of digestive enzymes for the study of the parasitism of Steinernema and Heterorhabditis in insect larvae

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The evaluation of the pathogenicity of *Steinernema* and *Heterorhabditis* (penetration and development) is important in laboratory experiments and field trials. However, dissection of insect larvae to count the nematodes and to determine the stages is difficult. Digestive proteins (pepsin and papain) are commonly used in veterinary research for the recovery of nematodes (Trichostrongylidae) embedded in the tissues of the gastrointestinal tract of ruminents. With some modifications, this technique can be succesfully applied for the recovery of L3 and adults of *Steinernema* and *Heterorhabditis* in the body cavity of insect larvae. Pepsin and papain have the same digestive activity but pepsin which tends to clear the solution allows best examinations. The quantification of nematodes in a *Galleria* treated with pepsin is rapid compared with the manual dissection. One another advantage of the method is the possibility to freeze the insects treated with pepsin for further investigations.

Procedure
pepsin solution: pepsin 8 g, NaCl 23 g, HCl 20 ml, H2O 940 ml
papain solution: papain 8 g, NaCl 23 g, HCl 20 ml, H2O 940 ml
30 *Galleria* in 3 ml solution, shaking 2 h.

Recovery of heterorhabditid nematodes from Irish and Scottish soils

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INTRODUCTION

An earlier survey of the Republic of Ireland for entomopathogenic nematodes (Griffin *et al.*, in press) recovered steinernematids from 10.5% of samples but *Heterorhabditis* was recovered from only one of the 551 soil samples tested. Similarly, *Heterorhabditis* was recovered from only one site in Britain (Hominick & Briscoe, 1990) and was not recovered at all from Northern Ireland (Blackshaw, 1988) while again *Steinernema* was prevalent in both these regions. The single Irish sample positive for *Heterorhabditis* was from a very sandy soil close to the coast. We wished to understand whether *Heterorhabditis* was indeed rare in these islands or was restricted to a particular habitat. We subsequently sampled from sandy soils around Ireland, both inland soils of glacial origin and coastal soils, and from coastal soils in Scotland.

METHODS

Sites with sandy soils were identified from maps (including the Soil Map of Ireland and satellite maps) and by local inspection. Samples consisted of a number of subsamples taken to a depth of about 10cm with a soil corer or with a small spade.

Samples were transported to the laboratory and were baited with *Galleria mellonella* larvae (Bedding & Akhurst, 1975) at a rate of 5 larvae per 500cc of soil at 15° or 20°C Bait insects were recovered after 10 (15°C) or 7 (20°C) days. Luminescent cadavers were incubated on damp paper and transferred to modified White traps for emergence of nematodes. Emerging nematodes were used to infect fresh *Galleria* larvae for identification and esablishment of cultures.

A selection of soil samples from which *Heterorhabditis* was recovered were analysed for pH and organic carbon by the Teagasc Soil Laboratory, Johnstown Castle. Determination of organic carbon was by the Walkley Black Method.

RESULTS

Sampling was carried out in Ireland from Autumn 1988 to June 1990. *Heterorhabditis* was not detected at any of the 31 inland sites sampled, but was recovered from 18 (or 13.7%) of the 131 coastal sites (Fig. 1). The genus was present at 2 (3.9%) of the 51 sites sampled in Scotland in

Fig. 1. Distribution of heterorhabditids in Ireland.

autumn 1990. The occurrence of *Steinernema* which was frequently present was not recorded in this survey.

All of the sites from which *Heterorhabditis* was recovered were within a few hundred metres of the sea; they were predominantly either within dune systems or in nearby grassland used for pasture or recreation (e.g. caravan parks, golf links). The only soils sampled in the present survey were sandy coastal soils. The organic matter content ranged from 3 to 7%, and pH from 4.6 to 8.1.

DISCUSSION

In our previous survey of entomogenous nematodes in Ireland, *Heterorhabditis* was recovered from only one of the 551 samples tested; this sample was from a sandy coastal soil. In the present survey, the genus was recovered from 14% of soils having a similar texture and location. It would appear from this that *Heterorhabditis* in Ireland is more or less restricted to sandy soils close to the sea. The same may be true for Scotland also, but we have not sampled inland in that country. From our results, it is not possible to say whether the preference of the genus in these islands is for sandy soils *per se*, or whether it is also related to the moderate temperatures of the coastal zone.

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