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Integrated Control of Soil Pest Subgroup "*Melolontha*"

Lutte Intégrée contre les Organismes du Sol Sous-groupe "*Melolontha*"

editor:

Siegfried Keller

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Working group "Integrated Control of Soil Pest"
Subgroup "*Melolontha*"

OILB / SROP

Groupe de travail "Lutte Intégrée contre les Organismes du Sol."
Sous-groupe "*Melolontha*"

PROCEEDINGS of the MEETING

COMPTES RENDUS de la RÉUNION

at/à

Sion (Switzerland)
19 - 21 October 1998

Edited by Siegfried Keller

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Introduction

The working group "Integrated Control of Soil Pest" subgroup "*Melolontha*" held its second meeting within the frame of IOBC. 38 participants from 9 countries met from 19-21 October 1998 at Sion, Switzerland. The local arrangements and a half-day excursion to *Melolontha* sites in the upper Rhone valley were successfully organised by Augustin Schmid.

17 oral and 5 poster contributions were presented dealing with the following topics: Damages, distribution, population development, integrated and biological control, current situation and prospects, other soil living scarabaeid larvae. The problems with *Melolontha* spp. are increasing in central and east Europe with some local exceptions. They are mainly due to *M. melolontha* L. and concern grassland, orchards and reforestation areas, occasionally vineyards and other crops. Two main non-chemical control measures have been developed: the placements of nets to protect expensive crops and the use of the entomopathogenic fungus *Beauveria brongniartii*. Both are successfully used but there are still some open questions concerning the efficacy of *B. brongniartii* in dry soils. Attempts with other pathogens failed, but good control of *Amphimallon* larvae were obtained with nematodes.

A highlight of the meeting was the excursion into the upper Rhone valley in the vicinity of Brig where white grubs cause severe damages in meadows on steep surfaces leading to a high risk of soil erosion (see contribution of A. Schmid). Representatives of the local extension service explained the problems in context with the local farming system. A machine designed to apply fungus material on steep surfaces was demonstrated and the points to be considered were discussed (see contribution of H. Matzke).

Future work of the group will concentrate on improving the existing control measures and exploring new ones. With respect to *B. brongniartii*, the quality of the material and of the application as well as the efficacy in dry soils must be improved. Therefore soil-fungus interactions need to be studied. There is also a need to standardize sampling procedures of white grubs and fungus. It was decided that the next meeting will be in Vallée d'Aosta, northwest Italy, in 2001.

Siegfried Keller
Convenor of the subgroup

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Table of Content

Biological control with *Beauveria brongniartii*

Miscellaneous

Population development, damages, integrated control

Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.3 - 4

Evolution de la situation relative au Hanneton en Vallée d'Aoste

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Resumé: Le ver blanc (larve de *Melolontha melolontha*) est le principal ravageur des vergers et des prairies de la Vallée d'Aoste. La surface concernée est de presque 8 000 ha. Aucune technique de lutte proposée jusqu'à présent ne s'est avérée satisfaisante. Pour suivre l'évolution de la situation, des sondages sont effectués toutes les années le long de l'axe de la vallée principale et tous les trois ans, à l'occasion du stade L2, également dans le sens perpendiculaire à l'axe principal. Pendant les trois ans du cycle, plus de 2 000 sondages sont effectués. Depuis la réunion précédente qui a eu lieu en 1993 à Freiburg, en Allemagne, la situation a empiré, car la moyenne sur les 8 000 ha concernés est passée de 12 à 21,3 larves (L2) par mètre carré.

Abstract: The evolution of the situation about the *Melolontha Melolontha* in Aosta Valley: The white worm (larva of *Melolontha melolontha*) is the main ravager of the orchards and meadows of Aosta Valley. An area of about 8000 hectares is concerned. Up to the present, none of the control techniques proposed was satisfying. In order to follow the evolution of the situation, surveys are made every year along the axis of the main valley. Besides, every three years, when the phase L2 takes place, surveys are made also perpendicularly to the same axis. During the three years of the cycle, more than 2000 surveys are carried out. Since the previous meeting, which took place in 1993 in Freiburg (Germany), the situation has got worse. In fact, in the 8000 hectares concerned, the average number of larvae (L2) per square metre rose from 12 to 21.3.

Introduction

Ce rapport représente une mise à jour sur l'évolution de la population du hanneton (*Melolontha melolontha*) en Vallée d'Aoste depuis la réunion précedente du groupe de travail "lutte intégrée contre les organismes du sol", sous groupe "Melolontha", qui s'était déroulée à Freiburg (Allemagne), du 23 au 25 octobre 1995.
Rappelons les éléments essentiels du problème du hanneton en Vallée d'Aoste.

- les infestations sont historiquement attestées car déjà en 1644, d'après l'Abbé Henry, une procession avait été faite pour libérer les campagnes de ce parasite. Le hannetonage fut financé d'abord par le "Cornice agricole", ensuite par l'assessorat de l'agriculture "jusqu'en 1989;
- la surface concernée est de 7830 ha;
- la densité moyenne de larves (L2) par m² était de 32 en 1987, de 11 en 1990; de 12 en 1993, de 21 en 1996.

Buts

- vérifier si l'aire de diffusion s'est modifiée;
- vérifier les eventuelles régressions au cours du cycle;
- comparer la densité des différents cycles;

- rechercher et établir l'eventuelle présence d'auxiliaires.

Modalités d'intervention

- moyens: pioches Blaisinger (lame de 16 cm); cartes et photographies aériennes;
- méthode: creusage d'une tranchée de 17 cm de largueur de 1 m, de longueur, correspondant à 1/6 de m² (Chessel *et al.*, 1984);
- distance des creusages: 100 m sur l'axe du fond de la Vallée et, en correspondance des stades L2, également sur les perpendiculaires à l'axe susdit. Au total 386 sondages ont été effectués en 1995, 1335 en 1996 et 386 en 1997;
- unité territoriale 22 photographies aériennes.

Résultats

- l'aire de diffusion est la même des cycles précédents, c'est à dire 7830 ha, de Pont-Saint-Martin (343 m) à Morgex (1000 m);
- les cultures infestées sont les prairies permanentes (7380 ha), les vergers (400 ha),et les vignobles (50 ha);
- le nombre moyen de larves par m² du cycle 1995/98 sur l'axe principal était de 45,9 en 1995; de 23,5 en 1996 et de 14 en 1997 (voir tableau);
- le nombre moyen de larves (L2) par m² dans les quatre derniers cycles a été de 32,4 en 1987, de 10,7 en 1990, de 12 en 1993 et de 21,3 en 1996 (voir tableau).
- le pourcentage de larves momifiées par la moisissure *Beauveria brongniartii* et repérées pendant les sondages a été de 0,38 en 1993, de 0,84 en 1996 et de 3,99 en 1997.

Conclusion et perspectives

Depuis des siècles, le ver blanc est le parasite le plus nuisible pour notre agriculture, l'arboriculture en est particulièrement touchée.

Les arboriculteurs sont dégoutés par le manque de remèdes efficaces. Notre production de pommes est fortement reduite; les pommiers atteints produisent des fruits de mauvaise qualité. Le remplacement des vieux vergers (10÷12 hectares/an sur un total de 400 hectares) est absolument insuffisant pour assurer un avenir à l'arboriculture valdôtaine.

Les produits chimiques se sont avérés polluants et peu efficaces, la lutte mécanique avec les filets est efficace mais trop coûteuse car un grand nombre d'heures de travail sont nécessaires pour placer et retirer les filets, surtout dans les vergers en pente et entre les lignes non régulières des arbres.

Dans le but de réduire les frais de main d'oeuvre nous expérimentons actuellement la pose d'un filet fixe.

L'emploi de nématodes entomopathogènes est pour le moment trop coûteux.

L'inoculation dans les nids de ponte de la moisissure *Beauveria brongniartii* avec le semoir conçu par la station expérimentale de Laimbourg pose des problèmes, car cette machine ne peut pas opérer dans nos vergers en pente. Les résultats obtenus avec l'épandage à la volée de graines enrobées, sur sol enneigé, demandent des vérifications ultérieures.

Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.5 - 9

The common cockchafer (*Melolontha melolontha* L.) in the Adige valley (Trentino): development of the population from 1987 - 1998.

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Abstract: In Trentino the first new centres of infestation by the common cockchafer (*Melolontha melolontha* L.) were reported in 1987. At that time detailed investigations were started to evaluate the population density and the diffusion of this plant pest. The affected area increased from two initial centres of infection in 1987 to about 100 ha in 1989-90 and with the flight of 1991 it reached about 150 ha. With the flight of 1994 the affected area increased to about 250 ha and with the flight of 1997 to 350 ha. In both these last periods the population density, however, decreased. As for apple trees and young vines of less than three years, the most severe damages are caused by the larval activity of this Coleoptera species, which inflicts serious injury to the root system.

Key words: *Melolontha melolontha*, population dynamic, damage

Introduction

The presence of the cockchafer in Trentino is documented by numerous bibliographic and popular reports (Bollettino Agrario 1896) and the latest reappearance has evidenced the particular need for finding means of control of this pest in horticulture and viticulture.

After a first approach of chemical control, which showed little effect, biological and physical means of control were used. In 1989 the first trials were started with the fungus *Beauveria brongniartii*, produced by the Agricultural Research Centre Laimburg (BZ), but the effects took place rather slowly, and the growers demanded faster means of control.

Thus in 1991 the first trials with covering the soil with plastic nets during the flight period were started, which led to very interesting results. The costs of this system were justified by the high value of the crops and in the affected areas the growers engaged themselves in organising replantings of the orchards and the placement of soil covers according to the flight periods of the cockchafer.

After the great efforts at the beginning of the '90ies for finding means of control in horticulture and viticulture a continuous strategy of control of the cockchafer has been maintained in this area of the Adige Valley in Trentino. In our province several foci of infestation have been reported, but the most conspicuous presence and the heaviest damages have always been observed in the area between the municipalities of Mezzocorona and S. Michele all'Adige.

Materials and Methods

Since 1989 samplings have been carried out in the areas affected by the cockchafer; the observations were carried out counting all the adults or larvae present in holes 50x50 cm wide and 50 to 70 cm deep. Four to six holes were dug per hectare and the soil was sieved in the

In 1991, 1994 and 1997 also visual controls of the flight were carried out, giving an evaluation of the duration and intensity of both the nutrition flight (towards the woods) and the oviposition flight (return flight).

Results

Situation of the cockchafer population until the flight in 1997

Since the first damages were reported in 1987 the affected area has increased with the various flights to a total area of about 250 hectares. In '89 and '91 there were increased reports of damages, in particular in orchards, whereas the wines proved to be susceptible only in the first years after establishment. Table 1 shows the increase of the area affected by the cockchafer from 1990 to 1997 (before the last flight), and the mean population density observed.

Table 1: Area affected by the cockchafer from 1990 to 1997 and the mean population density.

Diffusion of the cockchafer in Mezzocorona and S.Michele a/Adige		
year	**affected area**	**mean population/sqm**
1990	100 ha	22,8 ADULTS/sqm
1991	150 ha	20,9 larvae 1st year/sqm
1994	150 ha	13,4 ADULTS/sqm
1995	250 ha	4,3 larvae 2nd year/sqm
1997	250 ha	4,8 ADULTS/sqm

Considerations

The observation of the events of these eight years has lead to the following considerations:

- Each flight has brought about an inevitable increase of the affected area, although with a concomitant reduction of the mean population density per unit area;
- The area of expansion consists mainly of older vineyards and consequently there were less reports of damages;
- Several orchards situated in the affected area were covered with plastic nets during the flight of the adults which has led to a consistent reduction both of the damages and the cockchafer population.

Observations of the flight in 1997

The observations were started at the end of March. The flight towards the woods started on 1st April and was repeatedly interrupted by low temperatures which considerably disturbed the activity of the adult cockchafers. The peak of the flight was observed between 22 and 26 April and reached its end towards mid May. Compared to the previous flights (1991 and 1994) the intensity of the flight was reduced.

During the whole period of the flight no considerable presence of cockchafers on cultivated plants (apples and vines) was observed. The return flight started on 22 April, reached its peak around 25 April and continued until 15 May.

Situation after the flight in 1997

During 1998, the period of the most intense larval activity, only single cases of damage were reported. At the end of September 1998 the area affected by the cockchafer was monitored, examining both the traditional foci of infestation and the areas of recent expansion.

In the first areas the mean population was 3.6 larvae (2nd year)/sqm (minimum 0 and maximum 21 larvae/sqm). The areas of recent expansion (minimum 0 and maximum 12 larvae/sqm) and those adjacent to them (minimum 0 and maximum 9 larvae/sqm) had a mean population of 2.3 larvae/sqm.

Table 2 Situation in autumn 1998: larvae 2nd year

location	affected area	mean population /sqm
1st focus of infestation	100 hectares	3.6 larvae
Areas of recent expansion	50 hectares	2.3 larvae
adjacent areas	100 hectares	2.3 larvae

After the flight of 1997 the area affected by the cockchafer increased by about 100 hectares reaching thus a total area of 350 hectares.

Discussion

- The present population is in continuous decline; the mean population is in fact 2.5 larvae (2nd year)/sqm;
- In all three monitored areas however some small foci of infestation could be detected which can represent a potential risk for newly planted orchards;
- In 1997 only a few hectares were covered with nets.

Table 3 Diffusion of the cockchafer in Mezzocorona and S.Michele a/Adige

Year	Affected hectares	mean population/sqm
1990	100 ha	22.8 ADULTS/sqm
1991	150 ha	20.9 larvae 1st year/sqm
1994	150 ha	13.4 ADULTS/sqm
1995	250 ha	4.3 larvae 2nd year/sqm
1997	250 ha	4.8 ADULTS/sqm
1998	350 ha	2.5 larvae 2nd year/sqm

Table 3 summarizes the diffusion of the cockchafer from 1990 until autumn 1998. It can be noticed that the affected area has increased, although the population density is in continuous decline.

Present species

Also during the flight of 1997 two morphologically different types of cockchafer were observed, which seem to be *Melolontha melolontha* L. and *Melolontha hippocastani* F.. The two species were observed mating, but it could not been verified whether fertilization took place. It needs to be underlined that in our area the flights take place at three-year intervals and that during years within periods hardly any flight occurs; moreover, numerous samplings carried out in the field

have revealed larvae of the same age, and only in rare cases individuals of different stages of development were observed.

Effect of natural parasitization

Several investigations carried out during these years have never shown an incidence of natural parasitization to such a degree that the evolution of the cockchafer population was influenced. Table 4 lists the percentages of parasitization observed from 1990 to 1998.

Table 4: Percentages of parasitization by *Beauveria brongniartii*

% parasitization by *Beauveria brongniartii*						
year	1990	1991	1994	1995	1997	1998
location	adults	larvae 1st year	adults	larvae 2nd year	adults	larvae 2nd year
1° focus of infestation	8.9	2.3	0	0	6.1	4.7
areas of new expansion	0	1.2	0.9	0	0	17.1
Adjacent areas	0	5.3	0	0	0	1.5
TOTAL	8.2	2	0.4	0	2.5	6.7

Conclusions

The experiences about the cockchafer gained in our region are particularly interesting, because they represent an example of the ineffectiveness of the traditional methods of chemical control compared to the possibilities of physical and biological control.

In our region a decrease of the population density with time can be observed, although it is difficult to affirm with certainty that this is a durable process. The last two flights (1994 and 1997) actually took place in periods of unfavourable climatic conditions, which could have had consequences on the population development, and also a decline of the fitness of the females could be observed. It is therefore necessary to confirm this development of our cockchafer population also during the next flights, before being able to consider the cockchafer problem as effectively diminished.

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Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.11 - 13

Cockchafers (*Melolontha melolontha*) - The last 20 years in the Valais

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Abstract: The cockchafer situation in the canton Valais in the last 20 years is presented. It is characterized by a three and a four cycle, the latter exists at altitudes above 750-900 m. Although some populations have declined, there is a tendency for a general population increase.

Key words: *Melolontha melolontha*, distribution, population dynamics, damage

Distribution of the different developmental cycles of cockchafers in the Valais

The distribution of cockchafers in the Valais is related to the presence of permanent grassland (i.e. Brig and surroundings, the valley of Goms, Susten to Turtmann, Vollèges and St. Maurice to Evionnaz). In the central Valais from Martigny to Sierre this insect is mostly present in areas with tall fruit-trees, where fruits and hay are produced, as in Sion-Bramois.

Four different cycles of development are observed depending on the region. These cycles are shown in the appendix. The duration of one complete cycle is 3 years up to an altitude of 750 to 900 m a. s. l. and 4 years at higher altitudes. However, if several years with high temperature succeed, the cycle of 4 years is partially reduced to a cycle of 3 years, for example around Brig. It is very interesting to observe that the cycles are very constant. For example, it was described about a hundred years ago, that the flight in Vollèges was one year before that in another region in the central Valais as it still happens today.

Evolution of the density of the cockchafer population from 1979

In some regions, the density of the population was high over 20 years, independent of pest control measures (Susten, Bramois, St. Maurice). In some regions, the density of the population decreased (Lax, Bitsch). However, in most of the regions we observe an increase or a displacement of the zones with damage: from Ried-Brig to Termen, from Niederernen to Ernen; from Vollèges to Sembrancher; from St. Maurice to Massongex. The evolution of the density of the cockchafer population is difficult to explain and the fluctuation in the different zones are hardly predictable.

Damages caused by cockchafer larva

The economic damages are important especially on permanent grassland and on some fruit plantations. The office of agricultural consultation estimated the damages on permanent grassland over two cycles:

	damaged grassland	
	loss: 30 to 50 %	loss:more than 50 %
1984 to 88 resp. 1986-89	165 ha	100 ha
1988 to 92 resp. 1989-92	111 ha	250 ha

For the last two cycles (1992-96 resp. 1992 to 95 and 1996 to 98 resp. 1995 to 98), the area of the damaged grassland decreased. However, the losses were still very important in some regions.

Among the fruit cultures the raspberry and strawberry plantations were damaged strongly due to their little tolerance against cockchafer larvae. The losses in the orchards in the region from Bramois to St. Léonard were locally very important.

Success and failure of different pest control methods

Since 1980, several methods (chemical, biotechnical, biological and mechanical) are tried in the Valais to control cockchafer larvae or to reduce damage:

1. Deltametrin (Decis) in 1980

Brig, Ried-Brig on 3000 trees distributed over about 250 ha,
product applied with the mistblower Swissatom 3000.

Results:

⇨ Immediate, rapid and good effect on cockchafers.

⇨ Good protection of grassland against cockchafer larvae for the following years.

2. Inhibitor of synthesis of chitin: diflubenzuron (Dimilin) in 1984

The same region as mentioned above; product applied by helicopter.

Results:

⇨ no effect on cockchafers.

⇨ no significant decrease on the fertility of the collected cockchafers.

⇨ important losses on the grassland for the following years due to cockchafer larvae.

3. Blastospores of* Beauveria brongniartii, *applied by helicopter

1986: 7 ha in Bramois.
1988: 100 ha in Ried-Brig, Ernen, Lax.
1989: 30 ha in Turtmann.

Results:

⇨ 60 to 90 % of the cockchafers collected after the treatment on the trees and developed in laboratory died due to the fungus.

⇨ The cockchafers returned into the soil for depositing eggs, however they come out to die anywhere in nature at an important distance to the site of depositing eggs. For this reason the transmission of the *Beauveria brongniartii* fungus on the descendants did not happen.

⇨ The density of the populations of cockchafer larvae remained nearly at the same level as in the sites without treatment during the following years.

⇨ The level of mortality of the cockchafer larvae due to the fungus during the following cycles was very variable form one site to the other, however it was independent of the treatment (0 to 59 %).

⇨ The level of mortality of collected cockchafers during the following flights was 1 to 25 % independent of the treatment on the preceding generations.

4. Grains of cereals colonized with* Beauveria brongniartii *(fungus grains)

Several experiments on small surfaces since 1988:

⇨ Correct application of fungus grains in grassland is difficult to achieve with some machines (for example with the machine Eurogreen)

⇨ Incorporation of fungus grains in ploughed soil of orchards is easier

⇨ The efficiency on cockchafer larvae is vary variable, the results are sometimes contradictory, the level of mycosis as well as the density of population.

5. Protective nets

Experimental applications on small areas in 1992, on 20 ha in 1995 and on 30 ha in 1998 in the orchard of Bramois-Sion. Costs: Price of the nets: 3000.- SFr/ha and 60 hours of work at 20.- SFr. (1200.-SFr.) = 4200 SFr./ha. If the nets of the canton of Thurgau are hired the costs are lower.

Results:

⇨ very strong decrease of the density of cockchafer larvae population during the following years:
- without insecticides on the nets: reduction of the population of 80 to 95 %
- with insecticides on the nets: reduction of the population of 90 to 99 %

Conclusions

⇨ Evolution of the density of cockchafer larvae populations are variable and impredictable from one site to another.

⇨ Well applied chemical pest control during the flight of cockchafers allows to avoid or to reduce damages of this insect under certain circumstances. However, the effects on the rest of the fauna needs to be studied and discussed.

⇨ *Beauveria brongniartii* applied by helicopter does not give positive results neither for short term nor for long term.

⇨ *Beauveria brongniartii* applied as mycelium/conidia on cereal grains give random results. The chosen application method is important, but it does not always explain the positive or the negative results.

⇨ The protection of fruit cultures (orchards, raspberry, strawberry, aromatic plants) by nets is efficient but relatively expensive.

⇨ For permanent grassland an efficient control method still needs to be found.

Integrated Control of Soil Pest, subgroup "Melolontha"
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Possibility of the Integrated Control of *Melolontha* larvae

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Abstract: In the last years problems of *Melolontha* larvae has increased in Poland, especially in the case of a reforestation of non cultivated lands abandoned by agriculture, where pests occur soil habitat have comfortable conditions for a population increasing. Looking forward for new approaches to the control of *Melolontha* pests the bioassay was conducted in the order to recognise the possibility of the simultaneous application of biological agents and chemical insecticides.

In bioassay the entomogenous nematode *Heterorhabditis megidis* and entomopathogenic fungus *Beauveria brongniartii* as well as low doses of chemical insecticide (carbosulfan) were used for control of grubs. Results show that combine treatment with fungus, nematode and insecticide carbosulfan increase a mortality of *Melolontha* larvae. Also, combine treatment of only nematodes and fungus show higher efficacy than treatment of alone agent.

Key words: *Melolontha melolontha*, grub control, *Beauveria brongniartii*, *Heterorhabditis megidis*, IPM, mixed applicaton

Introduction

In Poland grubs population density increase because low productivity agricultural lands are uncultivated. High population density of grubs makes difficulties for the reforestation of uncultivated area. This is a reason that more than 20-30% uncultivated lands need crop protection undertakings (Malinowski *et al.*, 1996). Environmental protection principles of national forestry policy order to reduce the usage of chemical pesticides to protect the natural biodiversity of forestry landscapes. It indicates to search evironmental friendly solutions, like biological control or cultivation methods instead of chemical methods.

However, recent field tests with nematodes in genera *Steinernema* and *Heterorhabditis* and entomopathogenic fungus in genera *Beauveria* have shown that they can be effective biological control agents against these insects (Keller 1986, Klein 1990). Unfortunately, the separate application of entomopathogenic fungus or nematodes to control of *Melolontha* larvae shows that the efficiency could be unreliable (Zegler 1993, Vlug 1996). It is necessary to search more holistic approach in the control of *Melolontha* grubs.

Integrated Pest Management (IPM) is defined as applied pest control with combines and integrates biological and chemical control. Chemical control is used as necessary and in a manner which is least disruptive to biological control (Stern *et al.*, 1959). Pest should be reduced (as opposed to completely exterminated) only when their populations reach levels at which economic injury to the crop is expected. Economic threshold, set below the economic-injury level, sever to signal the need for action to keep the pest population from reaching the point at which economic injury would occur.

The founding principles of IPM are that natural the process can be manipulated to increase their effectiveness, and control fail to keep pests below economic-injury levels. There are defined two IPM strategies:

1. IPM strategy depends on pesticides as the primary management tools and have highlighted the need to develop systems that depend primarily on biological control organism, resistant plants, cultural controls, and other ecologically based tools (Edwards 1991).
2. IPM biologically intensive strategy relies on biologically based tools from those that depend primarily on conventional broad-spectrum pesticides (Edwards 1991).

In our study was investigated other apect of pesticides in the Integrated Pest Management strategy. In contradiction to the customary role of insecticides as exterminators of pests we emphasise the importance of eventually synergism between insecticides and biological agents use in the control of *Melolotha* larvae. To reduce the environmental impact of chemical factors in the action it was intended to minimise the concentration of insecticides as much as possible. We expect that even low concentration of the insecticide can weaken a defensive mechanism of insects what enter a way of entomopathogenic factors attact.

Materials and methods

Melolontha melolontha L. (*Scarabaeidae*) larvae of III stage was used in our study. Grubs were collected in June and July from the uncultivated area near Olsztynek, North of Poland. Larvae were kept separately in pots contain soil origin from the some area as grubs, and incubated in the temperature 19°C. They were fed with slices of a fresh carrot.

The entomopathogenic fungus *Beauveria brongniartii* (commercial strain com. Andermat from Switzerland) was utylized in the bioassay. We used a dose 14 mg biopreparat per 28 cm^2 of sand surface. It is equivalent of the recommended dose use in the control of grubs.

Heterorhabditis megidis (HSH2, strain isolated in Germany by Dr. Ehlers, Kiel) was treated individually or jointly with the fungus. Infective juveniles were reared on *Galleria mellonella* larvae (Dutky at al. 1964) and stored in tap water at 6°C. They were used for experiments within 2-3 weeks of being harvested. We use a suspention of 1400 EPN juveniles per 28 cm^2 of sand surface, as a equivalent of 50 Mil. EPN juveniles per 100 m^2, a dose used for the pests control.

We used carbofuran as 50g/kg GR (Furadan 5 GR, FMC - USA). This insecticide is applied for the control of *Melolontha* grubs in the Polish forestry. In the bioassay we treated carbofuran in dose that is recommended: 1,5 mg per 1 Kg of the sand.

The effect of mix application of entomopathogenic fungus, entomogenous nematodes with the low concentration of insecticide were examined in tests conducted under laboratory conditions in temperarure 19°C. *M. melolontha* larvae were placed individualy into the pot (top surface of ca 28 cm^2) contain 0,2 kg of a damp sterile sand with or without carbofuran. Insecticide was mixed with the sand 24 h before application of biological agents. Next the sand was treated with EPN juveniles and/or fungus. We tested 30 insects for one combination.

Mortality of grubs was checked: 7, 14, 21, 28 or 35 days after application of biological agents.

Results and discussion

The mortality of *Melolontha melolontha* larvae III stage treated with a biological agent alone reached the level of 60% for *H. megidis* and 70% for *B. brongniartii* (tab. 1). This confirms results of other researches that entomopathogenic fungus and entomogenous nematodes could be an effective factor of *Melolontha* grubs control under laboratory conditions (Keller 1983, Vlug 1996) The mortality was higher when insects were treated with mix application of *B. brongniartii* and *H. megidis* or with mix application of the biological factor and the low dose of carbofuran in one treatment (tab. 1). In the case of joint application fungus and nematode and low dose of carbofuran the mortality of insects was highest (100%).

Table 1. The mortality of *Melolontha melolontha* L. larvae III stage 34 days after treated by carbofuran, *Beauveria brongniartii* and *Heterorhabditis megidis*.

Treatment of Biological and chemical factors	**Mortality in %**
Control	20,0
Beauveria brongniartii	70,0
Heterorhabditis megidis	60,0
Beauveria brongniartii, Heterorhabditis megidis	75,0
Carbofuran 3 mg/kg soil	80,0
Beauveria brongniartii carbofuran 3 mg/kg soil	95,0
Heterorhabditis megidis carbofuran 3 mg/kg soil	90,0
Beauveria brongniartii Heterorhabditis megidis carbofuran 3 mg/kg soil	100,0

These results indicate that joint treatments done with nematodes and fungus significantly improve the efficacy of *Melolontha* larvae mortality. In that case pathogenic organisms sinergize each other. Prior investigation on the control of other insect species with nematode and fungus or nematode and virus revealed the similar reaction (Kamionek *et al.*, 1974, Bednarek 1986).

Also low doses of the insecticide (60 mg/Kg of soil) treated together with biological agents could stimulate pathogenic activity of entopathogenic organisms. These results suggest that integration of different biological and chemical factors could be a new approach in the control of *Melolontha* larvae. On the other side environmental effects should be much lower because lower concentrations of chemicals are applied into the soil.

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Integrated method of the control of *Melolontha* spp. larvae and adults in Poland

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Introduction

As a result of political and economic changes, thousands of hectares of agricultural lands were abandoned in Poland. Now these lands have to be afforested. Comfortable conditions have been found for the development of white grubs in non-cultivated post-agricultural lands. In recent years, populations of common cockchafer (*Melolontha melolontha* L.) and other soil pests have increased significantly. This results in serious problems during afforestation of those lands, because of the damage caused to the roots by larvae of *M. melolontha, M. hippocastani, Amphimallus solstitiale* and other soil pests. The adults of the above mentioned species can also cause significant defoliation of deciduous stands.

In the previous paper the occurrence and management of *Melolontha* spp. white grubs in Polish forestry were described (Malinowski at al. 1996). Now, we would like to present more detailed data on this subject with a special reference to the use of an integrated method of forest protection against soil pests.

Control of white grubs

Assessment of the threat

The assessment of the threat by pests of roots refers to the areas which will be afforested or reforested, the areas in which nurseries will be established, the areas of existing nurseries and plantations. The main method of assessment of the threat is the use of control pits, which are dug usually at the beginning of September. The white grubs found in soil derived from control pits are counted and determined. The control pits measurements are 1 × 0,5 m and their depth depends on the level of insect occurrence, and usually is 30-40 cm and not exceed 0,5 to 1 m. The moment of digging of control pits depends on climatic conditions. The optimal time is the period of the maximal white grubs occurrence in upper parts of the soil. Under Polish conditions, this time falls between 15 August and 15 September. The following number of control pits are required: for nurseries to 1 ha – at least 2 pits for each 100 m^2; for nurseries greater than 1ha – to 15 pits for each hectare; for areas which have to be afforested – 6 pits for each hectare.

In the case of agricultural plantations, the use of 32 control pits per 1 ha is recommended; the measurements of one pit are 25cm × 25cm and a depth of 30cm (totally 2m^2 per ha) (Piekarczyk 1993). Counting the white grubs in the control pits is a basis for making decision about different protection treatments. The established tresholds or so called critical numbers of grubs found in control pits may help us for making decision. The critical

numbers of grubs to forest nurseries and plantations is given in Table 1. The nurseries are the most susceptible, the second place is occupied by forest plantations on poore and dry soils. The scale of assessment of the white grubs (second stage) density in agricultural plantations that may help us in making decision about protection treatment is presented in Table 2. The treatments are needed when white grubs density reaches middle level. The threat of existing forest plantations and young stands by white grubs may by estimated using the scale presented in Table 3. In that case, the protection treatments ought to be done at the low to middle level of destroyed seedlings depending on the forest lands.

Table 1: Critical numbers of different developmental stages of soil pests to forest nurseries and plantations (accor. to instruction of forest protection in Poland, 1988)

Kind of area And type of Forest land	***Melolontha melolontha*** and ***M. hippocastani***				***Amphimallus solstitiale***		***Polyphylla fullo***		
	Number of individuals /0.5 m^2								
	grubs; years old:			pupae and adults	grubs; years old:				
	1	2	3		1	2	1	2	3
Nurseries	0.5	0.5	0.5	0.5	1	1	0.25	0.25	0.25
Plantations									
a)mixed coniferous and broad-leaved forests	5	4	3	3	10	8	3	2	1.5
b) fresh coniferous forest	3	2	2	2	6	5	1.5	1	1
c) dry coniferous forest	1	1	0.5	1	2	1.5	0.5	0.5	0.25

Table 2: Scale of assessment of white grubs density (second stage of development) in agricultural plantations (accor. to Piekarczyk 1993)

Plantations	**Degree of threat**		
	low	**middle**	**high**
Cereals	to 5 indiv./m^2	5-10 indiv./m^2	>10 indiv./m^2
Root plants	to 2 indiv./m^2	3-6 indiv./m^2	>6 indiv./m^2
Vegetables, orchard, nurseries	to 2 indiv./m^2	3-6 indiv./m^2	>6 indiv./m^2
Pastures	to 10 indiv./m^2	10-20 indiv./m^2	>20 indiv./m^2

The above mentioned critical numbers, as well as the presented scales of threat does not have an obligatory character. These numbers and scales cannot be used as the only criterion for making decision about protection treatments. The type of soil, species and a stage of the white grubs, the size of damages and other factors that can diminish the population of pests,

should also be taken into consideration. It seems to be important to underline the possibility of the natural reduction of pest population during the winter, especially of younger stages.

Table 3: Scale of threat of plantations and young stands by white grubs (accor. to Sierpiński 1975)

Scale	% of destroyed seedlings	Degree of threat
0	0	no
1	1 to 10	low
2	11 to 20	middle
3	21 to 40	high
4	>40	very high

Methods of white grubs control

In Poland, white grubs may be reduced with the use of different prophylactic, agrotechnical and chemical methods. The purpose of the prophylactic methods is to ensure the optimal conditions for seedlings or young trees development and unprofitable conditions for pests. The above conditions may be reached by a good cultivation of soil, appropriate fertilization and planting trees in optimal terms and in accordance with the requirements of respective species in relation to the environment. The afforestation of post agricultural lands requires the complete cultivation of the whole areas threatened using the agricultural plough on a depth of 20cm with an additional dredger working on a depth of 60 cm in planting rows only or using the special forest plough on a depth of 50-60 cm in some types of soils. The cultivation of soils in the way described above give the appropriate conditions for the development of young seedlings, because the sufficient amount of oxygen and water is ensured. The white grubs are quite susceptible to soil cultivation and to the sunlight. Many of them are destroyed during soil cultivation. The birds of different species may also play a significant role in diminishing the number of grubs in cultivated lands. After the cultivation of soil, the number of grubs was reduced even in the situation when they occurred in the high amount before. It ought to be noted that the prophylactic methods can work only when soil pests occur in a moderate density. In the case of mass occurrence of the pests, the use of more effective methods is needed.

The second group of methods applied in Poland against white grubs are the agrotechnical methods. These include the mechanical cultivation of soil in a specific way and the plantation of buckwheat (*Fagopyrum (sagitatum) esculentum*) with antifeeding activity. The agrotechnical methods were well known and applied in Poland and in other countries years ago (Różyński 1926, Ułatowicz 1932). The use of those methods was stopped about fourty years ago when chemical insecticides were introduced against soil pests. Now, the agrotechnical methods are used again in Polish forestry. It is also possible to apply of chemical insecticides in some cases. The insecticides which are registered and may by used against white grubs are presented in Table 4.

The data in Table 4 indicate that organophosphate insecticides as granules containing diazinon as active ingredient (Diazinon 10 G, Basudin 10 G) and as emulsifiable concentrates based on chloropyrifos (Dursban 480 EC, Pyrinex 480 EC) belong to insecticides characterised by the lowest toxicity to higher animals. Among these insecticides, only Counter 5 G containing terbufos as active ingredient belongs to the first class of toxicity and

aught to be used by persons having special certificates, that allow them to apply this group of substances. The granular carbamate insecticides based on carbofuran (Furadan 5 GR, Diafuran 5 GR) having the good activity against white grubs belong to the second class of toxicity and can also be applied by persons with certificates. The last carbamate insecticide, Marshal suSCon 10 CG, which is a special slow relaese formulation containg carbosulfan as active ingredient that change to carbofuran in soil is characterised – among this grup of insecticides – by the lowest toxicity to higher animals (third class).

Table 4: Insecticides registered in Poland to the protection of forest nurseries and plantations against soil pests

Commercial name	**Active ingredient, %**	**Toxicity class** *)	**Producer**
Organophosphates			
a) granules			
Diazinon 10G	diazinon, 10	IV	Nippon Kayaku - Japan
Basudin 10G	diazinon, 10	IV	Ciba-Geigy AG - Switzerland
Counter 5G	terbufos, 5	I	American Cyanamid Comp.
b) emulsifiable concentrates			
Dursban 480EC	chloropyrifos, 48	III	Dow-Elanco – USA
Pyrinex 480EC	chloropyrifos, 48	III	Makteshim-Agan - Israel
Carbamates			
- granules			
Furadan 5GR	carbofuran, 5	II	FMC – USA
Diafuran 5GR	carbofuran, 5	II	Mitsubish Kasei - Japan
Marshal suSCon 10CG	carbosulfan, 5	III	FMC - USA

*) According to Polish classification

The above mentioned insecticides may be applied in three ways:
1) on the whole surface (surface treatment) of infested soils,
2) on the planting rows only (row treatment), and
3) on the hole (point treatment) during or after planting young trees using special applicators.

The surface treatments on the whole areas are executed before the planting trees using the insecticides with the lowest toxicity to higher animals. Granular insecticides such as Basudin 10 G, Diazinon 10 G, and Marshal suSCon 10 CG at doses of 80-120 kg/ha, or liquid ones such as Dursban 480 EC or Pyrinex 480 EC at doses of 2,5-5 l/ha in 450-600 l of water (before planting) may be applied. The above liquid insecticides may also be applied during the vegetation season at the same doses (2,5-5 l/ha) but diluted in 2000 l of water because of the easier penetration of chemical into the soil and to eliminate the possiblity of its fitotoxic action. It is also possible to use granular insecticides in planting rows only: Marshal suSCon 10 CG at a dose of 80 kg/ha, Counter 5 G at a dose of 20 kg/ha, and Furadan 5 GR and Diafuran 5 GR at a dose of 30 kg/ha. The granular insecticides are also applied in the hole during or after planting trees using a handle applicator: Marshal suSCon 10 CG at a dose of 10 g/plant, Counter 5 G – 5-10 g/plant, and Furadan 5 GR and Diafuran 5 GR at a dose of 4-9 g/plant. The depth of application is 10-15 cm. All the treatments are more effective when

applied on the youngest stages of white grubs. The above mentioned insecticides (with exception of Marshal suSCon 10 CG) are also registred to control white grubs in agricultural plantations in Poland using appriopriate recommendations.

Integrated control of white grubs

According to the Polish Low dated 12 July 1995 on Plant Protection (Dziennik Ustaw Nr 90, poz. 446), the protection treatments ought to be applied with the use of mainly non-chemical methods (agrotechnical, mechanical, biological etc.) that allow to reduce the application of chemical insecticides to the minimal level. Taking these into acount, the agrotechnical methods are the first of all applied against white grubs in post-agricultural lands that have to be afforested. The use of these methods is recommended as the only methods on the areas placed near the reservoirs of water (rivers, lakes) or placed over the underground sources of water where the application of chemicals is forbidden. The agrotechnical methods were firstly applied by Regional Directorate of State Forest in Olsztyn (Puchniarski 1996) where many hectares of post-agricultural lands that placed the underground sources of water ought to be afforested.

Before the afforestation of lands abandoned by agriculture, the density of white grubs is evaluated. If the number of grubs exceeds the level tolerated by young trees (which is the level determined by established critical numbers), the delaying planting of seedlings for one or two years is needed. During one vegetable period, the soil is cultivated 4 to 5 times using disc harrow, agricultural or forest plough, normal or active harrow etc. In most cases one year period is sufficient to reduce the grubs to a non-dangerous level. In some cases, the above action have to be repeated next year or chemical insecticides have to be used. This takes place on 10-20 percent of the total area on which repeated soil cultivation is used.

The examples of the repeated soil cultivation with good results are given in Table 5. The results showed that the number of grubs diminished to the tolerated level. Another method which can be used alone or alternatively is the plantation of buckwheat on the areas threatened by white grubs. After one season, the number of grubs was reduced below the tolerated level (Table 6).

Table 5: Number of white grubs per pit before and after the repeated soil cultivation (1996)

Forest district	Area (ha)	Number of treatments	Average number of grubs per pit	
			before treatments	after treatments
Olsztynek*	15,2	6	4.0	1.4
Olsztynek*	18,7	4	4.8	1.3
Choszczno**	2,6	4	1.0	0.2
Trzebielino***	11,4	2	2.6	0.3
Szczecinek***	1,0	3	5.2	0.5
Łupawa***	3,8	5	1.4	0.2
Damnica***	16,5	4	2.0	0.26

* Regional Directorate in Olsztyn

** Regional Directorate in Szczecin

*** Regional Directorate in Szczecinek

Control of *Melolontha* spp. adults

In Poland, the control of *Melolontha* spp. adults is possible using the insecticides from pyrethroid and aryloproppyl ether grups given in Table 7. The above insecticides are recommended not only for *Melolontha* spp. adults control but also against leaf-feeding larvae of nun moth, pine moth, pine sawflies and others. The insecticides are recommended at minimal and maximal levels of doses. The higher doses are applied mainly against leaf-feeding larvae. The cockchafer adults are more susceptible to pyretroid insecticides than larvae of the above mentioned species of insects (Woreta, Malinowski 1998) and lower doses ought to be used against them. The insecticides are applied by helicopter or aircraft equiped with atomizers (Micronoir AU 5000). The total amount of spray solution is 2-4 l/ha and it usually contains the lowest recommended dose of insecticide mixed with Ikar 95 EC (mineral oil) at a dose of 0.7 l/ha and water at a dose of 1.3 to 2 l/ha.

Table 6: Number of white grubs per pit after the plantation of buckwheat during one season (1996). The number of grubs per pit before was over the tolerated level.

Forest district	Area (ha)	Average number of grubs per pit after one season of buckwheate plantation
Bytów*	0.5	1.4
Bobolice*	0.5	0.3
Mirosławiec**	2.0	0.75
Łuków***	0.5	0
Smardzewice****	0.6	0.5

* Regional Directorate in Szczecinek
** Regional Directorate in Piła
*** Regional Directorate in Warszawa
**** Regional Directorate in Łódź

The effectiveness of treatment depends on several factors such as optimal spray timing, areas of treatment, rainfall after treatment and others. The most important matter is the determination of optimal spray timing. The treatment executed too late is not fully effective because the significant amount of females could lay eggs to the soil. Treatment ought to be done as early as possible to avoid laying eggs by females. According to our knowledge, the population of grubs remains on the same level as the one before only in the case when 96 % females are destroyed. In favourable climatic conditions, the first treatments will be done 2 to 3 days after cockchafer adults leaveing soil, and final treatments 4 to 5 days later. Under unfavourable conditions, the appearence of adults is extended in time and treatments have to be repeated two or three times with intervals 10-14 days. The durability of pyrethroids action on treated leaves was evaluated as about 14 days (Woreta, Malinowski 1996).

In order to obtain the above efficacy, the treatments must cover all the areas where cockchafer adults occur. It seems reasonable that treatments against cockchafer adults ought to be the main method of grubs reduction in the next seasons. In that case, the direct treatments against grubs will be done rarely.

Table 7: Insecticides registered in Poland against *Melolontha* spp. adults and other leaf-feeding pests (accor. to Głowacka 1997)

Commercial name	Active ingredient, %	Toxicity class *)	Doses of commercial formulation (L/ha)	Carency period in days
Pyretroids				
Alfazot 05 EC	alphamethrin, 5	III	0.12 – 0.17	7
Fastac 10 EC	alphamethrin, 10	III	0.06 – 0.075	7
Decis 2.5 EC	deltamethrin, 2.5	III	0.15 – 0.25	7
Karate 025 EC	lambdacyhalothrin, 2.5	III	0.15 – 0.25	7
Sumi-alpha 050 EC	esfenvalerate, 5	III	0.15 – 0.2	7
Zorro 100 EC	zetacypermethrin, 10	III	0.06 – 0.075	7
Arylpropylether				
Trebon 10 SC	etofenprox	IV	0.3	3

*) According to Polish classification

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Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.27 - 32

The 1997 Control Campaign of *Melolontha melolontha* (L.) at the Kaiserstuhl area (Baden-Württemberg): Field Trials and Practical Experiences

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Summary: In 1991 and 1994 heavy flights of the Common Cockchafer (*Melolontha melolontha* L.) and subsequently heavy infestation of adjacent farmlands by White Grubs had occurred in the northern Kaiserstuhl area. According with its phenological emergence pattern an even more severe flight and increasing damages to agricultural crops had to be expected for the next flight and the following years. Therefore a control campaign had to be organised in 1997.

An ad hoc-committee under the leadership of the State Ministry for Landscape, Food, Agriculture and Fisheries agreed upon a set of integrated control measures, including polyethylene net covers, the entomopathogenic fungus *Beauveria brongniartii*, insecticide treatments of fruit-trees and hedgerows, as well as helicopter insecticide sprays at the edges of the forests.

Numbers of white grubs in the farmland adjacent to those parts of the edges of the forests being partly treated with insecticides because of conservancy reasons were clearly higher than in those fields near totally treated edges of the wood.

An area of about 50 hectares (orchards, grapevine, nurseries) were covered with nets, with very good results.

A three year lasting trial showed promising results with *Beauveria brongniartii*, but the effect of the large scale application of *Beauveria brongniartiia* can not be estimated finally still now.

Introduction

The current cycle of the Cockchafer in this area presumably began in 1978, when a wine-grower first informed an extension officer about some White Grubs in the boundaries of the village of Kiechlinsbergen. The local plant protection service reported the occurrence in their annual reports, calculating it insignificant. It was only towards the end of March in 1991 when the State Institute for Plant Protection has been asked for nets to protect a nursery plantation of nut trees. At this moment a heavy flight had already started. Therefore it was too late to initiate any control measure. As a result remarkably high densities of White Grubs in farmlands and house gardens (up to 288 grubs/m²) were found. In 1994 a further very heavy flight occurred. But it took over four years from the first sporadic approaches to form political pressure to the State Ministry, which was responsible for the decision of an insecticide application at the edges of the forests by helicopter. As a result of this an ad hoc-committee comprising the Ministry, the different extension and environmental services, counties, municipalities, farmers and professional organisations started a series of discussions. The following criteria were taken into consideration:

Tab. 1: Main criteria for the evaluation of an integrated control concept of *Melolontha melolontha*

Criterion	explanations
Geographical spread of the Cockchafer	Mainly infested: the farmlands of eight villages in the northern Kaiserstuhl area, the infestation is still exceeding
Extension of infestation	Number of white grubs per m² partly up to 300, likely to increase phase of the population cycle unknown,
Agricultural structure	Great variability of crops, relatively small allotments, susceptible crops (fruits, vineyards, nurseries, potatoes) are grown on 66 % of the farmlands
Species preservation	the area is one of the environmentally most sensitive region in the State, many rare insect and plant species are occurring
Acceptance of spraying by helicopter	suspected to be rejected by the conservancy service, wild-life representatives, non farmers and tourists
White grub control	Insufficient relating to the actual extension of infestation
Beauveria brongniartii	Seems to be not efficient enough so far; mostly because of lack of soil humidity
Financial compensation of damages	There is no fund at all

Based on the criteria mentioned above, the following principles for the control campaign were worked out by the committee:

- protection of nursery plantations and newly planted fruit-trees and vineyards by polyethylene net covers; to be done by the farmers.
- treatment of feeding trees in the farmland region (mainly stone-fruit-trees and hedgerows, infested by adults by insecticides; to be done by the farmers.
- application of the entomopathogenic fungus *Beauveria;* to be done by the farmers.
- application of insecticides at forest trees by helicopter; to be organised and carried out by the State Institute for Plant Protection.

For in springtime 1997 there was no insecticide registered to control the Common Cockchafer, it was decided to perform a trial to evaluate the efficacy of the active substances Phosalone and Azadirachtin. The costs for the campaign were covered to 60% by the State Ministry and to 40% by the municipalities of the communities concerned (tab. 2).

Tab. 2: Total Costs of the Control Campaign 1997

Complete Costs of the Campaign 1997		**Distribution of the Costs**
Nets 55 ha and Rollertubes Costs/ha: 3600,- DM/ha net + at least 20% „overlapping„ = 4300,- DM/ha (Sistex, I)	200.719,- DM	40% communities 60% state of Baden-Württemberg farmers: working hours
Beauveria 1400 kg 13,45 DM/kg * 30Kg/ha = 404,- DM/ha (Schweizer, CH) + application about 30 DM/ha	18.829,- DM	100% state of Baden-Württemberg farmers: application
helicopter 135 ha forest 2 times insecticide + application (Meravo, D)	67.794,- DM	40% communities 60% state of Baden-Württemberg
Total	**287.342,- DM**	

Oviposition Control by Nets: Preparations, Trials and Results

The method of covering farmland with polyethylene nets, which are normally used in the harvest of olives was discussed intensively during the OILB *Melolontha* Meeting in Freiburg in 1995. The results with net covering from younger orchards in the trials, started in 1994 in the region of the Kaiserstuhl, were such promising (fig.1), that the Melolontha working group commended the usage of the nets in a larger scale in the year 1997. Although the farmers had no costs for the net materials, only an area of about 50 ha was registered at the plant protection service. The mean arguments from the growers against the nets were the highly time-consuming handling and the impossibility in covering the whole great diversify region, to get a reduction of the *Melolontha* population in the region.

The farmers who used the nets for covering their orchards, younger vineyards or the arable land before planting the grapevines with the aim of an object protection, had no problems with white grubs afterwards.

After the use the nets were rolled up with a special machine, collected and stored by the communities for the next usage. Already in 1998 the nets were used a second time near Karlsruhe about 150 km from the region of the Kaiserstuhl.

***Beauveria brongniartii*: Preparation, Trials and Practical Experience**

A large scale field trial on an area of grapevine with about 10 hectare, which started 1994 with the first application of 30 kg/ha *Beauveria* grain, lasted until 1996, with one application/year in spring. Two times/year (spring and autumn) the infection rates were examined. The result showed increasing infection of the grubs with *Beauveria brongniartii* over the years (fig. 2). But infections in a similar range were found in the untreated areas as in the treated, showing that the fungus has already been established in the region.

In the control campaign 1997 *Beauveria brongniartii* should be applicated by the farmers in a larger dimension. About 45 ha farmland (grapevine, fruit-trees, nurseries) were treated two times. The controls showed unfortunately nearly anyone infection with *Beauveria* yet. Probably the reasons are the application technique on the one side and the very dry soil conditions in 1997 and 1998 on the other.

Beetles Control by Insecticides: Preparations and Results [1)]

With the preparation of the insecticide campaign the State Institute for Plant Protection and the local environmental conservancy service verified the edges of the woodland for reasons that exclude insecticide application by helicopter, e.g. buildings, high tension cables, open water resources and conservation reasons. At that time the environmental conservancy service intended to prohibit most of the surface presumably to be treated by helicopter. For this reason the State Institute for Plant Protection thought spraying insecticides by helicopter would be ineffective. When the first beetles had emerged the plant protection service and the local environmental conservancy service thoroughly controlled all the infested edges of the wood on the spot. This time the conservancy service withdrew a great part of his former restrictions. That's why the helicopter application of insecticides at edges of the forests finally was decided.

The emergence of the beetles started on April, the 8th. The day after heavy infestations already were observed first, at those woodland boarders spreading far into the farmlands. For the next two weeks cold and rather windy weather conditions retarded the increase of leaf mass as well as the feeding activity of the beetles. Nevertheless trees and bushes at the most prominent sites were defoliated in a single day. Because of permanent blowing wind and continuos movement of the canopy it was impossible to verify which parts of the woodland were infested by the beetles and ought to be treated. In spite of these adverse conditions some female beetles started oviposition. When a change of weather conditions has been forecasted, April, the 24th was timed for the helicopter application of insecticides. April, the 23th was the first windless day that allowed the determination which parts of the woodland had to be treated.

The application of the insecticides by helicopter has been performed on April, 24th with well-suitable weather conditions concerning wind and temperatures. The essential application data are shown in tab. 3.

After that treatment, further swarms of beetles started to migrate from arable land to the main host trees in the adjacent woodlands. Therefor a second a second insecticide treatment was necessary, which has been completed on May, the 7th, 1997. Application rates and carrier amounts were exactly the same as on the first application date. The total forest area which has been treated was about 135 hectares.

Tab. 3: Insecticides applicated with helicopter

Product		Application-Rate	Carrier Amount of Spray Solution
Name	Active Ingredient		
Rubitox-Spritzpulver	30 % Phosalone	2,5 kg/ha	75 l/ha
NeemAzal T/S	1 % Azadirachtin	3,0 l/ha	75 l/ha

The days after the treatments the efficacy of both insecticides was assessed to be highly efficient. Monitoring data in autumn 1997 and spring 1998 documented significant differences between grub numbers in farmlands near the edges of the forests partly treated for

[1)] the results concerning side-effects, wind drift and differences between the chemical products will be given in a separate paper

conservancy reasons and those totally treated. In farmlands influenced by partly treated edges of the forests 30 % of the holes controlled (0,25 m²) were found free of grubs compared with 55 % near totally treated woodland edges. Whereas the number of holes with high grub densities near partly treated edges of the woods clearly exceeds the corresponding dates from farmlands influenced by totally treated edges of the forests (fig. 3). There is no difference neither between the chemical products applied nor between the two assessments.

Conclusion

The control campaign showed clearly that an one generation lasting control of *Melolontha melolontha* to prevent valuable agricultural plants from acute damages by white grubs, is possible. Controls of the densities of white grubs clarified a strong reduction of the individuals. But it is to expected that the same control measures 1. **nets** to have fields totally free of grubs and 2. **insecticides by helicopter** to reduce significantly the number of grubs in a greater region), are necessary in the year 2000, at the next heavy flight, for the durable control with the *Beauveria* application in 1997 and 1998 has still disappointing results, although the large scale field trial 1994 - 1996 showed promising infection rates. For the application technique seems to be the reason, a new drill machine for grassland with a very exactly setting of the depth placing is used since autumn 1998.

Controls on all relevant points will be carried out further on until the next flight in the year 2000.

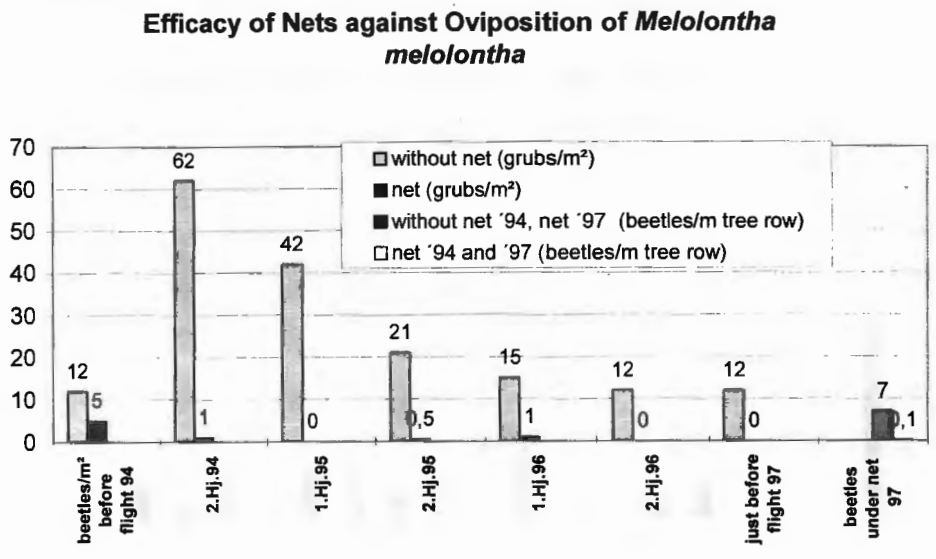

Fig. 1: The net prevented the treated area almost completely from oviposition of *Melolontha melolontha.*
Controls: 1.Hj. = spring; 2.Hj .= autumn

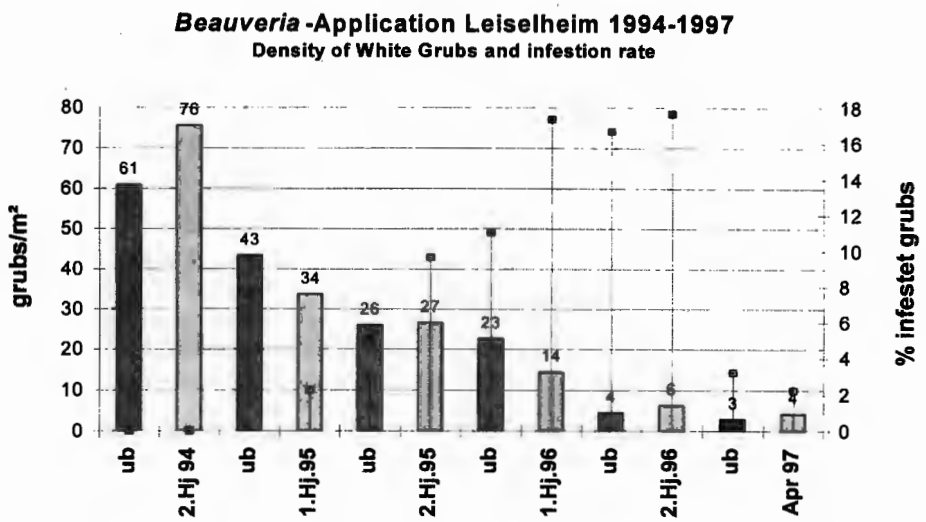

Fig. 2: Infection of grubs (in 1997 adults) after three yearly application of „*Beauveria*" grain. ub = untreated area, number of grubs/m² dark grey columns; treated area = light grey columns; infection rate = narrow columns with dots; Controls: 1.Hj. = spring; 2.Hj. = autumn

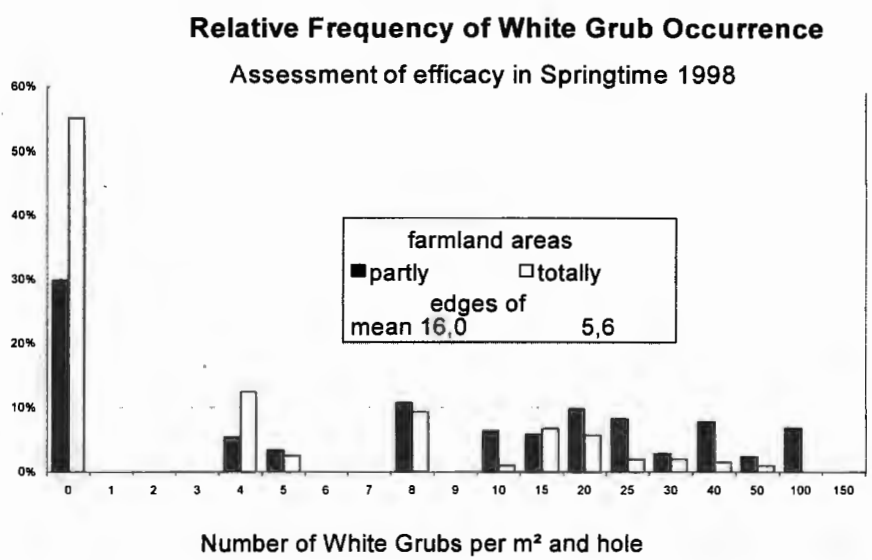

Fig. 3: Relative frequency of white grubs in the farmland areas around the partly and totally treated edges of forests; the mean shows the effect of the insecticide application

General biological control

Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.35 - 37

Potential for microbial control of scarabs and weevils in Danish forestry

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Introduction

Scarab larvae (*Melolontha* spp.) are considered as important pests in the production of Christmas trees and decoration green (*Abies nordmanniana*) in Denmark. Extensive damage has been documented in Northern and Southern Jutland, Funen and on Zealand (Harding, 1994). In the 1990's, weevils (*Strophosoma* spp. and *Otiorhynchus* spp.) also caused serious damage on Christmas trees and decoration green (*Abies nordmanniana* and *A. procera*) (Harding, 1993; Harding *et al.*, 1996).

Two species of *Melolontha*; *M. melolontha* and *M. hippocastani* occur in Denmark, the former is the most abundant. The life cycle of *M. melolontha* is 4 years, while it is 5 years for *M. hippocastani*. On Christmas trees and decoration green the weevil pests are mainly *S. melanogrammum* and *S. capitatum* as well as *O. singularis*.

The purpose of this paper is to give an overview of the history of *Melolontha* spp. as pest insects in Denmark and the present pest status together with fungal pathogens infecting Scarabid and Curculionid pests in Denmark.

History

Melolontha melolontha and *M. hippocastani* are among the oldest known pest insects in Denmark. From 1850 until the beginning of 1900, severe damage was caused by these scarab beetles, mainly on arable land, described by Boas (1904).

From the year 1887 the public people in Denmark were prescribed by legislation to collect cockchafers (both larvae and adults) at public costs. The amount of cockchafers in pounds (= 500 g) collected in each parish was recorded separately for each year. From 1887 to 1899 the cockchafers mainly appeared in the same regions; the southeastern parts of Jutland, the whole of Funen including most of the minor neighbouring islands, and some parts of, mainly eastern, Zealand. A local distribution was found, and there was no tendency of occupying new regions. *M. hippocastani*, which is restricted to the sandy regions, mostly in Jutland, occurred also in great numbers in 1892, 1897 and 1902 but only in the northern part of Jutland. After numbers of years cockchafers were reduced to a minimum, probably due to a bacterial disease.

Boas (1904) reported further that control experiments of *Melolontha* spp. were undertaken as early as in 1893, where *Botrytis tenella* (= *Beauveria brongniartii*) produced in France was released in a Danish nursery, but the control was not sufficient. Some years later several *M. melolontha* and *M. hippocastani* larvae were found killed by *Beauveria* sp. (Table 1).

Present pest status and control

Chemical control of soil-dwelling larvae of *Melolontha* spp. and curculionids are not allowed in Danish forestry. Use of mechanical control measures is not possible in these perennial crops and the growers have no options to control the larvae and prevent attacks. Weevil damages have so far been prevented by foliar application of insecticides (pyrethroids), but total prohibition of pesticide application in Danish forestry can be foreseen within some years.

Microbial control of *Melolontha* spp. and curculionids larvae with *Beauveria* spp. and *Metarhizium anisopliae* may be possible alternatives to chemical control, as these fungi are known to be effective against these pests (Zimmermann, 1992; Moorhouse *et al.*, 1993; Keller *et al.*, 1997).

In Denmark mainly *B. bassiana* has been found to infect *Melolontha* spp., *A. solstitiale* and *Strophosoma* sp. naturally, however only few individuals of *Melolontha* spp. and *Strophosoma* spp. have been examined. In the next years more insects will be collected and examined for insect pathogenic fungi and infection experiments will be preformed.

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Table 1. Insect pathogenic fungi naturally infecting Scarabidae and Curculionidae in Denmark

Insect host	Pathogen	Reference
Scarabidae		
Amphimallon solstitiale	*Beauveria bassiana*	Vestergaard & Eilenberg, unpubl.
Aphodius fimetarius	*B. bassiana*	Steenberg, 1995
Melolontha hippocastani	*Beauveria* sp., *B. bassiana*	Boas, 1904; Eilenberg, unpubl.
M. melolontha	*Beauveria* sp.	Boas, 1904
Curculionidae		
Apion dichroum	*B. bassiana*	Aalborg, unpubl.
Hypera variabilis	*B. bassiana, Metarhizium anisopliae, Paeciliomyces farinosus, Zoophthora* sp.	Steenberg, 1995
Otiorhynchus sp.	*B. bassiana*	Steenberg, 1995
Sitona spp. (1,2,3,4,5,6,7)*	*B. bassiana*	Steenberg, 1995
Sitona spp. (1,3,5)	*M. anisopliae*	Steenberg, 1995
Sitona spp. (1,2,3)	*Verticillium lecanii*	Steenberg, 1995
Sitona spp. (1,3,4,5)	*P. farinosus*	Steenberg, 1995
Strophosoma sp.	*B. bassiana , V. lecanii*	Vestergaard & Eilenberg, unpubl.

* *1 = S. lineatus, 2 = S. puncticollis, 3= S. hispidulus, 4 = S. humeralis, 5 = S. flavescens, 6 = S. lineellus, 7 = S. sulcifrons*

Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.39 - 45

Susceptibility of *Melolontha melolontha* to *Heterorhabditis bacteriophora, H. megidis* and *Steinernema glaseri*

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Summary: In laboratory bioassays larvae of *M. melolontha* were treated with *Heterorhabditis bacteriophora, H. megidis* and *Steinernema glaseri* at doses of 1500 and 5000 nematodes per larva. The best performing nematode species was *S. glaseri* causing significant mortality of 2nd instar larvae at 1500 nematodes/grub. Both *Heterorhabditis* species were equally infective. The susceptibility of different larval instars was assessed using *H. bacteriophora* and *H. megidis*. In 2nd and 3rd instars, mortalities significantly different from the control were only obtained at doses of 5000 nematodes of *Heterorhabditis* spp. per larva. Only in 1st instar larvae the lower dose of 1500 nematodes did cause significant mortality. *Heterorhabditis bacteriophora* and *S. glaseri* were equally infective when injected into the grubs haemocoel. In contrast, only *S. glaseri* caused grub mortality when the nematodes were applied orally. The results indicate, that the poor control of *M. melolontha* by *H. bacteriophora* is not due to the insect's immune response but due to an inferior host finding and/or penetration capability of *H. bacteriophora* compared to *S. glaseri*.

Introduction

The control of scarabaeid larvae with entomopathogenic nematodes has been successful for several species like *Popillia japonica* and *Phyllopertha horticola* (Georgis & Gaugler, 1991; Peters & Ehlers, 1998). However, two major pest insects, the June beetle, *Amphimallon solstitiale* and the European cockchafer, *Melolontha melolontha* have not shown sufficient susceptibility to nematodes to encourage field application (Deseö *et al.*, 1992, Smits, 1992). So far, the most promising nematode against *M. melolontha* has been *Steinernema glaseri* (H. Vlug, 1996). By comparing the infection of *M. melolontha* by *H. bacteriophora* and *S. glaseri*, the key factors limiting nematode efficacy against *Melolontha melolontha* have been investigated.

Material and methods

Larvae of *M. melolontha* were field collected in Northern Italy (Trentino and Val d'Aosta) and kept at 15°C in the laboratory in natural soil with carrot pieces as food.

Nematode strains were lab-reared in *Galleria mellonella* larvae at 20°C and stored at 10°C in tap water for no longer than 3 weeks before being used in bioassays. The strains used were: *H. bacteriophora* strains HI-191, HI-127, HI-23 and HI-273, all being isolated in Italy (Deseö *et al.*, 1988), *H. bacteriophora* NJ, a mixture of several isolates from New Jersey, USA, *H. megidis* HSH-2 isolated in Schleswig Holstein, Germany and *S. glaseri*, RS92 from New Jersey. In one assay *S. glaseri* was tested after being propagated in *M. melolontha* (Mel). All assays were done at 20°C.

The susceptibility of grubs was tested in plastic beakers 6.5cm ∅ filled with 100 ml of soil from the collection site to a height of 8 cm. The grubs were put on the soil and moved down to the ground of the beaker within 24 h when the nematodes were added in 2 ml of tap water at doses of 5000 or 1500 dauer larvae (DL) per beaker. Each beaker contained one grub. The number of grubs tested per dose is given in the results. Mortality was assessed 7, 17 and 24 days after treatment.

The susceptibility of the grubs to intrahaemocoelic injection of the nematodes was tested by injection of nematodes (*H. bacteriophora* NJ and *S. glaseri* RS92) with a glass capillary (see Ehlers *et al.*, 1997). Nematodes were injected in doses of 1, 5 and 10 DL per larvae. Mortality was recorded each day for up to 10 days post injection. Since control mortality was fairly high in these injection experiments, the susceptibility of the grubs to aqueous washings of surface-sterilised and non sterilised DL was assessed. The DL were surface sterilised in 0.1% hypochloride solution for 10 min. The washing was injected with a Hamilton syringe (10µl per grub) and mortality monitored daily for 7 days post injection.

Force feeding experiments were done with *H. bacteriophora* NJ and *S. glaseri* RS92. Nematodes were injected into the mouth of 3rd instar *M. melolontha* larvae with a blunt needle at doses of approx. 20 DL/larva. In the control, larvae were treated with the same needle but just with water. The treated grubs were put on a White trap (Dutky, *et al.*, 1964) to collect nematodes leaving the grub. Mortality was assessed 7 days after treatment.

Results

Efficacy screening of nematode isolates

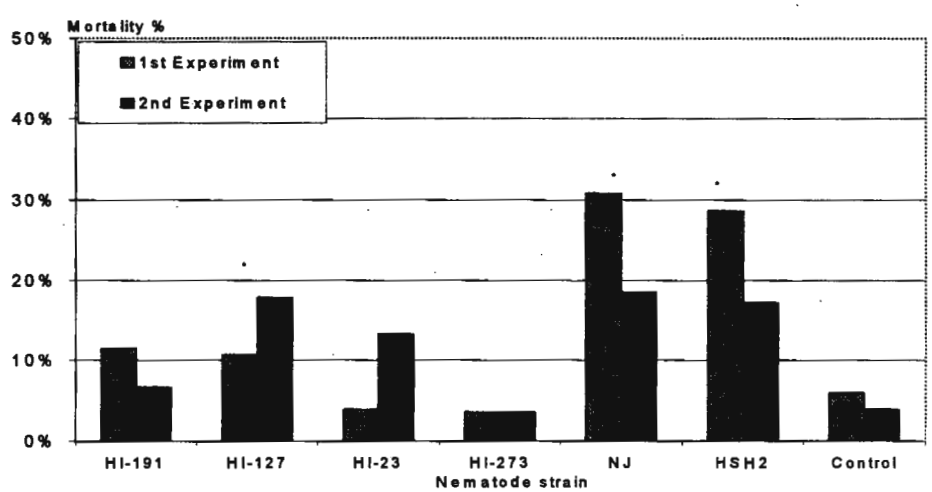

Figure 1. Mortality of *Melolontha melolontha* 2nd instar larvae 17 days after treatment with 5000 nematodes / grub. Two independent experiments. Asterisks mark mortalities significantly different from the control (Fisher's exact test, $p = 0.05$). (N=30 grubs per treatment).

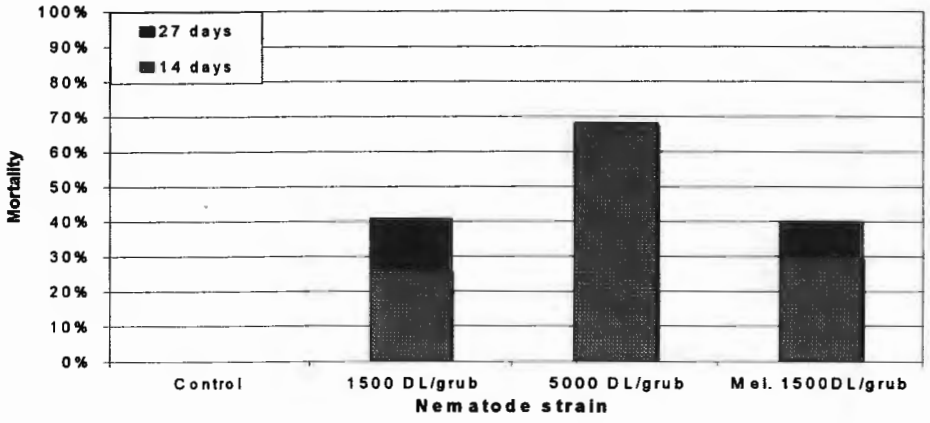

Fig. 2: Mortality of *Melolontha melolontha* 2nd instar larvae 14 and 27 days after treatment with *Steinernema glaseri* RS92 at different doses. Nematodes were propagated in *Galleria mellonella* or in *M. melolontha* (Mel.). Mortalities are significantly different from the control (Fisher's exact test, p=0.05). (N=30 grubs per treatment).

Of the nematode isolates tested, only *H. bacteriophora* NJ, *H. bacteriophora* HI-127 and *H. megidis* HSH caused significant mortality of *M. melolontha* L3-larvae (Fig. 1) at a dose of 5000 DL/grub. Accordingly, HI-127 had been the best performing amongst Italian isolates in earlier studies (Deseö *et al.*, 1992). It turned out, that mortality should be assessed at least 17 days after nematode treatment to account for total nematode caused mortality. In a separate assay *S. glaseri* RS92 proved to be signifcantly more efficient than *H. bacteriophora* NJ in killing the grubs (Fig. 2). In contrast to the results of Vlug (1996) the nematodes reared in *M. melolontha,* did not cause an increased mortality in the grubs.

***Instar susceptibility of* M. melolontha *to* H. bacteriophora *and* H. megidis**

First instar larvae proved to be more susceptible to nematode treatment than 2nd instars (Fig. 3). First instar larvae were killed most efficiently by *H. megidis* HSH2 even at a dose of 1500 DL/grub whereas *H. bacteriophora* NJ only gave significant control at a dose of 5000 DL/grub. No significant mortality was observed in the 2nd instar larvae even at a higher dose of 5000 *H. bacteriophora* per grub. No 3rd instar larvae were included in this assay, but from the results of the nematode screening, it can be concluded, that they are less susceptible than the 1st instar larvae at least against *H. megidis* HSH-2.

***Intrahaemocoelic injection of* S. glaseri *and* H. bacteriophora**

Second instar larvae of *M. melolontha* were equally susceptible towards intrahaemocoelic injection of *H. bacteriophora* and *S. glaseri* (Fig. 4). Whereas mortality after injection of 1 DL was not significantly different from control, 5 DL resulted in more than 50 % mortality within 7 days after treatment in the first assay and ≥80% mortality in the second assay. Control mortality was considerably high (>40% at 7 days after treatment). When only aqueous nematode washings were injected, 70 to 80% mortality was observed 5 days after treatment (Fig. 5). When the nematodes were surface sterilised their surface washings killed less than 20% of the grubs. The grubs hence seem to be very susceptible to microorganisms on the surface of the nematodes.

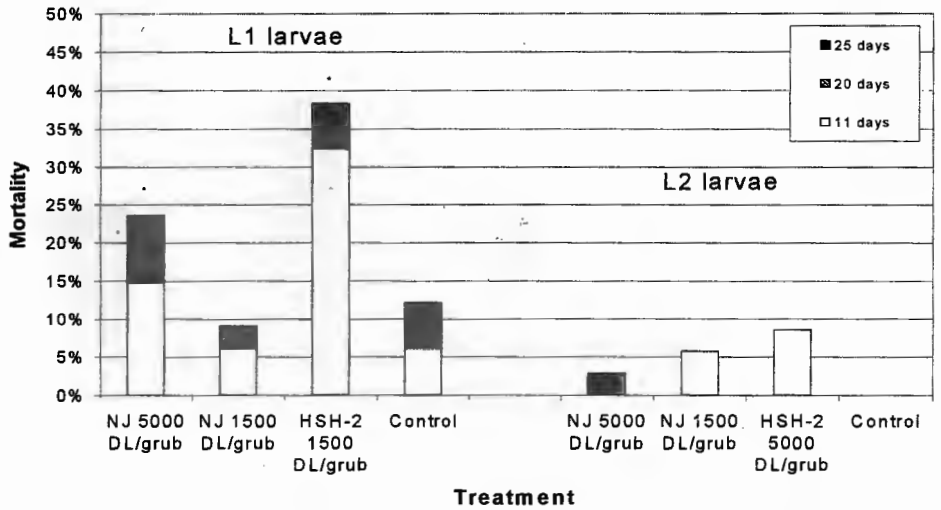

Fig. 3: Mortality of 1st and 2nd instar *M. melolontha* larvae to *H. bacteriophora* NJ and *H. megidis* HSH-2 at different doses. Mortality significantly different from the control is marked by an asterisk (Fisher's exact test, p=0.05). (N=35 grubs per treatment).

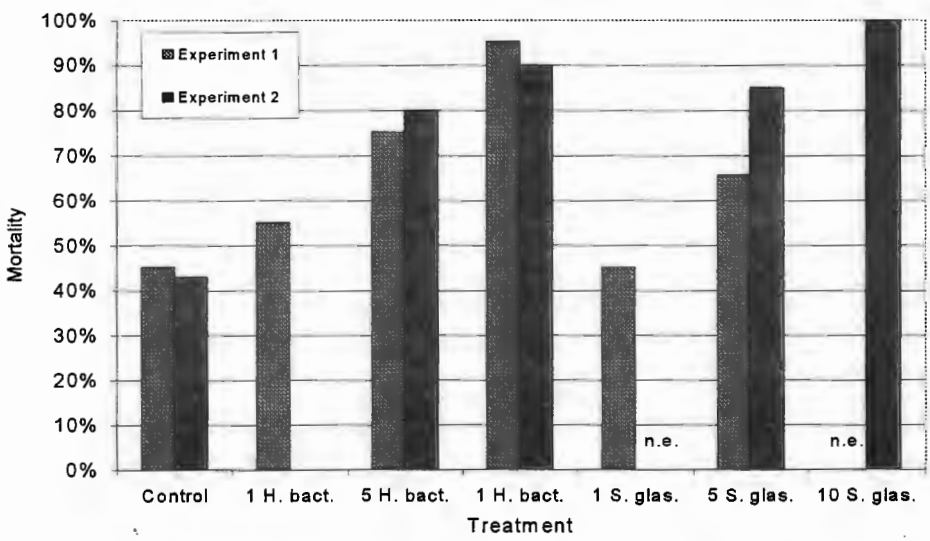

Fig. 4.: Mortality of *M. melolontha* 2nd instar larvae 7 days after injection of *Heterorhabditis bacteriophora* NJ and *S. glaseri* RS92. Treatments with 1 nematode per grub did not give significant mortality (Fisher's exact test, p=0.05). (N=20 grubs per treatment).

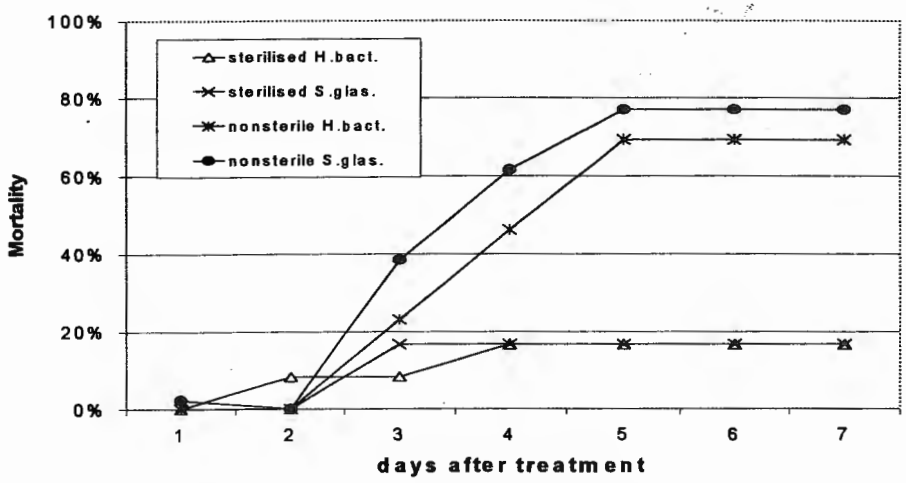

Fig. 5: Mortality of *M. melolontha* after injection of 10 µl aqueous surface washings of surface sterilised and nonsterile *H. bacteriophora* NJ and *S. glaseri* RS92 dauer larvae. (N=20 grubs per treatment).

***Force feeding of nematodes to* M. melolontha**

The mortality of *M. melolontha* L2 after oral nematode application was significantly higher for *S. glaseri* compared with *H. bacteriophora* which did not give any significant mortality (Fig. 6). Nematodes collected from the treated grub were mostly exsheathed. Presumably, the DL loose the sheeth when they are in contact with the gut fluids. This has also been observed for *H. bacteriophora* infecting *Tipula oleracea* (unpublished data).

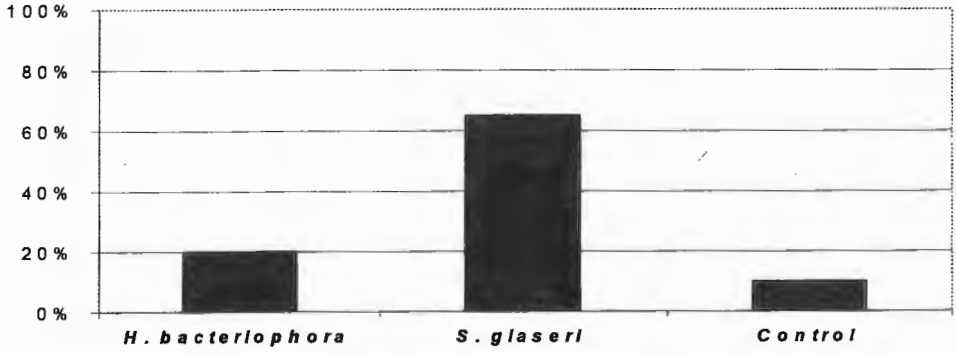

Fig. 6: Mortality of *M. melolontha* 2nd instar larvae after oral application of 20 dauer larvae of *H. bacteriophora* NJ and *S. glaseri* RS 92. N=30 grubs per treatment. Mortality was only significantly different fomr the control for the *S. glaseri* treatment (Fisher's exact test, p=0.05).

Discussion

The susceptibility of *M. melolontha* larvae to *Heterorhabditis* sp. is low, especially for the 2nd and the 3rd instar larvae. Deseö *et al.* (1992) reports 90 to 100% control of *M. melolontha* 2nd instar larvae after treatment with HI-127 and *S. glaseri* AUSTRALIAN but without stating the application dose. In field trials, a 40% reduction of "younger larvae" was observed after application of 1 Mio *S. glaseri*/m². In treatments against "older larvae" less than 20% reduction was observed. The doses used in the lab assays presented correspond to 1.5 Mio per m² (5000 DL/beaker) and 450000 DL/m². Losses of nematodes of 90-95% (Smits, 1996) during field application, however, do not occur in the laboratory. Accounting for 90% losses, lab doses of 5000 and 1500 would correspond to 15 Mio/m² and 4.5 Mio/m² field doses. Judging from these results, field control of *M. melolontha* with nematodes is yet far from being economically feasible.

Only when nematode propagation takes place in the few larvae infected by the DL sprayed, there is a chance for controlling the remaining grubs by the DL leaving the cadavers. This seems to be the mode of control of *Phyllopertha horticola* by *H. bacteriophora* in the field and explains that dead grubs can only be found 4 weeks after application (Ehlers & Peters, 1998). However, significant nematode propagation in *M. melolontha* was only found in *S. glaseri* and not in *H. bacteriophora* (data not shown). Field applications of *S. glaseri* against *M. melolontha* might be successful, but significant reduction of grubs are unlikely to occur before 1-2 month after application.

To improve nematode performance against *M. melolontha* the larval susceptibility should be investigated not only between but also within instars. In *Tipula paludosa*, for instance, there is a narrow window of high susceptibility of the 1st instar larvae approaching the 1st moult whilst the young 1st instars are not susceptible (Peters & Ehlers, 1994). There might be windows of high susceptility in *M. melolontha*, as well.

The difference between the susceptibility of *M. melolontha* against *H. bacteriophora* and *S. glaseri* is not due to their fate in the insect's haemocoel. Both nematodes can equally cope with the immune response of the grubs. *Steinernema glaseri*, however, is by far superior in penetrating the grubs gut wall. This has also been reported for *Popillia japonica* (Wang & Gaugler, 1998). Yet *H. bacteriophora* and *S. glaseri* achieve similar field results against *P. japonica* (Klein, 1990). Apparently, *H. bacteriophora* penetrates directly through the grubs' integument into their haemocoel preferably penetrating the fragile intersegmental membranes and the leg joints (Wang & Gaugler, 1998). It might be concluded, that *H. bacteriophora* can not penetrate these membranes as easily in the bigger larvae of *M. melolontha*. Whether *H. bacteriophora* is also inferior in its host finding and host recognition capability remains to be tested.

Grubs of *M. melolontha* proved to be quite susceptible to aqueous surface washings of both *S. glaseri* and *H. bacteriophora*. Since this effect disappeared when the nematodes were surface sterilised, microorganisms washed from the surface of the nematodes presumably caused grubs death. The nature or species of these microorganisms was not tested but it is likely, that *Xenorhabdus poinarii* and *Photorhabdus luminescens*, the symbionts of *S. glaseri* and *H. bacteriophora*, respectively, were present on the DL surface. It remains to be tested, whether the bacteria symbionts can kill the grubs by their own. In general, the grubs seem to be quite susceptible to bacteria infection in their haemocoel compared to *Tipula* spp. which are resistant against surface washings of nematodes (data not shown).

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Investigations on the infection biology of *Bacillus popilliae*

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Abstract: *Bacillus popilliae* (BP) causes the lethal milky disease of scarab larvae, which is characterised by a mass development of sporangia in the hemolymph. This pathogen produces a protein crystal during sporulation. We investigated the infection biology of the strain *Bacillus popilliae* subsp. *melolonthae* H1 (BPM H1), isolated from larvae of the common cockchafer (*Melolontha melolontha*).

Sporangia of this obligate pathogen were produced by injection of vegetative cells into grubs. The gene, which codes for the parasporal crystal protein, was cloned and sequenced (Zhang *et al.*, 1997). The protein (Cry18A) shows about 40% sequence identity to the Cry2A toxins of *Bacillus thuringiensis*. For production of Cry18A crystals the gene was expressed in a crystal free mutant of *Bacillus thuringiensis* ssp. *kurstaki* (BTK).

The infectivity of the BPM H1-sporangia is very low. Activation and germination of spores was investigated in gut juice *in vitro*. Sporangiolysis and germination only occur if the sporangia have been activated by heating (70°C) and incubated at a temperature of at least 20°C. The germinated cells are flagellated and very mobile for more than 12 hours after hatching from the sporangia.

The role of the crystal protein for the infection is unknown. The grubs show a reduced feeding rate after the ingestion of crystals. Beetles are more sensitive (LD-50 ≅ 22µg). Solubilization of crystals *in vitro* and addition of trypsin inhibitor increases the mortality of beetles.

These results show that BP is a low effective pathogen for *Melolontha* grubs. The toxic effect of the crystal protein on the beetles demonstrates that there is a chance to develop a microbial insecticide to control the *Melolontha* beetles by producing the BPM H1-crystal protein in *Bacillus thuringiensis*.

Key words: *Bacillus popilliae*, milky disease, *Melolontha melolontha*, grubs, crystal protein, Cry18A, activation, sporangiolysis, germination, invading cells.

Introduction

Bacillus popilliae (BP) is a gram-positive, spore-forming bacterium and the causal agent of milky disease of scarab larvae (Dutky, 1940). The name of the disease is characterised by the milky-white appearance of the infected larvae.

BP is an obligate pathogen for scarab grubs (Bulla *et al.*, 1978; Klein & Jackson, 1992) and forms a parasporal crystal protein during sporulation which role in pathogenesis remains unclear. Toxicity was detected when the intact or solubilized parasporal crystal protein were injected into hemolymph of grubs but not when fed to grubs (Weiner, 1978).

The infection process of BP for the European chafer *Amphimallon majalis* was investigated by Splittstoesser *et al.*, (1978). They observed a germination of the spores in the gut lumen and the subsequently traverse of the epithelial, basal lamina, and capsular barriers as vegetative cells to enter the hemolymph, despite the defence system of regenerative nidi, epithelial cells, and hemocytes. In the hemolymph, the vegetative cells evade phagocytosis by the hemocytes, proliferate, and sporulate (Splittstoesser *et al.*, 1978; Kawanishi *et al.*, 1978). The role of the parasporal crystal protein during this infection process is not discussed yet.

The aim of our approaches was to analyse the infection biology of *Bacillus popilliae* ssp. *melolonthae* H1 (BPM H1) and to find a way to apply BP as a microbial insecticide against grubs and beetles of *Melolontha* spp..

Material and Methods

BP-strain and Cry18A crystal protein

The strain BPM H1 was produced in larvae of the European cockchafer. The crystal protein Cry18A was expressed in a crystal free mutant of *Bacillus thuringiensis kurstaki* (Zhang *et al.*, 1997).

Investigations in gut juice

Using an in vitro system (Götz, 1987, modified) activation and germination of the spores was observed in gut juice under the light microscope. Therefore freshly isolated gut juice of grubs was mixed with BPM H1 sporangia and incubated on a microscope slide in a ring of Vaseline, covered with a cover slide. Sporangia were pasteurised by 75°C for 25min.

The flagella of the mobile BP cells were revealed with the electron microscope after negative contrast staining.

Grubs and beetles for bioassays

Grubs of the European cockchafer (*Melolontha melolontha*) and the beetles were collected in the field near Heidelberg and Freiburg and stored in the laboratory before using for the bioassays.

Bioassays

Grubs were placed in 35mm x 83mm culture vials, filled with of a soil-sand mixture, and fed with carrot slides. BPM H1-sporangia and the crystal protein Cry18A were applied *per os* directly. During bioassays, the grubs were kept at 25°C in darkness.

Beetles were placed in 100mm x 50mm culture vials filled with 10ml of soil, and fed with oak leafs. The crystals (Cry18A) were applied *per os*. The beetles were kept at room temperature. The feeding rate was estimated, the diseased or dead grubs and beetles were registered.

Results

Activation and germination in gut juice

During incubation of pasteurised sporangia in gut juice at a temperature of 30°C, the spores are getting dark i.e. activated, followed by a sporangiolysis. The activation reached 100% after 2 hours (fig.1A) and 40% free spores resulted by sporangiolysis (fig.1B). Untreated sporangia showed no activation, sporangiolysis, or germination under these conditions.

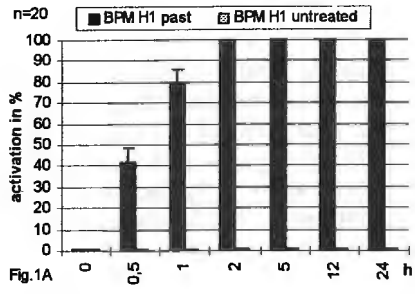

Fig.1A

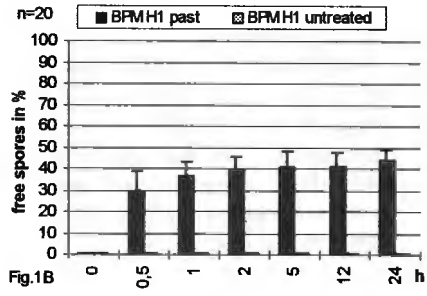

Fig.1B

Fig.1A: Activation of sporangia (pasteurised / untreated) and number of free spores (Fig.1B) after sporangiolysis at an incubation temperature of 30°C.

The germination only occurs at a temperature of at least 20°C (5% at 20°C) and increases with the temperature (table 1). At temperatures below, only activation but no sporangiolysis and germination occurs (data not shown) The germinated cells (named Invading cells) are flagellated and highly mobile. After 48h of incubation (20°C), the germination stops and the cells reduce movement. At higher temperatures the germination reaches the highest rate with 40% (30°C) after 5h and is getting lower at 12h of incubation. The vegetative cells stop the movement after 24h of incubation and start to divide.

Table 1: Germination of BPM H1 sporangia in gut fluid at different temperatures and after different incubation time.

incubation time	**10°C**	**15°C**	**20°C**	**25°C**	**30°C**
0,5h	-	-	-	-	-
1h	-	-	-	-	-
2h	-	-	-	-	20-25%
5h	-	-	-	0-5%	30-40%
12h	-	-	1-5%	30-40%	5-10%
24h	-	-	1-5%	30-40%	-
48h	-	-	1-5%	10-15%	-
72h	-	-	-	-	-

Characterisation of the crystal protein

The crystal protein (80 kDa) is completely soluble at pH ≥ 12. In gut juice, only partly solubilization is possible (data not shown). The gene, which codes for the parasporal crystal protein of BPM H1 was cloned and sequenced (Zhang *et al.*, 1997). 40% sequence identity to the Cry2A toxin of *Bacillus thuringiensis* was found. The role of the crystal protein during the milky disease infection was observed in bioassays using grubs, the toxic effect was investigated using beetles of the European cockchafer *Melolontha melolontha.*

Bioassays with grubs

The application of Cry18A crystals to grubs reduces the feeding rate to approximately 50%, compared to the control (fig.2A). The crystal protein is necessary for the milky disease infection. Purified BPM H1-spores (10^7 spores per grub), isolated in a sucrose gradient, are not infective. The application of 10^7 BPM H1-sporangia per grub causes an infection rate of about 50%. Vegetative BP cells (10^7 per grub) cause a low milky disease infection (10%), when fed together with crystals (1,5mg/ml). The vegetative cells, fed alone to grubs, are not infective (fig.2B).

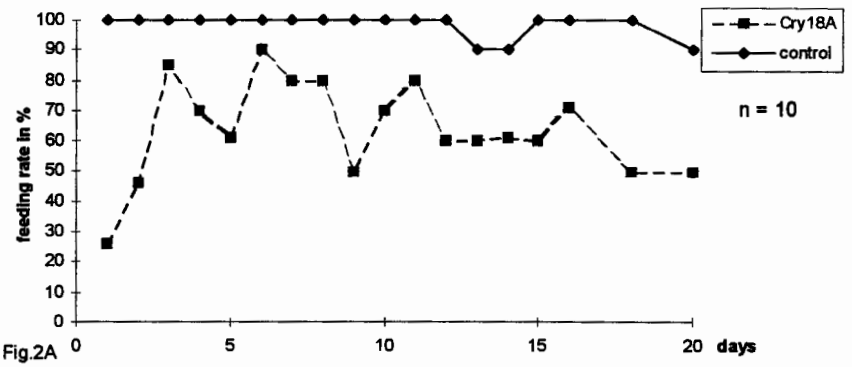

Fig. 2A: Feeding rate after the application of crystal protein (Cry18A) to grubs of *Melolontha melolontha.*

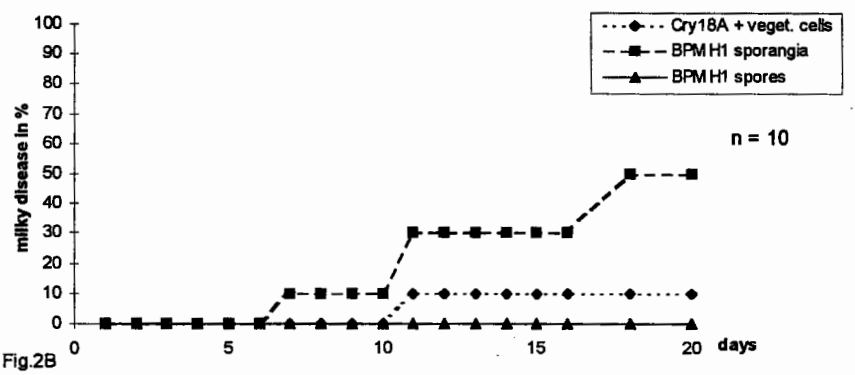

Fig. 2B: Development of milky disease infection after the application of BPM H1 sporangia, Cry18A with vegetative BPM H1 cells and BPM H1 spores (without crystal protein) to grubs of *Melolontha melolontha.*

Bioassays with beetles

The application of a high dose (40µg) of Cry18A crystal protein to beetles causes a long lasting feeding rate reduction and a mortality of about 80%. Using lower dose of 20µg or 10µg reduces the feeding rate also and causes a mortality of about 50%. Lower dose (5µg) had no effect on the beetles (fig.3A/3B). The LD-50 of crystal protein was determined (22µg). The solubilization of crystal protein *in vitro* rises the toxicity (LD-50 = 15µg) and additionally adding of trypsin inhibitor (1mg/ml) reduced the LD-50 to 11µg.

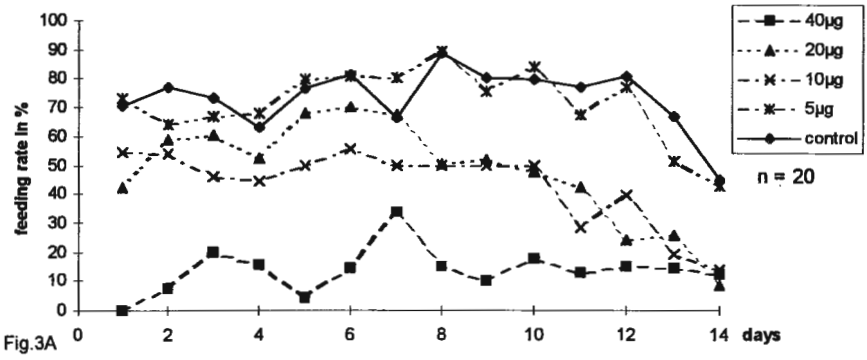

Fig.3A

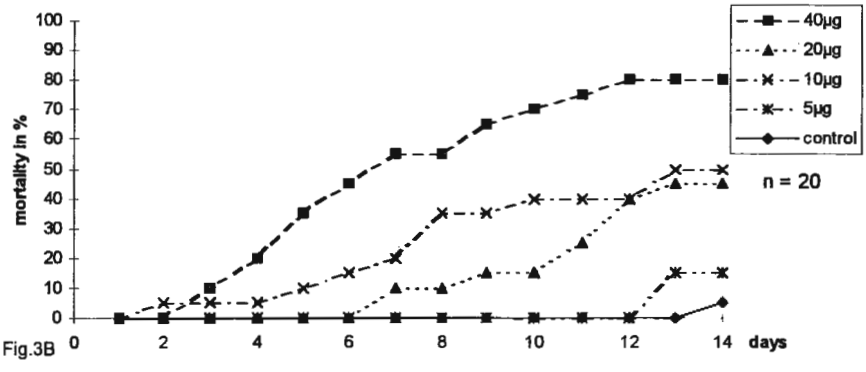

Fig.3B

Fig.3A/B: Feeding rate (A) and mortality (B) after the application of Cry18A crystal protein in different doses to beetles of *Melolontha melolontha*.

Discussion

The infection biology of *Bacillus popilliae* is not well understood. After the ingestion of the sporangia by the grub, the sporangiolysis occurs and the spores germinate in the gut lumen (Lüthy, 1968). The vegetative cells migrate with an unknown mechanism through the gut wall into the hemolymph (Flemming, 1968; Splittstoesser *et al.*, 1973, 1978; Kawanishi et al, 1978; Klein & Jackson, 1992; Stahly et al, 1992). Our *in vitro* investigations with gut fluid showed an activation of the spores, followed by a sporangiolysis and germination of spores. Similar

results are reported with *Bacillus thuringiensis* in gut fluid of the tobacco horn worm *Manduca sexta* (Wilson & Benoit, 1990). In contrast to *Bacillus thuringiensis,* the vegetative cells of BPM H1 are very mobile after hatching from the spores. The activation of spores is dependent on a heat activation of sporangia. Untreated BPM H1-sporangia showed neither activation nor sporangiolysis and germination. This results explain the higher infection rate after the application of heat activated sporangia to grubs (St. Julian & Hall, 1968; Krieger, 1994). However, the milky disease infection under natural conditions in the field will be further unclear.

Beard (1945) reported a supporting function of the crystal protein during the migration of the vegetative cells through the gut wall into the hemolymph. Similar results were demonstrated in our bioassays with grubs after the application of Cry18A crystal protein. A milky disease infection was found after the application of crystal protein together with vegetative cells. Crystal protein or vegetative cells applied separately did not cause any infection. Purified BPM H1 spores were also not infective. This results confirm an important role of the crystal protein during the milky disease infection. Additionally a feeding reduction and low toxicity of the Cry18A crystal protein was observed, which could be a sign for a cytotoxic effect by the crystal protein to the gut cells. Investigations to this question should be done in the future.

In the bioassays with beetles, a toxic effect of the crystal protein is conspicuous. Compared to the results of bioassays with grubs, the toxic effect is higher. This could be caused by a less regeneration capacity of the gut-cells of beetles. Solubilized crystal protein increases the toxic effect. This could be explained by an uncomplete solubility of the crystals in the gut. The combination of solubilized crystal protein with trypsin inhibitor increases the toxic effect additionally. This demonstrates that the crystal protein is partly inactivated in the gut system by trypsin-like proteases.

Conclusions

The germination in gut fluid of grubs occurs at a temperature of 20°C with a germination rate of 10% only. This explains the high needed dose of sporangia for milky disease infection (10^7 / grub). Together with the low toxicity after application of crystal protein and the temperature of the grub habitat, which is between 15 and 20°C, the practical use of BPM H1 against grubs in the field seems to be not possible so far.

The situation for beetles is different. The crystal protein Cry18A causes a long-lasting feeding reduction and is toxic to beetles, but in high dosages only. The solubilization and additional adding of trypsin inhibitor rises the toxicity. Because the crystal protein Cry18A can be produced in BT, it may be possible to develop a biological insecticide against the May beetle in future.

In further studies, a screening for new BP strains with higher infection rates for grubs and higher activity at lower temperatures, is necessary. Additionally, investigations of the mechanism of infection are useful. The mechanism of spore activation, binding studies and cytotoxic effects of the crystal protein, and the penetration of the invading cells through the gut wall to the hemolymph should be studied.

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Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.55 - 60

Why is the scarab specific *Bacillus thuringiensis ssp. japonensis* strain Buibui inefficient against *Melolontha melolontha*?

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Abstract: The Cry8Ca1 crystal protein of *Bacillus thuringiensis* ssp. *japonensis* strain Buibui (BTJ) shows larvicidal activity against some Scarabaeidae. In spite of this, bioassays with the European Cockchafer (*Melolontha melolontha*) only show little effects under natural conditions. A feeding reduction and a rising mortality in bioassays can only be reached at 31°C. This temperature-dependent effect correlates with an increasing growth rate of BTJ.

Crystals of BT are stable under neutral conditions. Although the gut pH of *M. melolontha* is about 8 the crystal protein of BTJ is dissolved by an unknown mechanism. Trypsin-like proteases cleave the 130 kDa-protein (Cry8Ca1) to fragments of at first 67 kDa and then 50 kDa within a few minutes. A metalloprotease degrades these trypsin-resistant core proteins to peptides < 10 kDa. These results could be observed for both the grubs and the adult chafers.

The inefficiency of the microbial insecticide could be due to the degradation of Cry8Ca1 by the metalloprotease of the gut fluid so that the classical mechanism of BT-toxins cannot work any more.

Key words: *Bacillus thuringiensis*, *Melolontha melolontha*, white grubs, gut fluid, crystal protein, Cry8Ca1.

Introduction

Bacillus thuringiensis (BT) is a spore-forming, gram-positive bacteria which produces insecticidal crystalline inclusion bodies (crystal proteins) during sporulation. After autolysis both the spore and the crystal are released into the media.

The crystal plays an important role in the biological pest control: Many insects have an alkaline gut pH, and under these conditions the crystal protein (130 - 140 kDa) dissolves. After that, the crystal is proteolytically digested to fragments between 50 and 70 kDa. These peptides are responsible for the insecticidal activity of the BT-toxins (Carroll *et al.*, 1997). A receptor within the gut membrane of the pest insect binds the activated toxin. After several conformational changes a channel is build within the gut wall by alpha-helical domains of the toxin. As a result, the epithelia cells get difficulties in their osmotic balance and they were finally knocked out. Thus, the barrier between gut lumen and hemolymph gets destroyed. Now, BT and gut bacteria cause a septikemia.

The crystal proteins work very specifically against susceptible insect and they are not toxic to non-target insects and man.

Bacillus thuringiensis ssp. *japonensis* Buibui (=BTJ) causes lethal infections in larvae of *Anomala* species, *Popillia japonica* and some other Scarabaeidae (Ohba *et al.*, 1992, Suzuki *et al.*, 1993, dito 1994). *Melolontha* species and *Amphimallon solstitialis* only show little sensitivity.

The aim of our investigations is to analyze why BTJ shows not or only low toxicity against these important pests in agriculture, horticulture and forestry.

Materials and Methods

BTJ and its insecticidal crystal protein Cry8Ca1

A suspension of crystals and spores of *Bacillus thuringiensis* ssp. *japonensis* Buibui (=**BTJ**) was made available to us by the company Mycogen. *In vitro* the bacteria were cultured in TSB-25 media (4,25g tryptone of caseine, 0,75g tryptone of soja, 0,625g $C_6H_{12}O_6$, 1,25g NaCl, 0,625g K_2HPO_4, ad 1 l dH_2O) at 25°C. Under these conditions autolysis occurs after 4 days. Crystals were separated from spores by using a density gradient at ultracentrifugation (Faloci *et al.*, 1990).

Pest insects tested in bioassays

Grubs of the European cockchafer (*Melolontha melolontha*, 3rd instar larvae) were collected in the field (Südhessen, near Heidelberg) and stored in the laboratory before starting the bioassays.

Bioassays

Grubs were individually placed in 35mm x 83mm culture vials, filled with a mixture of soil and sand, and fed with carrot slides. A suspension of crystals (Cry8Ca1) and spores were applied into the soil at the following concentrations: [spores] = $1,6*10^9$spores/g soil and 0,1 g crystal/g soil. The grubs were incubated at different temperatures (25°C to 31°C) for 31 days. Feeding rate and mortality were controlled. n = 40 grubs/test group were tested.

Solubilisation of the protein crystal

Crystals were dissolved at pH 10,5 and neutralyzed by 100 mM Tris/HCl to a final pH of 8 (according to the gut conditions of the white grubs). The pH-dependent solubilisation of Cry8Ca1 was tested in a 50 mM Citrat-Phosphat-Borat-Puffer (Franken 1994)

Proteolytic processing of Cry8Ca1

(1) Crystals were incubated with the gut fluid of *M. melolontha* for 1 minute at several dilutions. (2) For 90 minutes solubilized Cry8Ca1 was incubated with proteolytic active fractions which were prepared by gelchromatography of the gut fluid. The proteolytic reaction was stopped by denaturating the samples at 95°C for 1 minute. SDS-PAGE was used to detect the fate of Cry8Ca1.

SDS-PAGE

12,5-% SDS-gels were used with the PhastSystem (Pharmacia) according to the company's instructions.

Results

Bioassays

During a month in bioassays a temperature-dependent toxic effect of BTJ could be observed after 2 weeks. A feeding reduction and a quickly rising mortality only occurred in a test group with grubs which were treated with BTJ at 31°C (Figure 1, 2). All killed larvae showed a common appearance (see below). Using BTJ in the same concentration at lower temperatures no toxic effect could be recognized.

In additional bioassays (data not shown) with other BT strains no toxic effect could be observed.

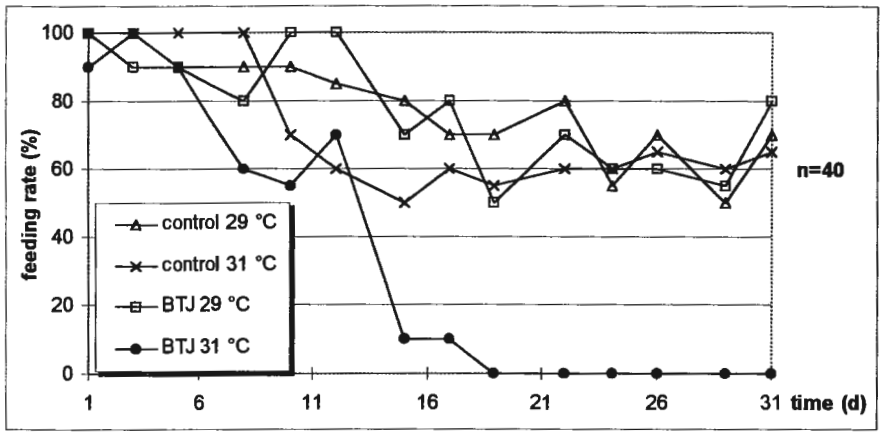

Fig. 1: The toxic effect of BTJ against *M. melolontha* is influenced by the temperature: The feeding rate of the white grubs which were treated with BTJ at 31°C decreases distinctly after 2 weeks. The negative control and the larvae at lower temperatures did not show this effect. (n = 40 grubs/group were tested.)

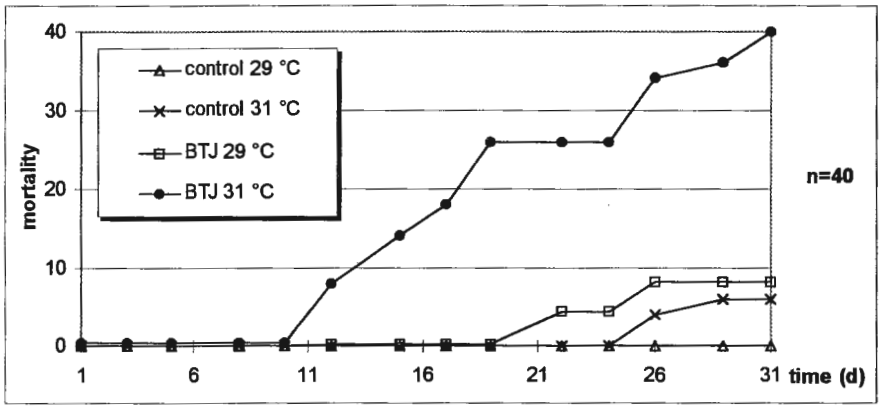

Fig. 2: Looking at the mortality, a toxic effect can only be found at 31°C, too.

The killed white grubs of our bioassays at 31°C all showed a common appearance: A week before dying the larvae show a glassy abdomen. During this period of time they do not devour any more. Post mortem they get darker and darker, but that also occurs in other grub diseases.

Investigations via light microscopy could not highlight BTJ in the hemolymph. The septikemia, which can be observed by the darkened grubs, is caused by the gut bacteria.

Temperature-dependent growth of BTJ

Our investigations show that there is a temperature-dependent growth of BTJ (Figure 3). Optimal incubation conditions can be observed at 34°C. Higher temperatures obviously hinder the bacteria development. Astonishingly, the soil bacteria BTJ shows a low growth rate at temperatures that represent the natural conditions (15-25°C).

In our bioassays we could demonstrate that a rising temperature correlates with a rising mortality of the white grubs of *Melolontha melolontha*.

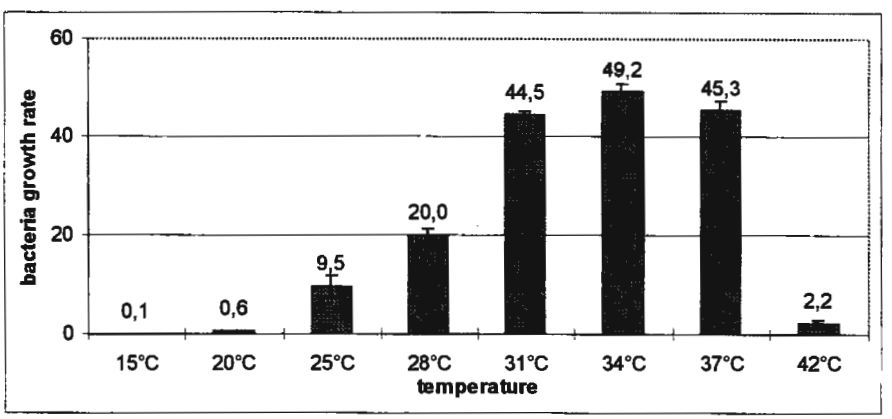

Fig. 3: Temperature-dependent growth-rate of BTJ in its vegetative phase in TSB-25 liquid culture. You can see the average number of cell divisions per hour during the log. phase.

Solubilisation and proteolytic processing of Cry8Ca1 in the gut fluid of the cockchafer

Crystals of BT dissolve under reducing and alkaline conditions (Figure 4). At neutral pH (physiological gut fluid conditions) only little solubilisation occurs. In spite of this the crystal is disassociated in the native gut fluid of *M. melolontha* (pH 8) by an unknown mechanism. Incubating Cry8Ca1 for only one minute the 130 kDa toxin is fragmented into polypeptides < 10 kDa.

After gel chromatography the proteases of the gut fluid could be tested individually. We found trypsin-like proteases with molecular weigths of about 21, 40 and 71 kDa (probably oligomers) and a metalloprotease of 15 kDa.

Incubating the crystal with these proteases no degradation could be shown. In spite of this solubilized Cry8Ca1 is cleaved to fragments of at first 67 and then 50 kDa by the trypsin-like proteases. The same molecular weights can be detected by using bovine

trypsin (Sugimura *et al.*, 1997). Testing the metalloprotease only peptides < 10 kDa could be detected after SDS-PAGE.

These results could be observed for the grubs and the adult chafers.

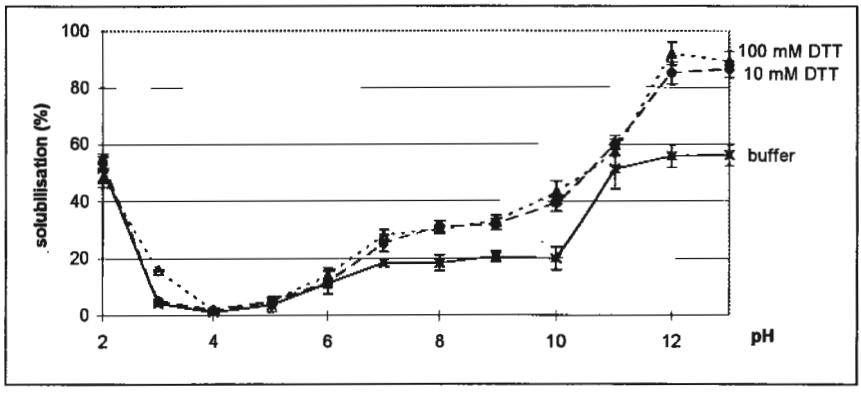

Fig. 4: pH-dependent solubilisation of the crystal protein of BTJ (Cry8Ca1). Under reducing conditions the dissociation of the crystal is increased about 50 %.

Discussion

The degradation of the toxin by the midgut metalloprotease could explain, why this scarab-specific *Bacillus thuringiensis japonensis* strain Buibui only shows little activity against grubs and beetles of *Melolontha* species under natural conditions. In contrast, the highly efficient crystal toxins of other *Bacillus thuringiensis* strains toxic for Lepidoptera, Diptera or Chrysomelids (Coleoptera) are activated by proteases in the gut fluid of target insects to fragments of a molecular mass of 60-70 kDa.

We assume, that small toxin fragments < 10 kDa are only active at a high concentration and during destabilisation of cell membranes by high temperature (31°C). Comparable results were found by Sugimura *et al.*, 1997: Bioassays with *Anomala cuprea* also showed a degradation of Cry8Ca1 to small polypeptides in the gut fluid, but the toxicity still remained. The missing of protease-resistent core peptids between 50 and 70 kDa, which are essential for the typical mechanism of pore formation in the gut wall of susceptible insects, suggests that there could be a new mechanism of toxicity of BT crystal proteins which is yet unknown.

The inhibition of the metalloprotease *in vivo* could not be managed so far. In addition, we have to prove if the fragments of 67 and 50 kDa, which can be found after digestion of Cry8Ca1 with trypsin, show toxicity in bioassays with organisms that are more sensitive against BTJ, e. g. *Anomala* species.

In further experiments it must be investigated, if the toxin binds on the gut cell membrane, like other BT toxins do. In addition, the search for new scarab specific *Bacillus thuringiensis* strains with higher toxicity against *Melolontha* species has to be continued.

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Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.61 - 63

Investigations on biological control of *Melolontha hippocastani*: Results on research activities 1997

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Abstract: Since 1986, increasing populations of the forest cockchafer, *Melolontha hippocastani*, were observed in southern parts of Hessen. This year, trees on about 7500 ha were heavily damaged or even destroyed in the forest areas of the cities Lampertheim and Darmstadt. Therefore, several research activities on biocontrol methods were conducted on the following topics: (1) diagnostic studies, (2) effect of deterrent plants and (3) use of entomoparasitic nematodes.

Key words: Insect pathogens, deterrent plants, *Melolontha hippocastani*, *Steinernema glaseri*

Diagnostic studies on the health state of cockchafer populations in Lampertheim and Darmstadt

In June and July, grubs were collected in three different sections each of both forest areas. The grubs were kept in the laboratory at 18°C for about 4 weeks seperately in small beakers filled with sterilized soil and fed with pieces of carrots. Dead grubs were dissected and diagnosed in light or electron microscopical studies for insect pathogens. The results of these investigations are shown in Table 1.

Table 1: Results of the diagnostic investigations on grubs of the forest cockchafer *Melolontha hippocastani* from different areas of Lampertheim and Darmstadt

Forest area	Section	Number of grubs	% Mort.	% *B. bro.*	% *R. melol.*	% *P. tenua*	% without pathogens
Lampertheim	306	206	25.2	15.0	2.9	0.5	6.8
	323/1	253	31.6	11.0	3.6	2.8	14.2
	323/2	94	33.8	21.3	2.1	2.2	8.5
Darmstadt	262	162	10.5	0	0	5.0	5.5
	270	181	10.5	1.1	2.8	0.6	6.1
	303	202	34.8	7.4	5.0	14.9	7.9

The main pathogens found in both populations were *Beauveria brongniartii*, the Lorsch disease, *Rickettsiella melolonthae*, and a microsporidium, preliminary described as *Pleistophora tenua* (Trzebitzky, 1992). In Lampertheim, the infection rate due to the fungus was much higher with 15%, 11%, or 21.3% compared to Darmstadt (0%, 1.1%, 7.4%). The mortality caused by *P. tenua* was 0.5 to 5.0%, in one section of Darmstadt even about 15%. The Lorsch disease was found in 4 of 5 sections, causing infections of up to 5%; however, in

three other sections in Darmstadt a mortality of about 40% was caused by *R. melolonthae*. Therefore, it is supposed that this disease will increase and spread within the cockchafer population in the following years. The diagnostic investigations will be continued in order to collect further data about the population dynamics of *M. hippocastani* and for prognosis with respect to possible chemical control methods.

Effect of deterrent plants

In laboratory experiments, the effect of deterrent plants on the behaviour and feeding activity of L-3 was tested. The following plant species were used: oil-rape, yellow mustard, winter rape, cabbage and French marigold (*Tagetes patula nana*). Fresh plants were cut and 50 g of each were mixed with 1 l of a soil/sand mixture. After three weeks, no mortality was recorded in all plants tested. Only in the *Tagetes* variant a 50% reduction in the feeding rate on carrots was noticed.

Laboratory studies on the efficacy of nematodes

Field collected strains of entomoparasitic nematodes from Germany showed only little or no parasitation of white grubs of *M. melolontha* and *M. hippocastani*. Also no parasitation was observed by L-2 of *M. hippocastani* 14 days after application of 2×10^6 infectives of commercially available *Steinernema carpocapsae*. Therefore, we chose an exotic nematode species for our tests, well known for its activity against white grubs in North America: *Steinernema glaseri*. Two strains sent to us from Wageningen have been tested: *S. glaseri* SG-0 (original strain) and SG-5, a strain passaged by 5 generations through white grubs of *M. melolontha*.

From both strains about 2×10^6 dauerlarvae / m^2 were applied to white grubs of *M. hippocastani* (L-2) reared individually in small cups (3 cm Ø, 5 cm high) filled with soil at 20°C constant temperature and in total darkness. The cups were checked for living and dead grubs about 7 and 14 days after application.

The preliminary results are shown in Table 2. Strain SG-5 killed about 100% of the *M. hippocastani* L-2, while SG-0 showed no effect. Nevertheless, SG-0 was still infective, as we could show by exchanging the still living white grubs by middle-aged larvae of the Greater waxmoth, *Galleria mellonella*. In a second test we applied the infectives, harvested from the dead grubs of the first test (SG-6) and multiplied on larvae of the Greater waxmoth, at 1×10^6 dauerlarvae / m^2 to *M. hippocastani* white grubs (L-2 and L-3). As with SG-5 all grubs and also the *G. mellonella* larvae were killed (see Table 2).

These tests demonstrate that entomoparasitic nematodes could be adapted to host insect species by passaging the nematodes over several generations through those hosts. – In 1999, we are planning field tests with *S. glaseri* multiplied on *M. hippocastani* white grubs over some additional generations.

Table 2: Efficacy of *Steinernema glaseri* strains against white grubs of *Melolontha hippocastani*

	Number			Number dead after		Number white grubs with nematodes
	G.m.	L-2	L-3	7 days	14 days	
S. glaseri (SG-5) [1]	–	10	–	8	10	10 (100%)
S. glaseri (SG-0) [1]	–	10	–	0	0	– [3]
Control	–	10	–	0	0	–
S. glaseri (SG-6) [2]	–	5	–	5	–	5 (100 %)
S. glaseri (SG-6) [2]	–	–	5	5	–	2 (40 %)
S. glaseri (SG-6) [2]	5	–	–	5	–	5 (100 %)
Control L-2	–	5	–	0	–	–
Control L-3	–	–	5	0	–	–

SG-5: *Steinernema glaseri* passaged for 5 generations through white grubs of *Melolontha melolontha,* SG-6 passaged through an additional generation of *M. hippocastani. G.m.*: middle-aged larvae of *Galleria mellonella.*

[1]) 2 x 10^6 infectives; [2]) 1 x 10^6 infectives; [3]) The activity of SG-0 was tested with larvae of *G. mellonella* after removing the still living white grubs. All larvae of *G. mellonella* had been killed within 3 days.

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Biological control with *Beauveria brongniartii*

Integrated Control of Soil Pest, subgroup "Melolontha"
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pp.67 - 71

Use of *Beauveria brongniartii* in Switzerland and its acceptance by farmers

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Abstract: In Switzerland, the fungus *Beauveria brongniartii* has been used to control the larvae of the May beetle *Melolontha melolontha* since 1991. From 1991-1998 over 36 tons of fungus kernels (barley grains colonised with *B. brongniartii*) were produced and distributed by two companies. About 70% of the material was used in five cantons where the control measures were organised by the official plant protection services. In these cantons a total of 363 hectares was treated, mainly grassland but also orchards. In autumn 1997 a questionnaire was sent to the farmers who applied *B. brongniartii*. They were asked to estimate their control success and to provide reasons for eventual failures.

A total of 228 fields could be assessed in this way. Between 69% and 100% of the farmers estimated the control success as very good or good, while 0-31% found it to be insufficient. The following reasons were given for insufficient control: Soil was too dry, applicator not correctly adjusted or fields were too steep. Insufficient control due to poor quality of the fungus can be excluded since each treatment campain in a canton was carried out with the same fungus batch. As a consequence of this inquiry it is recommended that the fungus is applied under humid conditions, and that the treatments are organised and surveyed by official plant protection services.

Key words: *Melolontha melolontha, Beauveria brongniartii*, microbial control, practical use, acceptance

Introduction

The fungus *Beauveria brongniartii* (Sacc.) Petch has been registered in Switzerland since 1990 as a bioinsecticide to control the larvae of the European cockchafer *Melolontha melolontha* L. Two companies, Andermatt Biocontrol AG and Eric Schweizer Samen AG, are producing the fungus since 1991. The majority of the product is used by farmers to control the pest in grassland. In most cantons the control measures are organised by the official extension services, which buy the product, organise storage and survey application. Treatments are normally done by local farmers experienced with the type of drill machine used in application. On plain lands, mainly Eurogreen 2000 and Psenner were used, while special constructions enabled the application on steep surfaces.

Material and methods

Data on production were obtained from the two companies, E. Schweizer Samen AG, Thun, and Andermatt Biocontrol AG, Grossdietwil. The cantonal extension services provided data on treated areas. In the cantons Thurgau, Obwalden, Nidwalden, Uri and Graubünden, a questionnaire was sent to farmers who applied the fungus from 1991 to 1997. For each field treated, they had to tick one of three answers: Have you been 1) very pleased, 2) pleased or 3) unsatisfied with the treatment. In the latter case they were also asked about the reasons they believed to be responsible for the bad results.

Results

Production and use

The fungus is produced on peeled barley grains (Aregger, 1992; Keller, unpubl.) and sold as dry kernels overgrown with fungus mycelium. The produced amount of fungus kernels is listed in Table 1 together with the amount used in those cantons with control measures organised by plant protection services. Table 2 summarizes the surfaces treated in the same cantons.

Table 1: Amount of *Beauveria* kernels commercially produced from 1991-1998 and amount of product (in kg) used in central and eastern Switzerland under the direction of cantonal extension services. The abbreviations refer to the following cantons: TG = Thurgau, UR = Uri, OW = Obwalden, NW = Nidwalden, GR = Graubünden.

year	1991	1992	1993	1994	1995	1996	1997	1998
produced	4600*	8090	1800	2710	8100	3500*	3500	4530**
used								
TG	4275	3830	0	1410	2760	0	0	
UR	210	3260	0	0	3200	0	0	
OW	0	0	1070	0	0	0	0	
NW	0	70	700	100	100	0	0	
GR	0	0	0	0	0	0	1055	
total	4485	7160	1770	1510	6060	0	1055	

*estimation
**provisional

Normally the fungus was applied in spring after the year of the flight which resulted in peak uses dependent on flight regimes. From tables 1 and 2 it is clearly visible that the cantons TG and UR as well as OW and NW have the same flight regimes. An exception from this rule is the application in the canton TG 1991 when orchards were treated in the autumn of the year of the flight.

Table 2: Area (hectares) treated in central and eastern Switzerland under the direction of the cantonal extension services. For abbreviations see Table 1.

year	1991	1992	1993	1994	1995	1996	1997
TG	82.5	53.7	0	7.0	53.5	0	0
UR	5.0	77.0	0	0	72.0	0	0
OW	0	0.5	16.1	0	1.9	0	0
NW	0	1.5	15.2	0	1.9	0	0
GR	0	0	0	0	0	0	23.5
total	87.5	132.7	31.3	7.0	129.3	0	23.5

Answers of the farmers

The questionnaire sent to farmers allowed to estimate the success of the treatment in 166 meadows and 62 orchards. 88 meadows were situated in the canton Uri (UR), 11 in the canton Nidwalden (NW), 22 in the canton Obwalden (OW), 29 in the canton Graubünden (GR) and 16 in the canton Thurgau (TG). Between 17% (GR) and 59 % (OW) of the rated meadows were very successfully treated and only between 0% (TG) and 31 % (GR) had insufficient control (Fig. 1).

The reasons for the insufficient control were mainly: Too late application in the season (9 cases), treatments with wrongly adjusted drill machines (8 cases) and too dry soil (7 cases). In four cases too steep fields or stony soils were mentioned. An incorrectly adjusted machine was responsible for most failures in the canton Graubünden where all treatments in a region done by the same person were unsuccessful. Treatments in summer or autumn as further reasons for failure must be seen in connection with dry soils and too long or inadequate storage. The quality of the fungus was never mentioned as reason for failures. This also can be excluded since usually all the treatments in a canton were done with the same fungus batch.

Discussion

Two approaches to control *Melolontha* larvae were developed. The treatment of adults with blastospores, using the females to carry the disease into the breeding sites, resulted in a long term control but needed more than a generation to induce epizootics (Keller et al., 1997). Since this was too long for the farmers, we developed in collaboration with the Entomological Institute ETHZ, a fungus product based on barley grains (Aregger-Zavadil, 1992; Fornallaz, 1992; Keller, unpubl). Production is simple, and the product can be applied with existing drill machines by individual farmers. It was quickly accepted by farmers since it is the only insecticide registered for white grub control in grassland. In orchards the farmers have the choice of applying chemical insecticides or nets to prevent females from oviposition (Brenner & Keller, 1996).

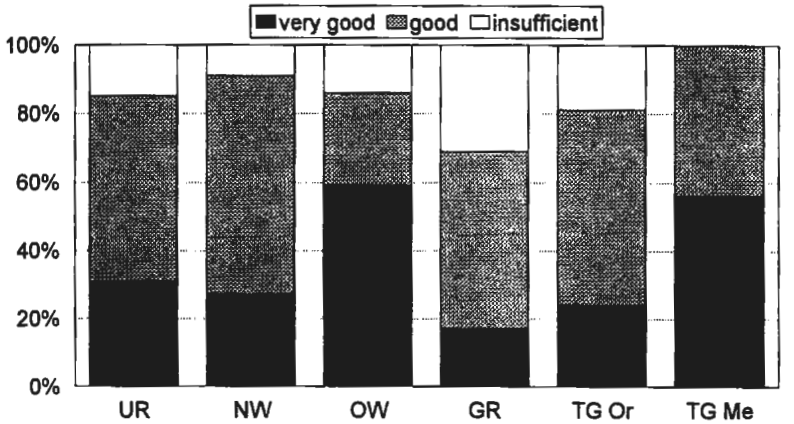

Fig. 1: Success of white grub control with *B. brongniartii* estimated by farmers. Or = orchard, Me = meadow. For other abbreviations see table 1.

B. brongniartii has not a big market in Switzerland. On average some 60 hectares per year have been treated from 1991-1997 but it fills an important market gap. It enables the farmers to avoid yield loss, and also soil erosion, which is often a problem in hilly regions after complete devastation of the plant cover.

The control success of *B. brongniartii* applied by farmers has never been assessed. After seven years of use we decided to start a follow up study on two levels. First, by surveying farmers and second, by identifying *B. brongniartii* isolated from soil samples from treated fields. Results of the latter are published by Keller et al. (in press) and David-Henriet et al. (this issue). The inquiry among farmers demonstrated that they were, in general, satisfied with the control success. In central Switzerland (cantons UR, NW, OW) control was insufficient in only 9-15% of the meadows. Good to very good control was achieved in the meadows in the canton TG, while 19% of the orchards were insufficiently controlled. This is a clear indication that orchards are more sensitive to white grub damage than meadows. The lowest success rate was achieved in the canton GR. The failure is mainly due to a single farmer who did not correctly adjust the drill machine, so that the fungus kernels were deposited on the soil surface. In no case was the quality of the fungus material a reason for failures since each treatment campain was carried out with the same fungus batch.

The good control success can mainly be attributed to the cantonal plant protection services who organised and surveyed the treatments, and to good fungus quality. It is crucial that all steps from strain selection to application are carefully done. Particular attention should be given to 1) the selection of the fungus strain which should be based on bioassays with white grubs from the population to be controlled, 2) the quality of the fungus product and 3) the application of the fungus grains which must be placed at the soil depth where white grubs are active.

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Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.73 - 78

Insect pathogenic soil fungi from *Melolontha melolontha* control sites in the canton Thurgau

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Abstract: From 1976-1988 a series of field trials were carried out to control the larvae of the European cockchafer, *Melolontha melolontha* with blastospores of the fungus *Beauveria brongniartii*. From 1989-1992 a commercial preparation (barley grains colonised by the same fungus) was used to control M. melolontha in orchards. Most populations were significantly reduced. In 1998 soil samples were taken from these treated areas and from untreated areas to determine presence and density of *B. brongniartii* and *Metarhizium anisopliae*. *B. brongniartii* was present in all areas except the one treated 1976. Densities and frequencies of this fungus were lower in the areas treated with blastospores compared to the area treated with the commercial product. The fungus was not found in soil from untreated sites. *M. anisopliae* was present in all soil samples with comparable frequencies. In bioassays we compared strains isolated from soils from treated areas with isolates from other regions as well as with the commercial strain before and after mass production. The highest rate of mycosis resulted from strains isolated from treated soils. There was evidence that the commercial strain lost part of its virulence during mass production, a fact which needs further careful investigations.

Key words: *Beauveria brongniartii, Metarhizium anisopliae*, Melolontha, biological control, fungus density, soil.

Introduction

The fungus *Beauveria brongniartii* (Sacc.) Petch (Deuteromycetes: Moniliales) is considered as the most important natural enemy of the European cockchafer, *Melolontha melolontha* L. (Coleoptera: Scarabaeidae). Its potential as biocontrol agent has been demonstrated in field trials using barley grains colonised by the fungus (fungus kernels) introduced into soils colonized by white grubs (Keller, 1992; Zelger, 1996), but also with blastospores applied on swarming beetles which subsequently carried the disease to the breeding sites. The latter method was extensively tested and used in the canton Thurgau, north-eastern Switzerland. Pilot trials were done in 1976 and 1982 with a tractor-pulled mistblower followed by large field trials in 1985 and in 1988 carried out with a helicopter (Keller *et al.*, 1979; 1989). At 13 out of 15 sites sufficient control was achieved to avoid damage by white grubs (Keller *et al.*, 1997). In this study we present data on the presence of *B. brongniartii* in the areas treated with blastospores in the years 1976, 1985 and 1988, but also from some orchards treated 1989-1992 with a commercial product (fungus kernels) of *B. brongniartii*.

Material and methods

Isolation of B. brongniartii from soils

Between March and September 1998 20 soil samples each were taken from meadows in the breeding area of adults treated with blastospores of *B. brongniartii* between 1976 and 1988 (Keller *et al.*, 1997) (Table 1, site numbers 1-17) and from untreated meadows devoid of *M.*

melolontha for more than 40 years (Table 1, site numbers 31-36) Further soil samples were taken from orchards treated with the commercial product of *B. brongniartii* between 1989 and 1992 (Keller *et al.*, in prep.) (Tab. 1, site numbers 21-25). We used a cylindrical soil sampler with an inner diameter of 5.5 cm. Soil samples were taken from a depth between 5 and 18-20 cm. The samples were stored in a cool room for not longer than 4 months.

Prior to the isolation the soil samples were mixed and 20 g/sample of fresh soil were shaken for 12 h in 200 ml Erlenmeyer flasks with 100 ml tap water containing 1.8 g/l Na-diphosphate to favour disaggregation of the soil. After 15 seconds of sedimentation 0.1 ml the suspension was distributed with a Trigalsky spatula on a Petri dish with selective media and intensively rubbed in (Fornallaz, 1992). Three replicates/soil sample were prepared. After 8-10 days at 20°C the colonies of *B. brongniartii*, which had a typical appearance and stained the medium red-brownish, were counted and a selection of colonies were isolated in tubes with Sabouraud-glucose-agar with 0.6 g/l Streptomycine. The soil suspension was not diluted, even if there were very high fungal densities exceeding 600 colonies/Petri dish, the maximum that could be counted. The minimum of colony forming units (CFUs) that could be determined with this method was 0.8/g soil.

We used a semi-selective medium adapted from Strasser *et al.*, (1997) with the following composition and preparation: 10 g peptone from meat pancreatically digested, 20 g glucose, 18 g agar, all dissolved in 1 liter destilled water and autoclaved at 120 °C for 20 minutes. At a temperature of 60 °C 0.6 g streptomycin, 0.05 g tetracycline and 0.05 g cyclohexamide previously dissolved in distilled, sterile water and 0.1 ml Dodine were added. The medium proved to be suitable for the isolation of Metarhizium anisopliae as well.

The same soil samples (20 samples /site) were used for the isolation of *B. brongniartii* with the *Galleria* bait method (GBM) (Zimmermann, 1986). 60 g of soil/sample sieved through a metal sieve with 5 mm meshes was filled in a cylindrical plastic tube with a diameter of 4.5 cm and a heigth of 6 cm and 4 larger *Galleria* larvae added. The samples were kept at room temperatures between 20 and 24°C. During the first four days the tubes were turned daily to keep the larvae moving in the soil. After 14-18 days the larvae were examined and attributed to one of the four categories „healthy", „infected with *Beauveria*", „infected with *M. anisopliae*" or „death from other reasons" like nematodes. *Beauveria* species were isolated on selective media and kept for further investigations. The data were analysed with ANOVA using the LSD-test. The number of CFUs was logarithmically transformed prior to the analysis.

Bioassays with* Melolontha *larvae

Bioassays were carried out with 18 strains of *B. brongniartii*. Eight of them orginated from the canton Thurgau isolated with selective media or with the GBM from soils from the sites treated 1985 and 1988. Two originated from central Switzerland, one from a site treated with the commercial product and the other from an untreated control plot. Five strains originated from other countries. These 15 strains were compared with three isolates of the commercial strain, one taken before mass production and two isolated from barley kernels. The concentration of the suspension was 4.6-10.6 x 10^6 blastospores x ml^{-1}. For each treatment 30 white grubs of the second instar were dipped in the suspension. After allowing the excess liquid to drip off, the larvae were placed individually in 80 ml plastic boxes half filled with damp peat. One control was left untreated, the larvae of the other control were dipped in water. The white grubs were fed with carrots and checked weekly for mortality during four weeks.

Results

Isolation of B. brongniartii *and* M. anisopliae

B. brongniartii could be isolated from all trial areas except from the site treated in 1976 (Table 1). Although there was a tendency to more CFUs and higher proportions of positive samples in the 1988 area as compared to the 1985 area, the differences were not significant (Table 2, Figure 1). The values from orchards were the highest and differed sometimes significantly. No *B. brongniartii* was found in check plots, while *M. anisopliae* was present in comparable densities in all areas. The two methods (selective medium and GBM) resulted in slight, non-significant differences concerning the proportion of soil samples where *B. brongniartii* was found (positive soil samples).

Table 1: Presence and density (colony forming units per gram soil, CFU/g) of *B. brongniartii* and *M. anisopliae* in soil samples from treated and untreated meadows (BS and check) and orchards (CP). 20 soil sample per site. BS = blastospores; CP = commercial product; sel. med. = selective medium; GBM = *Galleria* bait method.

Site nr.	treatment	*B. brongnartii*			*M. anisopliae*		
		CFU/g	% positive soil samples		CFU/g	% positive soil samples	
		mean	sel. med.	GBM		sel. med.	GBM
01	BS 1976	0	0	0	0	0	80
02	BS 1985	0	0	30	0	0	85
03	BS 1985	0	0	30	72.5	10	60
04	BS 1985	40.9	30	45	613	20	50
05	BS 1985	0	0	0	111	5	80
06	BS 1985	0.8	5	0	73.4	10	45
07	BS 1985	0	0	0	0	0	20
11	BS 1988	35.0	5	5	0	0	75
12	BS 1988	0	0	5	1970	90	70
13	BS 1988	19.1	25	15	0	0	65
14	BS 1988	70.0	45	15	796	95	50
15	BS 1988	0	0	0	190	15	35
16	BS 1988	2.5	10	0	366	55	60
17	BS 1988	0.8	5	10	181	15	85
21	CP 1989	18.4	25	20	0	0	30
22	CP 1991	91.7	5	45	70.9	5	65
23	CP 1991	3.34	5	40	422	10	25
24	CP 1992	46.7	20	25	169	15	50
25	CP 1992	32.5	30	70	71.7	10	35
31	check	0	0	0			50
32	check	0	0	0			50
33	check	0	0	0			80
34	check	0	0	0			40
35	check	0	0	0			35
36	check	0	0	0			70

Table 2: Mean colonny forming units (CFUs) and % soil samples with *B. brongniartii* and *M. anisopliae*. Different letters behind the numbers indicate statistical differences (LSD-test, $p<0.05$). GBM = *Galleria* bait method.

Treatment	*B. brongniartii*						*M. anisopliae*	
	Mean CFU/g		Mean % positive soil samples (% pss)				Mean % pss	
			Selective medium		GBM		GBM	
BS 1985	6.95	ab	5.8	abc	17.5	abc	56.7	a
BS 1988	18.2	bc	12.9	abc	7.1	ac	62.9	a
CP	38.5	c	17	b	40	b	41	a
Check	0	a	0	c	0	c	54.2	a

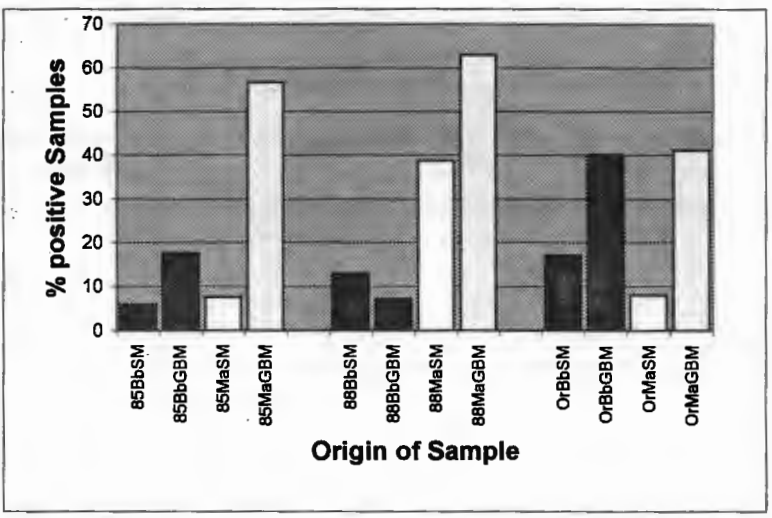

Figure 1: Comparison of the proportion of soil samples with *B. brongniartii* (Bb, black) and *M. anisopliae* (Ma, white) from areas treated 1985 (85) and 1988 (88) and from orchards (Or) either using selective media (SM) or the *Galleria* bait method (GBM).

Bioassays

The tested strains resulted in different fungus induced mortalities (Figure 2). Mortalities higher than the commercial strain (isolate number 14) were caused by strains isolated in the areas treated 1985 and 1988 and by two strains isolated in central Switzerland. Among the isolates with low mortalities are those from other countries. The strains isolated from commercially produced material (isolates numbers. 3 and 9) resulted in no or lower mortality than the original commercial strain.

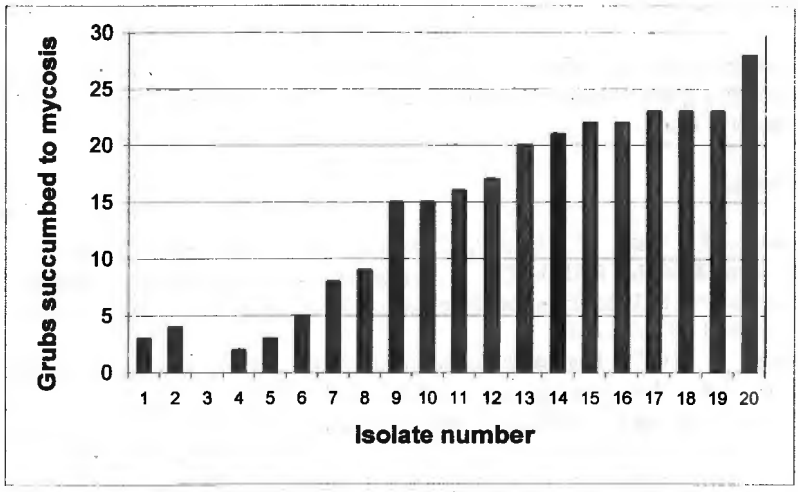

Figure 2: Number of white grubs succumbing to mycosis within four weeks. Isolate 1 and 2 corresponds to the control, isolate 14 is the commercial strain.

Discussion

B. brongniartii was isolated only from areas where the fungus had been applied either as blastospores on adults or as fungus colonised barley kernels. This does not mean that the fungus only exists in areas where it has been applied. *B. brongniartii* is considered a specific pathogen of *Melolontha* spp. and its presence can basically be expected at sites colonised by its host. Studies that demonstrate this close coexistence under undisturbed conditions do not exist but are planned. In areas treated with blastospores the densities and frequencies are below those treated with the commercial product. This is mainly due to the fact that the soil application of the fungus leads to a much higher fungus density than the treatments of swarming females which subsequently "inoculate" the breeding areas. The year of the treatment has only little influence as shown by the data from 1985 and 1988.

The longevity of the fungus in the soil can not be assessed from these data. In all the investigated areas white grubs were present on which the fungus could multiply. To study the longevity of the fungus host-free sites must be chosen. Further, we can not conclude that the strains isolated from treated areas are identical with those used for the treatments. Studies allowing the differentiation between naturally occurring and introduced strains basing on genetical markers (Cravanzola *et al.*, 1997; Neuvéglise *et al.*, 1994; 1997) are initiated.

The *Galleria* bait method proved to be a more sensitive method to detect *B. brongniartii* and *M. anisopliae* in the soil than the selective medium. However, selective media are used for the determination of the fungus density in the soil.

The bioassays demonstrated that more virulent strains of *B. brongniartii* can be found and, more important, that the fungus eventually can lose virulence during mass production. This fact must be carefully investigated and, if verified, a solution to overcome this problem must be found. In any case the quality of the final product must be guaranteed and a correspon-ding quality assessment is to establish.

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Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
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RAPD derived markers: a rapid method for identifying *Beauveria brongniartii* strains*

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Summary: The use of specific RAPD markers facilitated the molecular characterization of 5 highly virulent strains of the entomopathogenic fungus *Beauveria brongniartii* (BbC/2, BbF, BbK/2, BbN/3, BbW/2), selected for biological control trials of *Melolontha melolontha* in Valle d'Aosta (Italy).

These markers have been used to verify the potential natural occurrence of the selected strains in the experimental fields chosen for the biological control trials, located in different areas of Valle d'Aosta, before the soil treatments.

M. melolontha larvae infected by *B. brongnairtii* recovered from soil soundings in the different experimental fields were collected. The isolates of *B. brongniartii* obtained from these larvae were analyzed by RAPD, and their amplification profiles were compared with the profiles of the 5 selected strains of the fungus.

Forty isolates of *B. brongniartii* were obtained from the mycosed larvae of cockchafers recovered in the soundings and of these 8 showed the genotypes specific to strains BbC/2 (1 isolate), BbK/2 (3 isolates), BbW/2 (4 isolates). Strains BbF e BbN/3 were not recovered. The remaining 32 isolates were of different genotypes.

The results obtained are a demonstration of the usefulness of RAPD-PCR for the rapid tracking of entomopathogenic strains inoculated in the soil as biopesticides.

Introduction

RAPD-PCR (Welsh and McClelland, 1990; Williams *et al.*, 1990) has been used for the molecular characterization of strains of the entomopathogenic fungus *Beauveria brongniartii*, isolated from larvae of *Melolontha* spp. (Cravanzola *et al.*, 1997). Twentyeight different RAPD profiles were found within a group of 58 isolates obtained from infected larvae of *M. melolontha* recovered in Valle d'Aosta, north-west Italy (Piatti *et al.*, 1998).

RAPD profiles provide genetic markers which can be used for the rapid tracking of *B. brongniartii* isolates inoculated in the soil as biopesticides.

On the basis of these results, it has been possible to design experimental trials of biological control of *M. melolontha* in Valle d'Aosta. These trials are aimed at evaluating the influence of different soil and climatic conditions on the activity and efficacy of highly virulent strains of *B. brongniartii* which can be identified by RAPD markers.

It was necessary to verify the potential natural presence of the selected strains in the soil of the experimental fields, prior to inoculation.

In this paper we present the results of the preliminary investigations.

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Materials and methods

The strains of *B. brongniartii* Sacc. (Petch.), selected for the programmed trials of biological control of *M. melolontha* L. (Coleoptera, Scarabaeidae), are listed in Table 1. The year of isolation, the isolation site and the level of virulence are given for each strain. In the case of strain BbF, obtained from a *M. melolontha* larva found affected in Valle d'Aosta, the exact isolation site is unknown. Nevertheless, it has been included among the strains selected, as it showed a very high level of virulence against cockchafer grubs in repeated laboratory pathogenicity assays (Cravanzola, 1996; Cravanzola *et al.*, 1997, Piatti *et al.*, 1998)

For the controlled experiments 10 fields were selected, which contained apple orchards, and were located in areas of the Valle d'Aosta characterized by different soil and climatic conditions. The soil infestation levels of *M. melolontha* grubs in these fields were between 8 e 28 larve/ m^2. The locations of the experimental fields in the valley are shown in Fig. 1.

The following methodology was adopted in order to check if any of the strains selected for the biological control trials occurred in the soil of the experimental fields.

M. melolontha grubs infected by *B. brongniartii* were collected in each field from soil soundings of the cultivated layer (0-25 cm depth) of 0.04 m^3 (0.16 x 1 x 0.25 m) obtained with a Blaisinger hoe (Bondaz, 1996). In total 10 soil samples were examined for each of the 10 fields. The soil surface sounded in each field was 10000 m^2.

A pure culture of a *B. brongniartii* isolate was obtained from each of the mycosed grubs on Sabouraud Dextrose Agar (Oxoid) culture medium containing 0.25 g/l of actidione and 0.5 g/l of chloramphenicol (Veen and Ferron, 1966).

The isolates of *B. brongniartii* obtained were analyzed by RAPD-PCR to compare their amplification profiles with the profiles of the 5 selected strains being tested.

The DNA of each of the isolates recovered was extracted from a culture grown for 36-48 hours on Sabouraud Dextrose Agar with the Invisorb™ Genomic DNA Kit (Invitek, Berlin, Germany). This DNA was used for the RAPD amplification with the primers OPM-02 (5'-ACAACGCCTC), OPM-03 (5'- GGGGGATGAG), OPM-06 (5'- CTGGGCAACT) and OPM-14 (5'-AGGGTCGTTC), according to the method of Piatti *et al.* (1998).

The amplification profiles of the 5 strains selected and their specific RAPD markers are shown in Fig. 2.

Results

In the soil soundings carried out in the 10 experimentl fields 40 larvae infected by *B. brongniartii* were collected. The maximum number of grubs affected in any field was 9, in the fields at Saint-Pierre and Quart (Table 2). In one of the two fields of Saint-Pierre and of Gressan no mycosed larvae of *M. melolontha* were recovered (Table 2).

The results of the RAPD analysis of the 40 isolates of *B. brongniartii* are presented in Table 2.

The RAPD fragment I, characteristic of the strain BbC/2, was detected in one isolate obtained from the field at Jovençan (Table 2).

The simultaneous presence of bands III and IV in the RAPD profile produced by the OPM-14, which was specific for the strain BbK/2, was detected in 3 isolates, all from the field at Quart (Table 2). The Quart area is also the area in which the strain BbK/2 was isolated in 1996.

Fragment VI, which was characteristic for the strain BbW/2, was found in 4 isolates, 2 from the Aymavilles field and 2 from the Gressan field (Table 2). Fig. 3 shows an example of

the amplification obtained with the OPM-06 and illustrates the presence of the RAPD marker VI in 2 of the analyzed samples.

Table 1 - The strains of *Beauveria brongniartii* obtained from *Melolontha melolontha* larvae in Valle d'Aosta and selected for the biological control trials.

Strain	Year of isolation	Isolation site	Virulence*
BbC/2	1996	La Salle	85
BbF	1993	-	90
BbK/2	1996	Quart	90
BbN/3	1996	Nus	90
BbW/2	1996	Saint-Nicolas	90

* Virulence determined as percentage of larvae of *Melolontha melolontha* killed by *Beauveria brongniartii* within 45 days after inoculation (Piatti *et al.*, 1998).

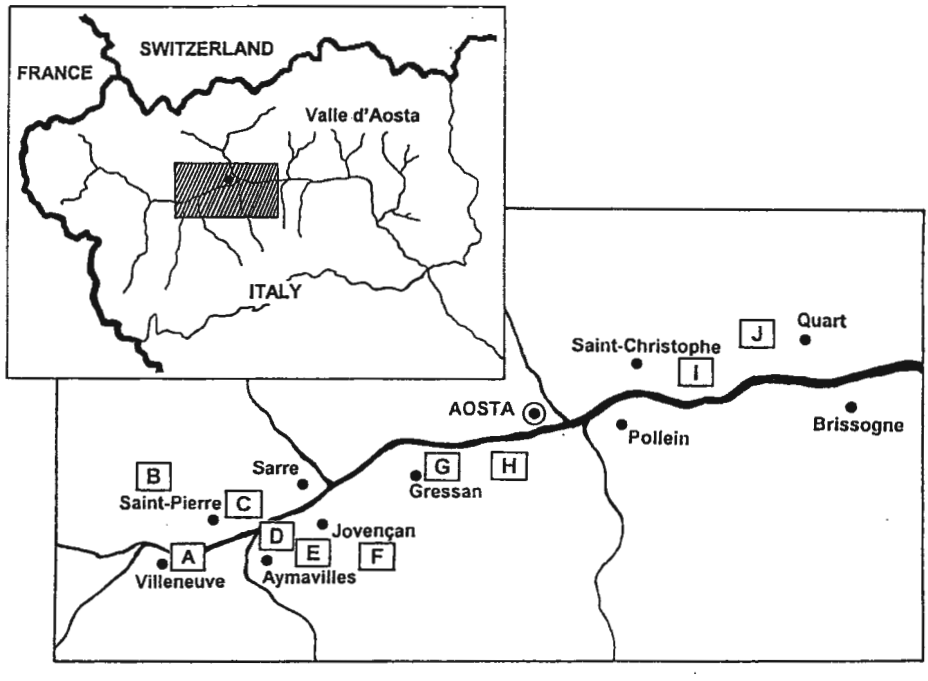

Figure 1. Locations of the experimental fields in Valle d'Aosta.

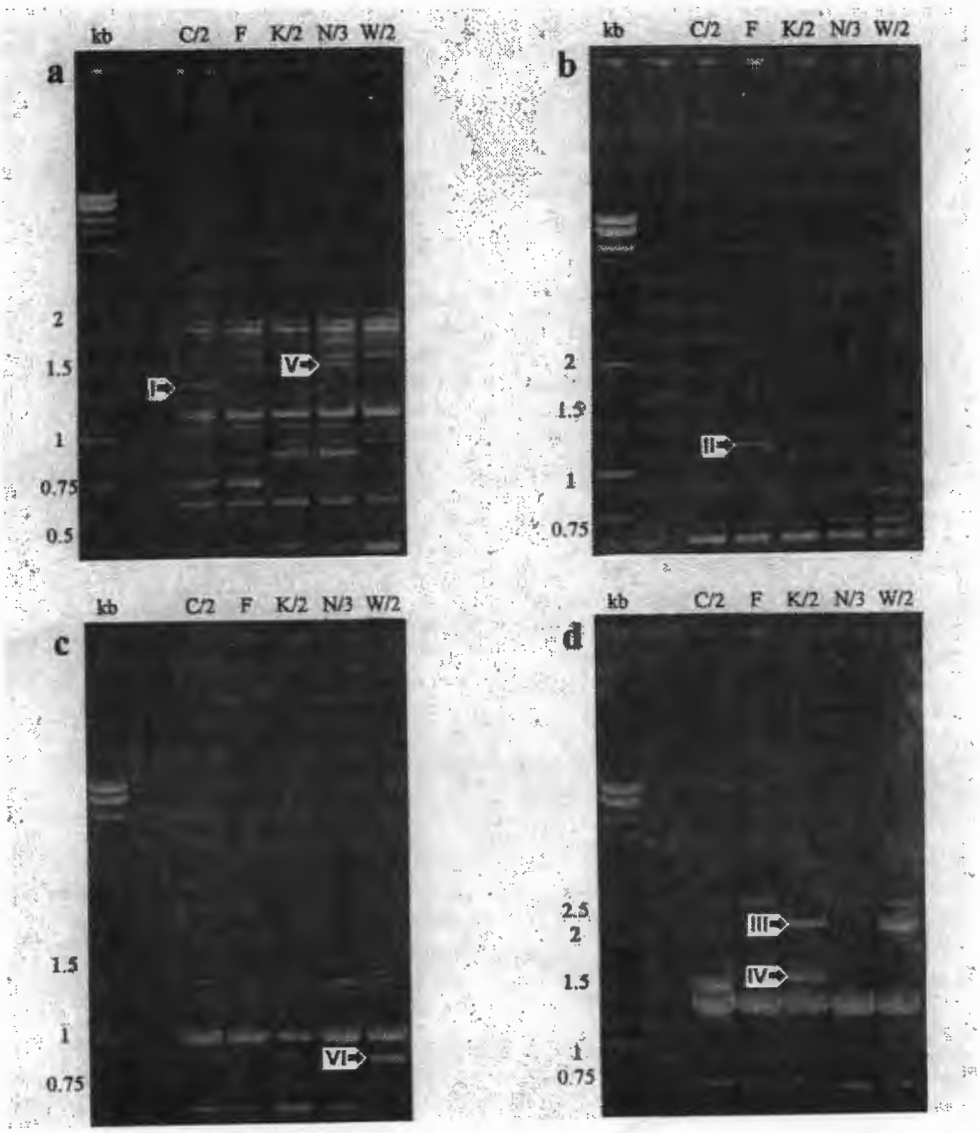

Figure 2. RAPD profiles of the 5 selected strains of *Beauveria brongniartii* obtained with the primers OPM-02 (a), OPM-03 (b), OPM-06 (c) and OPM-14 (d). The RAPD markers specific for the 5 strains are indicated with roman numbers. Band I (1.45Kb): specific for the strain BbC/2 (a). Band II (1.3kb): specific for the strain BbF (b). Simultaneous presence of bands III (2.4kb) and VI (1.6kb): specific for the strain BbK/2 (d). Band V (1.65kb): specific for the strain BbN/3 (a). Band VI (0.85kb): specific for the strain BbW/2 (c).

Table 2. The RAPD markers detected in the isolates of *Beauveria brongniartii* obtained in the different experimental fields.

	Experimental fields	number of isolates	RAPD markers detected					other genotypes
			I	II	III e IV	V	VI	
A	Villeneuve	1	-	-	-	-	-	1
B	Saint-Pierre	-	-	-	-	-	-	-
C	Saint-Pierre	9	-	-	-	-	-	9
D	Aymavilles	4	-	-	-	-	**2**	2
E	Aymavilles	7	-	-	-	-	-	7
F	Jovençan	3	**1**	-	-	-	-	2
G	Gressan	4	-	-	-	-	**2**	2
H	Gressan	-	-	-	-	-	-	-
I	Saint-Christophe	3	-	-	-	-	-	3
J	Quart	9	-	-	**3**	-	-	6
	TOTAL	**40**	**1**	**-**	**3**	**-**	**4**	**32**

Bands II and V, specific to the strains BbF and BbN/3 respectively, were not recovered in any of the 40 isolates examined.

The genotypes of the remaining 32 isolates analyzed gave profiles different from the profiles of the 5 selected strains.

Conclusion

The use of specific RAPD markers allowed the verification of the natural occurrence of the strains of *B. brongniartii* selected for biological control trials of *M. melolontha* in Valle d'Aosta in the 10 experimental fields selected for the study, before the soil treatments.

In the group of 40 isolates of *B. brongniartii* obtained from the mycosed larvae of cockchafers recovered in the soundings in the experimental fields, 1 isolate was genotypically identical to strain BbC/2, 3 isolates to strain BbK/2 and 4 isolates to strain BbW/2. The presence of the strains BbF and BbN/3 was not detected. The remaining 32 isolates showed different genotypes.

These results, even though obtained in a preliminary investigation, with a limited number of isolates, provide a further demonstration of the efficacy and the usefulness of the RAPD technique in biological control programmes for the rapid monitoring of entomopathogenic strains useful as biopesticides.

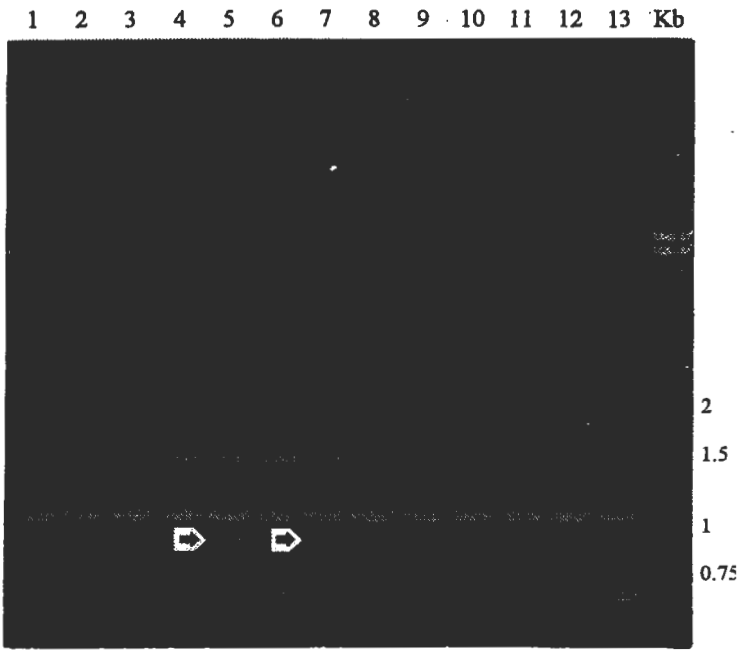

Figure 3: Example of RAPD amplification of 13 of the 40 isolates recovered with the OPM06. The arrows indicate in lanes 5 and 7 the presence of the marker VI, specific for the strain BbW/2.

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Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.87 - 92

Field Application of *Beauveria brongniartii*, established on peeled barley kernels

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Introduction

The control of *Melolontha melolontha* with the fungus *Beauveria*, placed on barley grains, is a method that is working well. The analysis of negative results with this product shows that problems are often caused by:

- insufficent structures
- unsuitable machines
- problematic locations

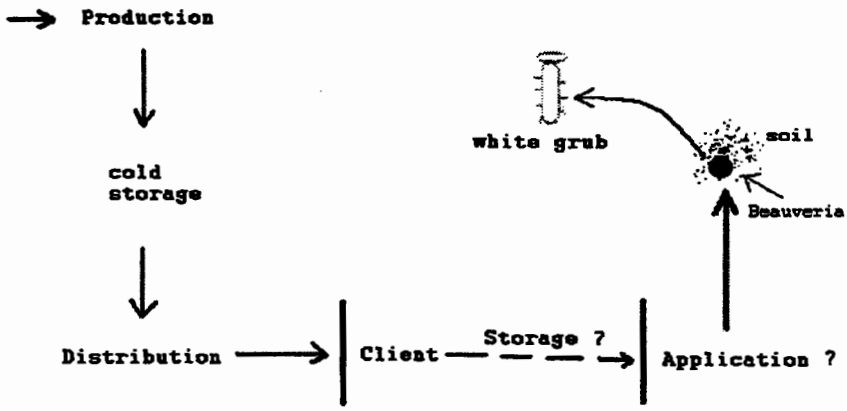
For a practical, satisfactory control of *Melolontha melolontha* you need to do more than create a product which only works well in the laboratory.

Basic problems

1. Is it really a white grub of "may beetle", or is it a white grub of another insect species?
The client has to be asked this questions before the product is dispatched, because *Beauveria* is specifically used for *Melolontha* and doesn't control any other white grubs. The supplier is able to identify the white grubs by their morphological symtoms and to advice the client.
2. Is it possible to produce fresh *Beauveria* material quickly?
If the official offices and research centers are following the correct procedures, the prompt delivery of *Beauveria* shouldn't be a problem. The problem is, that some private clients don't order the product until they notice damage in the field or they find some white grubs in the ground. Unlike chemical insecticides, *Beauveria is* not able to be stored for years. That's why it has to be guaranteed that the manufacturer is able to produce large units in a short time (employes, installations...) and eventually store the material (cold room).
3. Is the producer able to distribute large units (for example 25 - 50 kg bags) for field application and also small units (5 kg bags) for small areas or for transport to mountain locations?
Before distribution, the client must state how much of the product he would like to have, and how big the units should be. Care should be taken that the client does not receive too much, or that he is able to store the surplus.
4. Is the client able to apply the product in the correct manner?
Does the client have the right machines to apply the product into the ground correctly ? Are there enough people to help working in steep areas (for example: hillside locations).
5. Choose the right time for application.
Field experiments in Swizerland show, that the ideal time for application of *Beauveria* is in spring. At this time the growing conditions are warm and effective, and also the ground is humid enough for a good application.

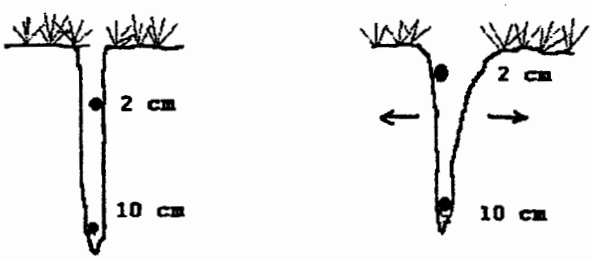
6. Repeat sowing to minimize damage and losses.
The product *Beauveria* is not a seed. It is not possible to simply apply it a second time like a normal seed. You have to work the product into the ground. Therefore it is useful to control the effect of the application.

Progression of the fungus from production to its destination, the white grub

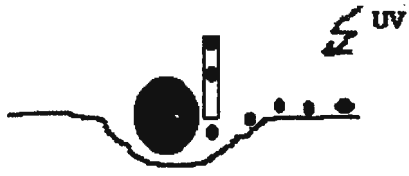


Problems with the application

The product has to be sown 8-10 cm deep into the ground. WHY ?
When the weather becomes dry after application, the soil hardens and the fungus grains will lay on the surface of the ground. They will be destroyed by sunlight or eventually eat by birds.

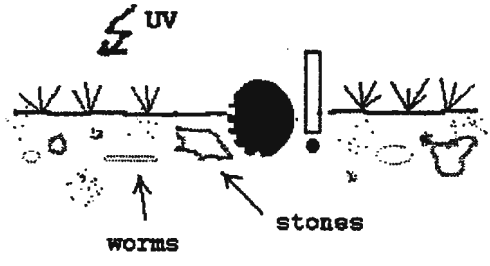


Problem: Compacted layer of the lawn
The compacted layer of the lawn prevents the machine getting deep enough into the ground, because this compact layer makes penetration difficult. So the barley grains with *Beauveria* will, as before, lay on the surface of the ground. The fungus will be destroyed by direct contact with UV-light and heat. After moving the lawn, the product is cleared away.



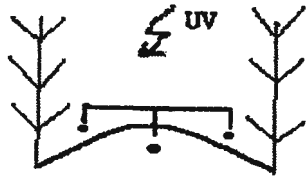
Problem: compacted layer of the lawn

Problem: stability of the machines
Sowing machines have to be stable, easy to repair and be of sufficent weight to get into the ground.



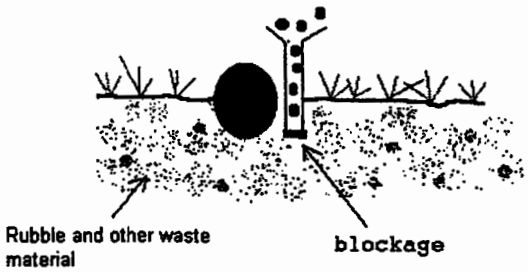
Problem: stability of the machines

Problems with vehicle tracks
The machines will rotavate the product only between the vehicle tracks. On both tracks, the product is falling onto the surface (as with vine cultures). Another problem is access to small areas, where it is too narrow for the machines to get through.



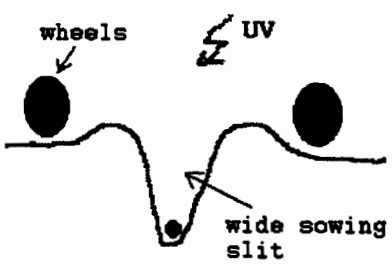
Problem: vine cultures

Problem: blockage of the sowing system
Rubble and other waste material in the ground may easily block the sowing mechanism.



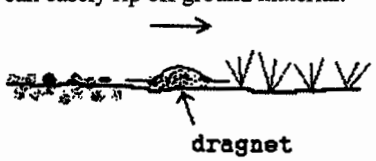
Problem: blockage of the sowing machine

Problem: sowing slits
Wide sowing slits have to be filled up because of the UV-light.



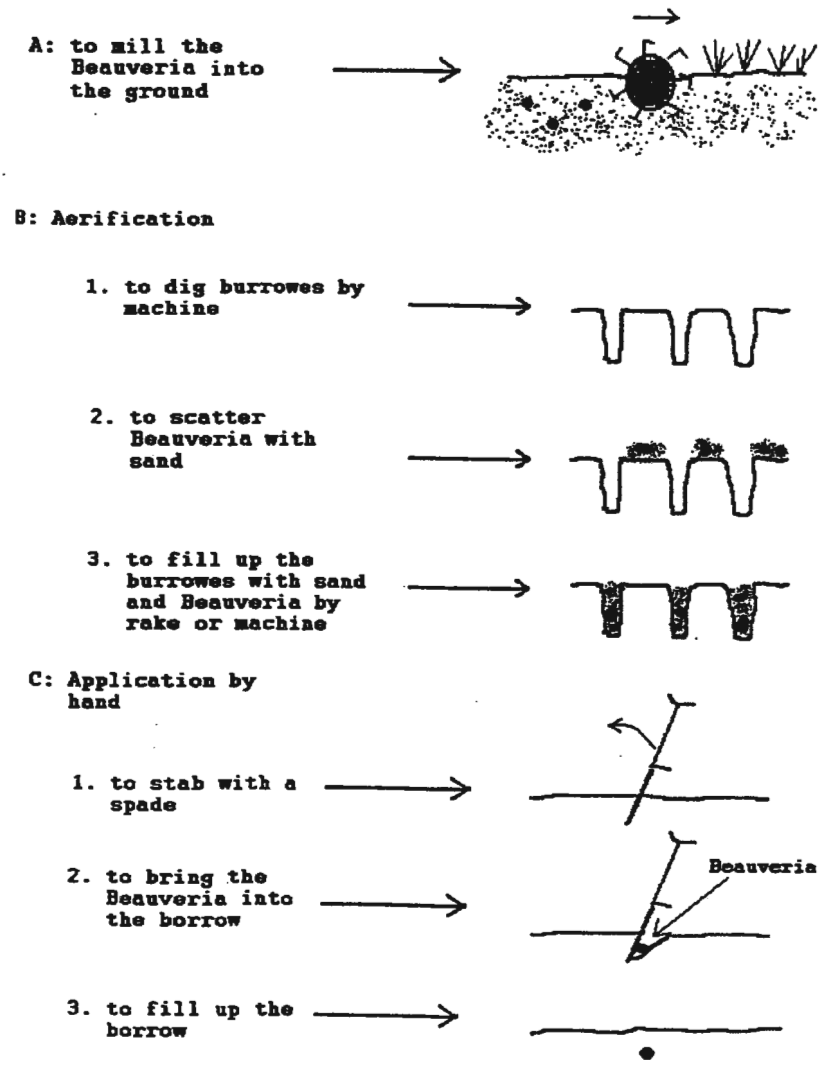
Problem: sowing slits too wide

Problem: dragnet
The problems of dragnets: They can easely rip off ground material.



Problem: dragnet

Special problems with house gardens and small areas
Big machines don't fit through gateways. The treatment of small and irregular areas with big machines is difficult.



Steep slopes in the mountainous areas
Steep slopes often have access problems. Also, big machines may be unstable. Such locations can spread over very large areas. Another problem is the transport of the product and the machine in cable railways.

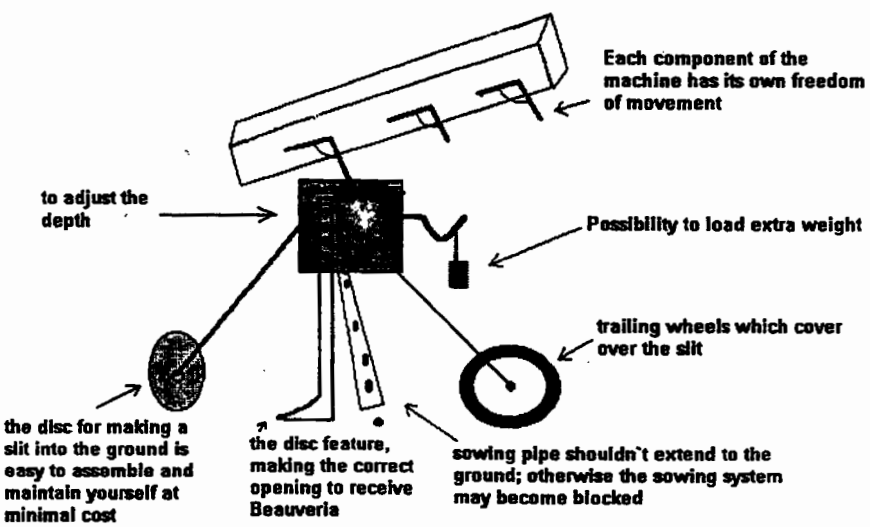
Design intention: Easy asssembly and dismantling of the machine;
Transport of the components in a backpack or in a vehicle boot;
Fitting of the components on difficult terrain

Application at hillside locations: Application always downhill

Application on flat areas: Installation of weight on front of the machine

By applying *Beauveria* with machines, you always have to watch that the components are installed correctly. As a result, there is proper control of the delivery of the product, and also the *Beauveria* reaches 8 - 10 cm into the ground. You can check this with a knife or a spade.

Principle of a sowing machine for Beauveria



Beauveria is not a product with an "knock-out-effect' 'like other chemical products are. It works after 3-4 weeks. On treated areas, you have to pay attention early to reduce the loss of grass by sowing a seed. Sowing seed early is not only preferable for the agriculture and aesthetics, but also to prevent erosion, at least at hillside locations.

Integrated Control of Soil Pest, subgroup "Melolontha"
IOBC wprs Bulletin Vol. 23 (8) 2000
pp.93 - 97

Progress Report on the Registration of *Beauveria brongniartii*

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Abstract: The biological plant protection agent MELOCONT® fungus-barley was developed by the Institute of Microbiology, Leopold-Franzens University Innsbruck, for the biological control of cockchafers (*Melolontha melolontha*). In spring 1998, the Austrian company F. Joh. Kwizda GmbH, who commercializes the biocontrol agent, submitted the bioinsecticide for registration. In 1998 MELOCONT®-Pilzgerste was temporarily authorized for application on the petition § 13 of the Austrian plant protection legislation (PMG/1997) - "authorized in danger of delay". The registration process is still in progress. However, the comments made by the Austrian authorities lead us believe that the bioinsecticide will be registered in 1999.

Key words: *Beauveria brongniartii*, microbial control, microbial product, registration

State of the Art

In April 1998, *Beauveria brongniartii*, a successful antagonist against the cockchafer has been filed for registration in Austria. The mitosporic fungus *Beauveria brongniartii*

Tab.1: Information regarding registration of *Beauveria brongniartii* in Europe

Trade name / Distributor	Country	Registration number	Date of approval
Betel® Natural Plant Protection (Calliope)	France	93 00094	1995
Beauveria -Schweizer® Eric Schweizer GmbH	Switzerland	W4574	1990
Engerlingspilz® Andermatt Biocontrol GmbH	Switzerland	W 4573	1990
MELOCONT®-Pilzgerste: F.J. Kwizda GmbH	Austria	2582	1998 (only for 4 months)

has been registered in Switzerland, and conditionally, in France (under the old synonym *Beauveria tenella*, Tab 1.). Based upon laboratory and field examinations the Austrian company F. Joh. Kwizda GmbH submitted the host specific insect killing fungus *Beauveria brongniartii* against the cockchafer (all stages of development) for registration in accordance with § 10 PMG (BGBL. I, Nr. 60/1997). Furthermore, with the request for registration, which

was formulated according to the EU guideline 91/414/EEC Annex II and III - Part B, the petition "authorization in danger of delay" in accordance with § 13 PMG (BGBL. I Nr. 60/1997) was filed simultaneously. This was necessary because extensive damage is to be expected for this and the following year in the cockchafer infested areas Inn valley (Tyrol), Drau- and Gail valley (Carinthia) and in the Rhine valley (Vorarlberg), and no other effective means of control exists. MELOCONT® - Pilzgerste has been authorized for application on the petition § 13 Austrian plant protection legislation (PG/1997) from 1st June 1998 to 30th September 1998, so the official use of *Beauveria brongniartii* has been realized in an EEC-member state.

Requirements for the registration of the biocontrol agent

- Can *Beauveria brongniartii* be classified as an "old" active substance?

Even mycologists have problems with the systematics of Beauveria species. For instance, there are different opinions concerning the seperation of the two taxa *Beauveria bassiana and B. brongniartii.* To this day the name *B. tenella*, a synonym of *B. brongniartii*, is used for registration as a biocontrol agent although only three species of Beauveria have been defined (Domsch & Gams 1980). Because the Austrian Beauveria production strains cannot be unambiguously assigned to one species, experts in whole Europe were asked for help (DOC 7846/VI/97). It was the opinion of the working group on plant protection products (European Comission DG VI) that based on the chemotaxonomic studies carried out on DNA, *B. bassiana* and *B. brongniartii* have to be considered as a "collective" species. However, the morphological differences and the specific quality of the host lead to consider the two species as separate. Since France has authorized both Beauveria strains, the Austrian authorities decided to grade *B. brongniartii* as an existing active substance.

The registration procedure is based on the national Austrian plant protectant legislation (PMG), which orients itself on the EEC directives 414/91 (Tab. 2). Despite the unsatisfactory discussion, no EU-member state raised objections against the Austrian arguments.

The registration procedure will be executed by the Austrian legislation - B. brongniartii is an "old active substance"!

Tab. 2: Registration of MELOCONT® - Pilzgerste in accordance with the Austrian and European plant protectant legislations:

- BGBl. I Nr. 60/1997 Austrian Plant Protection Legislation (§10 and §13)
- EU-Directive 91/414/EEC Annex II and Annex III - Part B: Commission of the European Communities - Directorate-General for Agriculture
- EU - Administrative Order 2092/91: Annex II, Part B: List of plant protection products
- Administrative Order Nr. 1488/97 of the Commission: Annex II, Part B: II: Microorganism and biological control agents "Microorganisms: (bacteria, "virus" and fungi)" - genetic modified organisms are excluded.

• *The following Austrian authorities are involved in the national registration procedure:* Austrian Federal Ministry of Agriculture and Forestry (assisted by the Federal Research Institute for Agriculture in Alpine Regions, Gumpenstein; Federal Office and Research Centre for Agriculture; Federal Office of Agrobiology, Linz); Federal Environment Agency - Austria and Bundeskanzleramt - Section VI (former Federal Ministry of Health).

From the beginning of our research work all officials (see above) were involved to define the requirements for a successful registration of the bioinsecticide MELOCONT®. Additionally, to guarantee the use of fungal colonized barley in organic farming on the basis of EU regulation 2092/91, the following offices and departments were contacted and asked to make a statement concerning the use of the biological plant protection method in accordance with § 13 PMG from "ecological agriculture" point of view: The Federal Ministry of Agriculture and Forestry, Austria Bio Garantie, Biocontrol Tirol, Federal Section of Österreichischer Ernteverband and Ernteverband Tirol. At present there are three written statements by Austria Bio Garantie, Biocontrol Tirol and Ernteverband Tirol available which did not put forward an objection concerning the use of the biocontrol agent. Also BMLF and the Federal Section of Austrian Harvest Association welcomed the biological plant protection method and confirmed the conformity with the EU regulation.

Based on the results of our laboratory and field experiments, expert authorities do not doubt the efficacy of the bioinsecticide. (Tab. 3).

Tab. 3: Laboratory and field-studies conducted to achieve the goal for registration

THREE YEAR FIELD STUDY IN KRAMSACH/TYROL (SINCE 1994)
- Determination of *M. melolontha* infestation
- MELOCONT® application: 50 kg per ha and year
- Evaluation of *B. brongniartii* density twice a year
- Study of vertical distribution of *B. brongniartii* in soils
- Assessment of autochthonic microorganisms density in treated soils
B. BROGNIARTII - PRODUCTION STRAINS:
- Virulence control (Bioassays)
- Morphological and physiological characterisation
- Optimization for mass production (scale -up; formulation)
- Quality control (storage stability, shelf life, colonization grade of barley kernels)
RISK ASSESSMENT
- Secondary metabolites (estimation in different substrata)
- Biosaftey tests (plant resistance testings; leaf dip assay - to study repellent / antifeedant acitivity, proliferation assays - to study the cytotoxic activity

However, it was clear that there would be less information on toxicological and environmental fate and behaviour of the fungus itself and its secondary metabolites excreted into the environment. Therefore, we were focused to meet the following registration criteria defined in the EEC diective 414/91 (Tab. 4):

Tab. 4: Request for microbial insecticides: "Critical evaluation of mycotoxins" defined in 91/414/ EEC Directive - Plant Protection products

ANNEX II
2.7: Information on the production of secondary metabolites (esp. toxins)
4.4: Methods to determine and quantify residues (metabolites, toxins); Analytical methods: have still to be developed!
5.1: Step I - Basic studies (acute toxicity, pathogenicity and infectivity)
7.: Fate and behaviour in the environment
ANNEX III:
5 : Analytical methods of relevant metabolites
7: Step I - Basic acute toxicity studies (cell culture and animal bioassays)

Many questions concerning the risk assessment of microorganism and its "mycotoxin" have sufficiently been answered (Strasser & Schinner, 1996; Strasser et al. 1996; Strasser et al. 1998, Strasser, 1998 and Abendstein & Strasser, 1999). However, there are still a few issues which have to be studied in more detail in the future. The expert authorities assert the need to do some more new testings, which can lead to delays in putting the needed biological control product on the market.

The following statements have been made, which show that additional information has to be provided, in order to meet the "standards" the authorities require for registration (extract of remarks):

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

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

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

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

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

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

Prospectives

It has to be stressed again that with respect to the effects of *Beauveria brongniartii*, the fungus ought to be recognized as an effective and safe natural prevention agent. There is a need of scientists, industry and usres to stay in permanent contact with the authorities. The goal is to finalize the registration process immediately and to not delay the marketing of this suitable control agent. For the near future we have to realize a harmonization of the regulation by streamlining the regulatory pathways to commercialization of biological products (Panetta, 1992). Many experts know that a great deal could be learned about health and environmental safety of microbial insecticides if more products are commercialized. Hopefully, we can formulate a policy on the release of microbial biocontrol agents. In this sense MELOCONT®-Pilzgerste should become registered as soon as possible.

Acknowledgement

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Considerations on "toxic" metabolites produced by *Beauveria brongniartii*

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Abstract: In Austria the registration of *Beauveria brongniartii* colonized barley kernels as plant protectant is endeavoured by the Austrian company Kwizda GmbH (product name: Melocont®-Pilzgerste). Although this control agent has been deployed sucessfully, more information on toxicological and environmental effects of the product is needed. As *B. brongniartii* is known to synthesize the secondary metabolites beauvericin, bassianin, tennelin and oosporein, the presence of those metabolites on different substrata was investigated. Oosporein was the only fungal secondary metabolite that could be detected in submerged culture (max. 0.27 g/l), on the final product Melocont®-Pilzgerste (max. conc. 3.2 mg/kg) and in cockchafer larvae (*Melolontha melolontha*) infected by *B. brongniartii* (max. 0.23 mg/larva). Furthermore, the adsorption and accumulation of fungal secondary metabolites was estimated in the indicator plants *Lepidium sativum* and *Phleum pratense*, which were grown on soil treated with the prospective bioinsecticide. Additionally, cytotoxic repellent as well as antifeedant activity of oosporein was examined. Based on the results of our investigations the matter of fungal secondary metabolites is discussed.

Key words: *Beauveria brongniartii*, production, metabolites, toxins, registration

Introduction

Beauveria brongniartii, a host specific entomopathogenic fungi, is known to be an effective organism in the biological pest control of cockchafers (*Melolontha melolontha*). Several experimental and commercial applications of *B. brongniartii* on cockchafer infested areas in France, Switzerland, Italy and Austria have been performed successfully. The complexity of interactions between the fungal pathogen and the insect host make it difficult to distinguish between impacts of particular virulence factors, but it is conceivable that mechanical destruction and enzymes as well as fungal primary and secondary metabolites are involved in the mycose (Gillespie & Claydon, 1989). However, the particular role of fungal secondary metabolites in pathogenicity is still not identified.

Fungal secondary metabolites are defined as low molecular substances, which are not used for anabolic and catabolic processes by the fungus (Vinning, 1990). Entomopathogenic fungi are known to produce various secondary metabolites with varying toxicity in submerged culture (Tab. 1). Therefore, detailed toxicological data on those substances are required for the registration of entomopathogenic fungi as bioinsecticides. In Austria it is endeavoured to register *B. brongniartii* colonized barley kernels as plant protectant agent by the Austrian company Kwizda GmbH (product name: Melocont®-Pilzgerste). As *B. brongniartii* is known to synthesize the secondary metabolites beauvericin, bassianin, tennelin and oosporein, we investigated the presence of those substances on several substrata. The release of those metabolites in submerged culture and on the final product Melocont®-Pilzgerste on three

B. brongniartii strains was determined through HPLC-analysis. Furthermore, the absorption and accumulation of fungal secondary metabolites was estimated in *B. brongniartii* infected cockchafer larvae and in the indicator plants *Lepidium sativum* and *Phleum pratense*, which were grown on soil treated with Melocont®-Pilzgerste. In order to estimate the toxicological potential of oosporein, the cytotoxic activity towards mamalia-cells and the repellent as well as the antifeedant activity of larvae of the diamondback moth (*Plutella xylostella*) and of the mustard beetle (*Phaedon cochleariae*) were investigated.

Tab. 1: Secondary metabolites of some entomopathogenic fungi

Fungi	Secondary metabolites
Cordyceps militaris	Cordycepin
Beauveria bassiana	beauvericin, beauverolide, bassianolide, bassianin, tenellin, oosporein
Beauveria brongniartii	beauvericin, beauverolide, bassianin, tenellin, oosporein
Entomophthora virulenta	azoxybenzenoid-compounds
Fusarium solani	fusarubrine, javanicin, fusaricacid
Metarhizium anisopliae	destruxine, cytochalasine
Tolypocladium cylindrosporum	Tolypin
Tolypocladium inflatum	Tolypin
Verticillium lecanii	bassianolide, dipicolinicacid

Material and methods

Formation of secondary metabolites

For submerged culture *B. brongniartii* was cultivated in a 14 l - stirred tank reactor with Sabouraud-2%-glucose media. *B. brongniartii* colonised barley kernels were produced in a modified way according to the procedure of Aregger-Zavadil (1992). Indicator plants *L. sativum* and *P. pratense* from a "Linzer Substrat-Test" and *B. brongniartii* infected larvae (all instar stages) were analysed for their possibility to accumulate fungal secondary metabolites.

Secondary metabolites were extracted with ethylacetat (El Basyouni & Vinning; 1968, modified) and detected through HPLC-analysis (Abendstein *et al.*, 1998).

Cytotoxicity of oosporein - proliferation assay

The cytotoxic activity of oosporein was estimated with a proliferation-assay (MTT-assay) with two different cancer cell-lines (leukemia-cells HL 60 and GTB). Cells were incubated with oosporein (0.01; 0.05; 0.1; 0.5; 1 and 2 µM). Soluble 3,4,5-dimethylthiazol-2,5-diphenyl-tetrazoliumbromid (MTT) was added. In contact with viable cells MTT precipitates to insoluble formazan. Formazan is dissolved in DMSO. The quantity of formazan - as an indicator of the cytotoxic activity of a substance - was estimated through photometric analysis (multiwell spectrophotometer, Elisa plate reader).

Repellent/ antifeedant activity of oosporein - leaf dip assay
The leaf dip assay is a standardized test to check substances for their repellent and antifeedant properties. Cabbage leaves (Ø 1.5 cm) were dipped in a oosporein-solution (1 and 2 mg/ml; solved in aceton/methanol) and dried. Reference leaves were only dipped in aceton/methanol. Moisted filter-paper with 2 oosporein leaves and 2 control leaves were put into a petri-dish. Larvae of *P. xylostella* and *P. cochleariae* were put into the middle of the test-system. Repellent activity of oosporein was permanently detected by observing the behaviour of the larvae. The aimed removal of the larvae from the oosporein treated leaves indicates an repellent activity of oosporein. Furthermore, the feed-behaviour of the insects was estimated after 24 and 48 hours.

Results

Formation of secondary metabolites
Oosporein was the only secondary metabolite which was accumulated in submerged culture, on Melocont®-Pilzgerste and in the cockchafer larvae. No accumulation of fungal secondary metabolites was observed in the two indicator plants (see Materials and methods).

The amounts of oosporein in submerged culture ranged between 30 and 240 mg/l. The submerged cultute was used as inoculum for the production of Melocont®-Pilzgerste (40 ml per kg of barley kernels). Therefore, one kg of Melocont®-Pilzgerste was contaminated with 1.2 to 9.6 mg of oosporein after inoculation. In the final product Melocont®-Pilzgerste, which was applicated in the field (50 kg/ha), an oosporein concentration of 2.0 to 3.2 mg per kg was detected (Fig. 1). Based on this result an enrichment of oosporein between one to two ng per m^2 soil can be expected (Fig. 2). Additionally, a natural oosporein accumulation occurs in the soil, when larvae are infected by *Beauveria*. Analysing *B. brongniartii* mummified larvae small amounts of oosporein (0.03 to 0.23 mg/larva) could be observed. Refering to those results a maximum enrichment of 18.4 mg of oosporein per m^2 of soil can be postulated, when 80 larvae per m^2 are infected (Fig. 3).

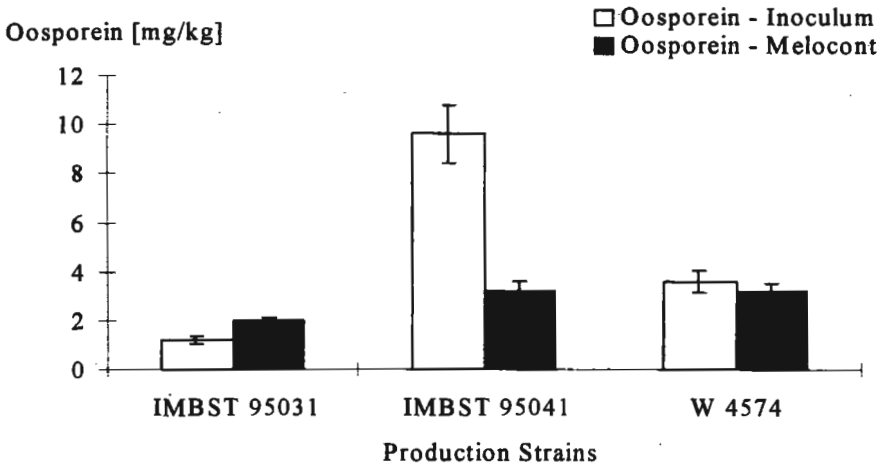

Fig. 1: Oosporein accumulation by three *Beauveria brongniartii* strains (IMBST 95031, IMBST 95041 and W4574) in submerged culture broth and in the final product Melocont®-Pilzgerste.

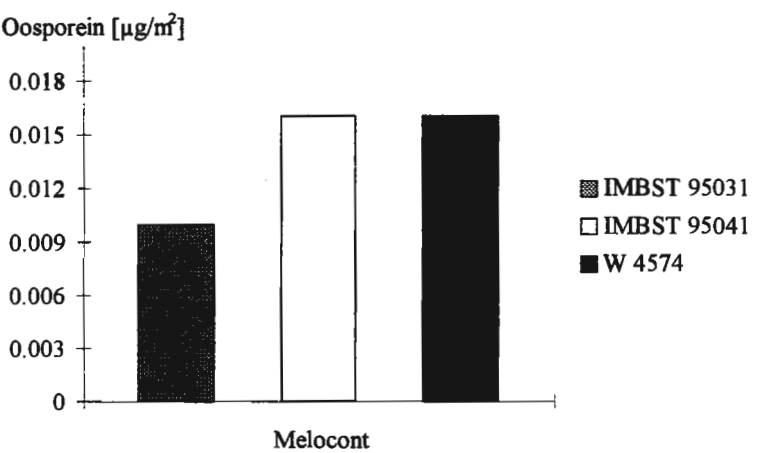

Fig. 2: Oosporein-enrichment per m^2 soil after application of Melocont®-Pilzgerste (50 kg/ha). The bioinsecticide was produced with three *Beauveria brongniartii* strains (IMBST 95031, IMBST 95041 and W4574).

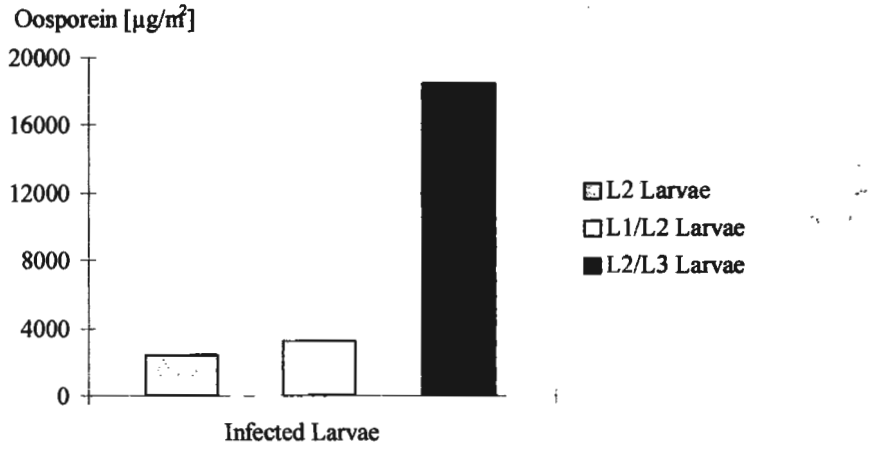

Fig. 3: Estimation of the oosporein enrichment in soil, when 80 *Melolontha melolontha* larvae per m^2 (L2, L1/L2 and L2/L3) are infected by *Beauveria brongniartii*.

Cytotoxicity of oosporein

Table 2 shows that the cytotoxic activity of oosporein was at any concentration lower than the inhibtiory effect of Helenalin and the DMSO-control, respectively. Regarding the two types of mamalia-cells oosporein is not a cytotoxic compound.

Tab. 2: Cytotoxic activity of oosporein and helenalin to leukemia cells HL 60 and GTB

Test-system	Substance	Inhibition [%]					
		0.01 µM	0.05 µM	0.1 µM	0.5 µM	1 µM	2 µM
HL 60 cells	oosporein	n. i.	n. i.	n. i.	n. i.	n. i.	1
	DMSO-control	5	2	8	10	2	11
	Helenalin	10	4	5	73	100	100
GTB cells	oosporein	10	13	13	6	15	17
	DMSO-control	22	30	13	3	2	0
	Helenalin	22	7	16	100	100	100

n. i. = not inhibited

Repellent / antifeedant activity of oosporein

Oosporein showed no repellent activity neither to *P. xylostella* nor to *P. cochleariae* larvae. Futhermore, oosporein seems to have no antifeedant activity to these insects. There was no difference in the feed-behaviour of the larvae between the oosporein treated leaves and the

control leaves. In comparison, destruxin E - a metabolite synthesized by *M. anisopliae* - shows an antifeedant activity at a concentration of 0,1 mg/ml (Amiri *et al.*, 1998).

Conclusion

Entomopathogenic fungi are efficient and important natural control factors for pest management. The assessment of metabolites synthesized by entomopathogenic fungi is necessary in order to estimate toxicological effects to the environment by the application of fungal bioinsectides. Oosporein was the only secondary metabolite that could be detected in submerged culture, on the final product Melocont®-Pilzgerste and in infected larvae.

Presently, scientific opinion on specific fungal "toxic" metabolites of the known entomopathogenic fungi is changing. They are no longer considered as toxins in the meaning of mycotoxins in food and fodder such as aflatoxins, citrinin, ergot, patulin and zearalanone, which are known to cause serious health damages. On the contrary, fungal secondary metabolites are considered to be "bioactive substances" in pathogenesis. Our toxicological studies do not indicate that oosporein is a toxin in the meaning of mycotoxin. No cytotoxic, repellent or antifeedant activity could be observed. However, preliminary studies have shown that oosporein has specific antimicrobial properties.

Secondary metabolites are meant to support the pathogenic fungus, aside from virulence factors, in knocking down the insect. It is supposed that some of these bioactive substances reduce host-resistance mechanism. For instance *M. anisopliae* synthesizes the immunsuppressing substance destruxin E, which stops the immun response by paralyzing the insectual haemocyts (Hajek & St. Leger, 1994). Because of their antibiotic properties some secondary metabolites are considered to bring the host-specific microflora down (Khachatourians, 1996). In this manner entopathogenic fungus can displace the intestinal flora from their ecological niche and manifest itself without competition in the host.

These observations give us new insight in the estimation of the toxic potential of fungal secondary metabolites. An enhancement of strains which synthesize high amounts of secondary metabolites may guarantee the developement of more effective biological control agents. Furthermore, because of their selective efficacy secondary metabolites seem to be a new source of environment friendly pesticides, which can show repellent, antifeedant or antimicrobial effects. Specifity of secondary metabolites might be improven by chemical modification. The goal for the future is to synthesize compounds, which operate only at a specific target site (e. g. mid-gut, haemolymph).

Nethertheless, investigations are needed to get more information about the toxicological potential of secondary metabolites. Fungal metabolites should offer a broad new field of bioactive substances in the near future.

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Estimating chitinase activity of *Beauveria brongniartii* in submerged culture

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Abstract: In order to characterize virulent *Beauveria brongniartii* strains in submerged cultures, the activity of the chitinolytic system (chitinase and N-acetyl glucosaminidase) as well as the alternative degrading path of chitobiose to glucosamine were examined. To accomplish this the monomer components of the remaining chitin substratum - N-acetylglucosamine (HPLC) and glucosamine (photometry) - were measured and compared including the strain specific mortality rate (standardized biotest with *Melolontha melolontha* larvae). A possible connection between the virulence and an increased activity of the chitinolytic systems of the entomopathogenic fungi isolates could be demonstrated.

Key words: *Beauveria brongniartii*, enzymes, virulence

Introduction

The entry of entomopathogenic fungi through the insect cuticle is considered to occur through a combination of mechanical pressure and enzymatic degradation (Charely, 1984). Up to now the relative importance of the two mechanisms is not known. Studies on aggressive entomopathogenic fungi have shown that three enzyme systems - such as chitinase, lipase and protease - are released by the germ tube during perforation of the insect cuticle.
The role of the enzymes may not only be limited to degrade the cuticle, but also to liberate monomers that can be metabolized by the germ tube in order to continue to grow into the host. It was also reported, that chitinase-negative and lipase-negative strains of *B. brongniartii* were not able to infect *M. melolontha* (Paris & Ferron, 1979). These facts focused our interest on studying the chitinolytical degrading enzyme system of *B. brongniartii* by detecting - chitinase and chitobiase. The goal of our research was to establish methods of identifying the chitin degrading enzymes by estimating those enzyme degrading products [glucosamine; N-acetylglucosamine (NAG)] as specific monomers in the supernatant of the culture broth, when chitin was the only carbon-source. Furthermore, the strains were checked for their virulence against *M. melolontha* larvae with a standardized biotest.

Material and methods

Beauveria brongniartii

The tested Beauveria production strains (V10 and V11) were reisolated with a modified isolation technique from *M. melolontha* infested greenland in Kramsach / Tyrol (Austria). In this study both strains were grown from single conidia stem cultures, which were cultivated on chitin-peptone nutrient media (pH 6,8) at 25°C and 80% RH.

Shake-flask experiments

B. brongniartii strains were cultivated in 100-ml Erlenmeyer-flasks containing 20-ml nutrient solution, where chitin as sole carbon source was added. The nutrient solution consisted of the following (g l^{-1}; Rechcigl 1978, modified): 20 Chitin (Practical grade, Sigma); 1 $NH_4 NO_3$; 1 $KH_2 PO_4$; 0,5 $MgSO_4 * 7 H_2 O$; 0,1 NaCl; 0,13 $CaCl_2 * 2 H_2 O$; 0,5 ml trace element solution; pH 6,8.

With the exception of the control group, the culture media were inoculated at a concentration of $5 * 10^5$ spores per ml. The flasks were cultivated at a temperature of 25°C on a gyratory shaker at 200 rpm for 16 days. The supernatant of the culture broth was measured after 0, 1, 2, 4, 8 and 16 days. For sampling three flasks of each strain were taken.

Standard-biotest

The *B. brongniartii* strains were examined for their virulence by bioassaying against *Melolontha melolontha* - larvae (second and third instar). The inoculation of the larvae took place with homogenous grown mycelium-agar rondell (8 * 10 mm) of both strains and incubated at 20 °C since all larvae were killed. For each strain 15 larvae were checked out. The larvae were fed carrot pieces once a week.

Analytical methods

Estimation of glucosamine (Sawicki et al. 1961, Ride & Drysdale 1972) modified:

The solved glucosamine and chitosan in the supernatant were desaminated with $NaNO_2$ and $KHSO_4$ and split to 2,5 - anhydromannose. The aldehyde of these anhydrosuggar reacts with MBTH (3-methyl-2-benzo-thiazolinon-hydrazon, Sigma) and $FeCl_3$ to a blue colour complex (λ_{MAX} = 650 nm) which can be detected photometrically (Beckmann DU 50, programme Nr. 6, Modul Quant II).

Reference: D(+)glucosamine hydrochloride (Sigma) dissolved in aqua dest.. The reference solution was 1 : 3 diluted with culture broth (see above, without chitin).

N-acetylglucosamine

The production of N-acetylglucosamine was determined by HPLC (column: AMINEX-HPX-87-H-cation exchanger with micro guard cation H+ precolumn; mobile phase, 4 mN H_2SO_4; flow rate 0.5 ml min^{-1}; temperature, 30 °C; UV-detector at 213 nm (range: -0,004; + 0,036). Reference: N-acetyl-D-glucosamine (Fluka) dissolved in aqua dest.

Results and discussion

Both Beauveria strains showed a high chitinolytic enzyme activity in submerged culture. A good utilization of chitin, as the sole source of carbon, through the fungus could be detected by demonstrating chitin-monomer N-acetylglucosamine (NAG) and glucosamine in the remaining substratum (fig. 1 and 2).

By increasing the duration of incubation over a testing period of sixteen days a continuous increase of the glucosamine concentration for the isolates V10 and V11 were determined. The highest concentration of glucosamine was found in strain V10 at 0.25 mM and in Strain V11 at 0.21 mM after sixteen days. The NAG monomer concentration of strain V10 increased during the first eight days to a concentration of 0.2 mM and 0.42 for strain V10, respectively. After that, however, a NAG decrease was determined for both isolates with 0.18 mM for V10 and 0.3 mM for V11 on the sixteenth day of experimentation.

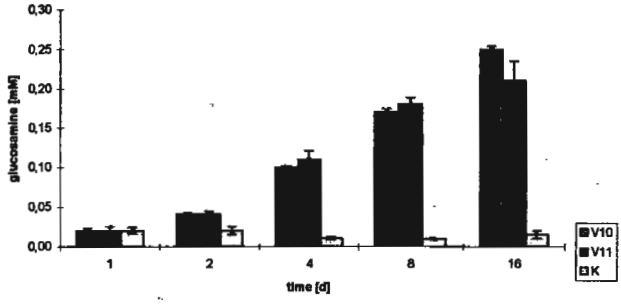

Fig. 1: Evaluation of the glucosamine - concentration [mM] of *Beauveria brongniartii* - isolates V10 and V11 over a duration of 16 days.

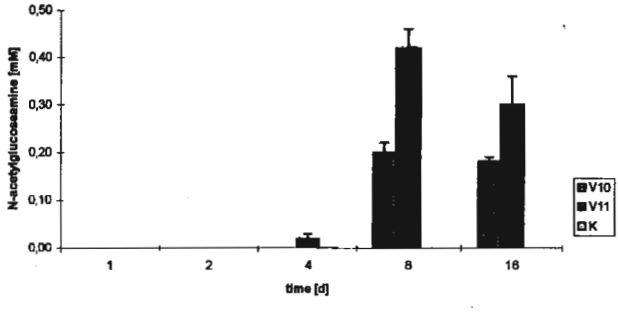

Fig. 2: Evaluation of N-acetylglucosamine - concentration [mM] of *B. brongniartii* - isolates V10 and V11 over a duration of 16 days.

Both strains showed a high mortality rate against *M. melolontha* while testing the strains in a standard biotest (Fig. 3 and 4). Die LT_{50} (50 % mortality rate) was reached for strain V10 after 12 to 13 days and after 9 to 10 days for strain V11.

In comparing the results mentioned above, a possible correlation may exist between an increased N-acetylglucosaminidase-, or chitinase-activity (chitinolytical - system) - demonstrated through the monomer NAG in the remaining culture - and an increased mortality of *M. Melolontha*, shown through the standard biotest (Fig. 3 and 4). Two days after the detection of NAG in the remaining culture the mortality of larvae was observed. The virulent Strain V11 activated its chitinolytical system earlier than strain V10 did. According to Samson et al. (1988), this difference in time is closely related to virulence.

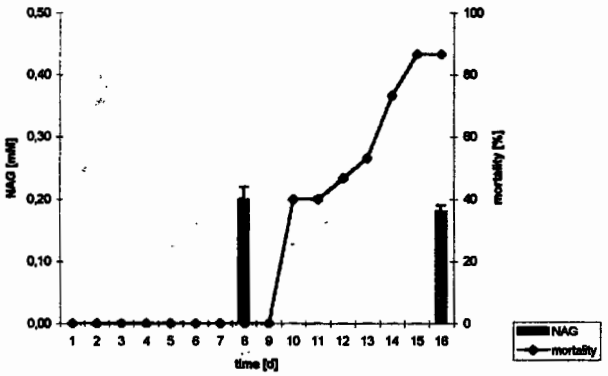

Fig. 3: Mortality rates of *M. melolontha* - Larvae (L2, L3) over the test period with regard to the N-acetylglucosamine - concentrations detected in *B. brongniartii* strain V10.

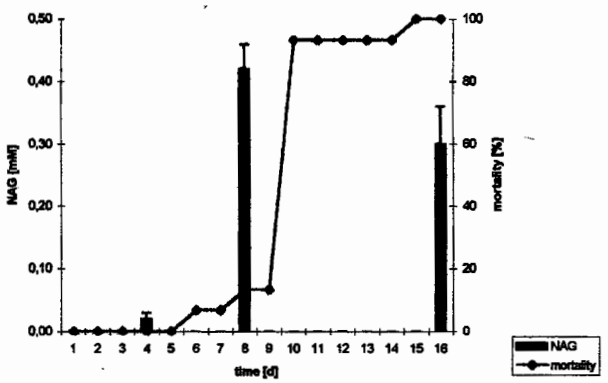

Fig. 4. Mortality rates of *M. melolontha* - Larvae (L2, L3) over the test period with regard to the N-acetylglucosamine - concentrations detected in *B. brongniartii* strain V11.

Readings on the fourth day of testing show a NAG-concentration of 0.02 mM for strain V11 and, in comparison to the biotest, a beginning mortality which is two days delayed of 6.7%. On the eighth day of testing a NAG- concentration of 4.2 mM was measured, and in comparison to the biotest, the mortality rate was 93.3% with a two day delay. Readings of strain V10 show a NAG-concentration of 0.2 mM only on the eighth day of testing. In comparison to the biotest, the mortality rate is 40% after ten days of testing.

This comparison seems to indicate a direct correlation between the detected NAG concentrations, an indirect measure of the beginning of the enzyme activities, and the mortality rates: the faster enzymes appear and the higher the measured NAG concentrations are, the higher the mortality rates seem to be at the given times in the comparison biotests.

A similar connection could not be identified between the measured concentration of glucosamine as an indirect measure for the chitinase-deacetylase-activity and the virulence of a certain test strain. Depending on the location of sampling, the relationship of the amount of glucosamine concentration is more or less the same in both test strain systems. At the beginning of the test up to 0.02 mM glucosamine was released in the culture broth because of the autoclave process. Comparing these initial rates with the detectable ratios in the flasks, the enzyme was active after two days.

Conclusion

With the detection of NAG, a high activity of the chitinolytic enzyme system could be shown for *B. brongniartii*. Those enzymes could be interpreted as key enzymes to identify virulent fungal strains. Further investigations with avirulent and virulent isolates should confirm this positive correlation concerning virulence and chitin degradation. With the help of this information screening test-kits for virulence control should be developed.

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Integrated Control of Soil Pest, subgroup "Melolontha"
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Oosporein, a fungal secondary metabolite with antimicrobial properties

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Abstract: Oosporein is described as a metabolite, showing a demonstrable effect on gram-positive, but only little effect on gram-negative bacteria. Fungizid efficacy could be observed on *Fusarium proliferatum, Phytophtora infestans* and *Mucor* sp.. However, detailed studies on the antimicrobial potential of oosporein are still missing. Therefore, our interests are focused on this substance, in order to find a new specific fungizide as alternative to current chemical pesticides.

Key words: *Beauveria brongniartii*, metabolites, oosporein, toxin

Introduction

Seed treatment is necessary to protect seedlings from fungal diseases or insect pests. Currently, the agricultural industry is confronted with the problem that fungicides have to be phased out because of their inefficacy against target pathogens. Therefore, new alternatives of fungicides are demanded, which guarantee a safe agricultural production of healthy food for man and animals.

Beauveria brongniartii production strains IMBST 95031, IMBST 95041 and W4574 synthesize the secondary metabolite oosporein which shows fungicide efficacy (Abendstein & Strasser, 1999). The aim of our research is to characterize and improve oosporein as a potential fungicide and/or insecticide for seed production industry.

Characterization of Oosporein

Oosporein can be classified as follows:

- red coloured dibenzoquinone, synthesized by many fungi e.g. *Acremonium* sp., *B. bassiana*, *Chaetomium aureum*, *C. trilaterale*, *Oospora colorans*, *Phlebia mellea*, *P. albida* and *Verticillium psalliotae*
- polymorph with variable physical and chemical properties (melting point between 245°C and 300°C, varying solubility in polaric solvents, etc.)
- oxidizes proteins and aminoacids by changing SH-groups, which causes malfunctions of enzymes (e.g. carboxylase, katalase and lipooxidase)
- damages mitochondria and inhibits erythrocyte membrane ATPase activity
- antibiotic effect on gram-positive bacteria like *Staphylococcus aureus* and *Bacillus subtilis*, but little effect on gram-negative bacteria described
- fungicidal effect observed on *Fusarium moniliforme*

Research Activities

Oosporein was extracted from culture broth of *B. brongniartii* after 4 and 7 days, respectively. The metabolite was checked for its purity by HPLC, ESI/MS and X-ray diffractometry (Strasser et al., 1998). Preliminary studies showed a specific fungicide activity against *Fusarium proliferatum*, *Phytophtora infestans* and *Mucor* sp. (Tab. 1).

Following these results, oosporein and several derivatives of oosporein will be investigated for their fungicide effects on common phytopathogenic fungi such as *Fusarium* spp., *Tilletia* spp. and *Botrytis* spp.. Presently, the phytotoxic potential as well as antibiotic and cytotoxic activities of these metabolites are characterized.

Tab. 1: Fungicide activity of oosporein, a secondary metabolite synthesized by *Beauveria brongniartii*

Fungi	Description	Oosporeinconcentration [mg/ml]			
		0.1	1	2	control
Aspergillus ochraceous	saprophytic	-	-	-	-
Cladosporium sp.	saprophytic	-	-	-	-
Coriolus sp.	saprophytic	-	-	-	-
Coriolus versicolor	saprophytic	-	-	-	-
Fusarium moniliforme	saprophytic	-	-	-	-
Fusarium proliferatum	saprophytic	-	+	+++	-
Mucor sp.	saprophytic	-	++	+++	-
Poria placenta	saprophytic	-	-	-	-
Sporobolomyces roseus	saprophytic	-	-	-	-
Beauveria bassiana	insectpathogenic	-	-	-	-
Beauveria brongniartii	insectpathogenic	-	-	-	-
Metharizium anisopliae	insectpathogenic	-	-	-	-
Paecilomyces fumosoroseus	insectpathogenic	-	-	-	-
Phytophtora infestans	phytopathogenic	-	+++	+++	-

Legend: + ... inhibited by oosporein - ... not inhibited by oosporein
control ... aceton/methanol/water solvent

Prospects

The large number of fungicide-resistant pathogens shows the need for the development of specific designed seed-dressings. Because of its properties (e.g anti-microbial efficacy, polymorphism, solubility) oosporein could be a new useful, ecologically beneficial plant protectant for the design of appropriate formulations. Beside oosporein other fungal secondary metabolites should be screend for their fungicide potential in the near future.

Acknowledgement

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Miscellaneous

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Melolonthine pests of sugarcane in Australia: a history of research and management

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Abstract: Canegrubs, the larvae of melolonthine scarabs, are the most important pests of sugarcane in Australia. Eighteen endemic species occur in sugarcane, but differ in their distribution and importance as pests. Staff of the Bureau of Sugar Experiment Stations (BSES), the principal research organisation for the sugar industry in Australia, began a program of research on the biology and control of canegrubs in 1911. Research from then until the mid 1940s was focused on the biology of canegrubs and their natural enemies and insecticides. A number of biological control agents were introduced but were not effective. In 1946, benzene hexachloride (BHC) was found to be a cheap and very effective insecticide for canegrubs. Following its commercial introduction in 1947, research in the next three decades was essentially limited to defining the use of BHC in sugarcane and investigating new insecticides. Controlled-release technology for insecticides was developed in the 1980s and led to the introduction of suSCon® Blue, the most widely used insecticide in Australian sugarcane today. Research in the 1980s was initiated on pheromones and *Metarhizium*. In 1987, the use of certain organochlorines in sugarcane was banned. Research on canegrubs since 1987 includes: (1) canegrub biology; (2) varietal tolerance of sugarcane to canegrub damage; (3) the introduction of novel genes to sugarcane to provide resistance to canegrubs; (4) the investigation of alternative farming systems and their effect on canegrubs; (5) the development of *Metarhizium* as an insecticide and the effect of other diseases on canegrubs; and (6) further work on insecticide formulation, application and new active ingredients. BSES remains the principal research provider for canegrubs with other organisations such as CSIRO and insecticide companies often involved in collaborative projects.

Introduction

Sugarcane is a semi-perennial crop, harvested annually for 3-6 years or more before being ploughed in and replanted. Most Australian sugarcane is grown on the subtropical and tropical east coast of Queensland. Sugarcane is milled to produce raw sugar, mainly for export. Australia is one of the largest exporters of raw sugar in the world. The gross value of raw sugar exports from Queensland in 1997 was US$1.15b.

Sugarcane was first grown commercially in Australia in 1864 and in the next 20 years, the industry became established along the coast of Queensland (Bell, 1956). Within 10-20 years of the introduction of sugarcane to many localities, canegrubs, the larvae of endemic Melolonthini, became significant pests (Illingworth and Dodd, 1921). Canegrubs feed on the roots and stool (underground stalk material that produces new shoots) of sugarcane plants, reducing plant vigour, and crop yield and sugar content. Where damage to the stool is severe, the ability of the plant to regenerate and produce subsequent crops (ratoons) may be severely impaired.

Destruction of roots by canegrubs deprives the plant of anchorage in the soil and may result in the inadvertent removal of the stool during mechanical harvesting. The removal of stools at harvest can reduce yield from the subsequent ratoon crop, and also contributes to undesirable levels of soil and other extraneous matter entering mills. In 1997, cane grubs cost cane farmers US$8m in lost production and insecticide. Actual costs to the industry are much greater as indirect costs to cane farmers of reduced yield in subsequent ratoons and of premature replanting, and costs to millers and harvesting contractors have not been calculated.

Eighteen endemic species of canegrub, belonging to the genera *Antitrogus*, *Dermolepida*, *Lepidiota* and *Rhopaea*, are pests of sugarcane (Allsopp *et al.*, 1993). Species differ in distribution according to latitude and, at a local level, according to soil type. Canegrubs have life-cycles of 1 or 2 years, and two or more species may occur in the same field of sugarcane. Many species are occasional or minor pests, but collectively, canegrubs are the most important insect pests of sugarcane in Australia. The species that cause most damage are greyback canegrub, *D. albohirtum* (Waterhouse), Childers canegrub, *A. parvulus* Britton, French's canegrub *L. frenchi* Blackburn, negatoria canegrub, *L. negatoria* Blackburn and southern one-year canegrub, *A. consanguineus* (Blackburn).

Canegrub research and management can be separated into three more or less distinct periods. Between the 1890s and 1945, significant research occurred on canegrub biology, and biological, chemical and cultural control. However, canegrubs remained significant pests. The second period, 1946-1987, began with the discovery that benzene hexachloride (BHC) successfully controlled greyback canegrub, the most important canegrub pest. Research on canegrubs in this period was largely related to insecticides. The period ended when the major organochlorine insecticides used to control canegrubs, were banned by the Australian government. The third period, 1988-present is characterised by an expansion of research into forms of control alternative to insecticides, while maintaining research on improving the range and efficiency of insecticides. Here we summarise the research and management on canegrubs since 1890s, based on that division into three periods of activity.

Period 1: 1890s-1945

In 1895, the Queensland government entomologist, Henry Tryon, became involved in canegrub research at the request of growers (Tryon, 1896). Significant research began in 1911 with the appointment of the first Bureau of Sugar Experiment Stations (BSES) entomologist to work in this period. BSES was created by statute in 1900 to provide research for the development of the Queensland sugarcane industry, and remains the principal provider of research today.

Seven species of canegrubs were identified between 1912 and 1915 and a further species identified in 1927. The biology of three of the most important species of canegrub, greyback canegrub (*D. albohirtum)*, French's canegrub (*L. frenchi*), and Childers canegrub (*A. parvulus*), was studied, and some information collected on the biology of other canegrub species. Natural enemies of *D. albohirtum* and to a lesser extent other canegrubs, were identified (Illingworth, 1921). Many control measures were trialed, largely without success. They included the destruction of feeding trees, the application of insecticides such as arsenicals to feeding trees, hand-collection of

beetles, light-trapping and the application of chemicals such as potassium cyanide, creosote, borax and arsenicals to the soil (Jarvis, 1923). Some success was achieved with the fumigant carbon bisulphide, sometimes mixed with paradichlorbenzene. Application was by a hand-held injector. It was recommended that fields be sampled prior to treatment to determine the distribution of canegrubs and fumigated when canegrubs occurred at densities of at least three /stool (Knust, 1934). The cost and labour-intensive method of application inhibited widespread adoption (Mungomery, 1949).

There were several attempts at the biological control of canegrubs. The fungus, *Metarhizium anisopliae* (Metschnikoff) Sorokin was cultured by Illingworth (1921) on corn meal and applied against greyback canegrub in a field trial. The trial was inconclusive as it coincided with an epidemic of *Metarhizium* that spread throughout the experimental site. The work ceased with Illingworth's departure from Australia. A tachinid parasite of *Phyllophaga* spp., *Microphthalmia michagensis* Townsend, was imported from Canada and released during 1931 and 1932 for the control of Childers canegrub in south Queensland. The parasites apparently failed to become established as they were never recovered from canegrubs in the field. In 1935, the cane toad (*Bufo marinus* L.) was introduced from Hawaii for the control of canegrubs (Mungomery, 1935). Cane toads were reared and released throughout most cane-growing districts between 1935 and 1937. Although cane toads feed on cane beetles, no control of canegrubs by cane toads has been demonstrated, and the cane toad has since been recognised as a threat to the native vertebrate fauna.

Period 2: 1946-1987

In 1946, BHC effectively controlled greyback canegrub in a field trial (Mungomery, 1949). Subsequent trials determined the optimum rates of application, and placement, and from 1947, BHC was commercially applied to sugarcane to control greyback canegrub. BHC was trialed successfully against *L. frenchi* but less successfully against *A. parvulus* and *L. crinita* (Wilson, 1950). Control of greyback canegrubs in particular, was so successful that in 1956, the Queensland Canegrowers Council presented a bronze plaque to BSES in recognition of "the major service performed to the canegrowers of Queensland in initiating and carrying to fruition the research which led to the successful control of the canegrub.." (Bates, 1957). Other organochlorines (aldrin, heptachlor, chlordane and dieldrin) were applied in trials against canegrubs with success but BHC remained the most effective insecticide. BHC and other organochlorines were relatively cheap, and consequently were applied prophylactically to large areas particularly in central and north Queensland. Wilson (1969) estimated that over half of the harvestable area of sugarcane in north and central Queensland was treated with BHC.

Organochlorines began to lose favour in much of the world during the late 1960s and 1970s. Research into alternative insecticides to control canegrubs began in the 1970s. In the early 1980s, BHC failed to control the canegrubs *A. consanguineus* (Bull, 1986) and possibly *D. albohirtum* (Chandler, 1984). Resistance was suspected but was not confirmed. Heptachlor replaced BHC as the preferred treatment for canegrubs in north Queensland in the early 1980s as a result of concerns that BHC was

losing its effectiveness. BHC, dieldrin and heptachlor were banned for use in sugarcane after October 1987.

Controlled-release formulations of chlorpyrifos (suSCon® 140G and suSCon® Blue) were developed in the 1980s. SuSCon® 140G was registered and first used commercially in 1984; suSCon® Blue was registered in 1985 (Allsopp and Chandler, 1989). Considerable research defined the optimum granule size, application methods and rates against a number of species. The granule is a plastic matrix about 2 mm in length, encapsulating 140 g/kg of chlorpyrifos, which is released slowly into surrounding soil. Chlorpyrifos occurs at lethal concentrations in the soil to most canegrub species during a 3-year period. Both products were readily adopted due partly to the ease of handling and application, low hazard, and in some cases, better control than the organochlorines. Mocap® (100 g/kg ethoprophos), an insecticide with short-term, knockdown effects, was trialed successfully against canegrubs, and was registered for application in ratoon crops to control five species.

There was limited research on the biology of canegrubs and on alternative forms of control during 1947-1987. Eight further species of canegrubs were identified as pests of sugarcane during this period. Research on the potential of *Metarhizium* to control canegrubs began in the 1980s. Strains were isolated from soil and from canegrub cadavers and tested for pathogenicity against a number of canegrub species (Milner, 1992). In the 1984 and 1985, the nematode *Steinernema glaseri* was applied to sugarcane, in small field trials, against *A. consanguineus*, *L. negatoria* and *L. frenchi* (Hitchcock, 1983). No control of *L. frenchi* occurred. Fewer larvae of *L. negatoria* and *A. consanguineus* were recovered from treated plots compared with untreated plots in one year but no effect was found in the second year. No further development of nematodes as biocontrol agents has occurred.

Period 3: 1988-present

The loss of BHC and heptachlor for canegrub control in 1987 has generated significant research on a variety of alternative control measures. Management of canegrubs was recently reviewed, and a research program for integrated pest management (IPM) of canegrubs was outlined (Robertson *et al.*, 1995). The development of IPM of canegrubs consists of three phases: (1) improving the efficiency of current control measures, (mainly synthetic insecticides), (2) substituting more benign insecticides and alternative products for synthetic insecticides, and (3) redesigning the cropping system to integrate a range of cultural, biological and chemical control measures (Robertson *et al.*, 1995). Here we divide the research on canegrubs since 1988 into these three phases (efficiency, substitution, and redesign).

Efficiency

In comparison with BHC and heptachlor, their replacement insecticides, suSCon Blue and knockdown insecticides, are more costly (Bull, 1987) and generally less effective. To target insecticide application more efficiently, or to reduce the need for insecticide application, it is necessary to be able to estimate canegrub abundance. The within row distribution of third instars was defined for a number of canegrub species and a sequential sampling plan developed (Allsopp & Bull, 1989). Pheromones of cane beetles in south Queensland were investigated as a possible tool for monitoring the abundance of beetles, and to eliminate adults. However, attempts to isolate

pheromones from six different canegrub species were largely unsuccessful. Sequential sampling of canegrubs has been used in further research to determine levels of abundance of canegrubs. However, no regular monitoring occurs in commercial sugarcane, partly because sampling to detect canegrubs in sugarcane is physically difficult.

In 1992, control failures with suSCon Blue were reported in the Burdekin district in north Queensland. In the following years, application of suSCon Blue declined despite increasing levels of damage due to greyback canegrub (Fig 1). Analysis of the chlorpyrifos content of suSCon granules and surrounding soil from many sites in the Burdekin and other cane-growing areas showed that accelerated loss of chlorpyrifos had occurred. Surveys linked rapid degradation of the active ingredient chlorpyrifos with its use in the previous crop and the use of lime to improve water penetration of soils. High soil pH (>6.5) is associated with rapid hydrolization of chlorpyrifos, and soil microbes are thought to be involved with rapid degradation of the hydrolysis product (Robertson *et al.*, 1998). The failure of suSCon Blue to control greyback canegrub in the Burdekin, where soil pH is naturally high, led to the removal of its registration in this district. However, the premature degradation of chlorpyrifos can be ameliorated by the application of acidification agents, such as sulphur and ammonium sulphate. Use of suSCon Blue has increased recently in the Burdekin (Fig 1), probably largely due to treatment in newly infested areas. With the success of acidulation, further increase in the use of suSCon Blue may occur in the future.

Substitution

Significant research has occurred on new insecticides and new formulations since 1987. Rugby 100G (100g/kg cadusafos) was registered for the control of *A. consanguineus*, *A. parvulus* and *L. negatoria* (Allsopp & McGill, 1997). Research on alternatives to synthetic insecticides has been on *Metarhizium*, on identifying cultivars of sugarcane tolerant to canegrub feeding, and on introducing non-sugarcane genes that produce canegrub antimetabolites, to selected sugarcane cultivars.

Research to evaluate the potential of *Metarhizium* as a biocontrol agent for canegrubs began again in 1985, after the inconclusive work in the 1910s and 1920s. Isolates from soil and cadavers of canegrubs were tested in bioassays for pathogenicity to several canegrub species. The best isolates were tested in field trials in a variety of formulations. Most trials in the late 1980s and early 1990s were unsuccessful. More recently, the *Metarhizium* isolate, FI-1045, formulated as a rice grain-based granule (BioCane™), successfully controlled greyback canegrubs in field trials (Robertson *et al.*, 1997). BioCane will shortly enter the process of registration as a microbial insecticide and may be available for commercial use in the year 2000. Details of the research and development of FI-1045 are given in the concurrent paper by Logan *et al.*

The potential of tolerance or resistance in sugarcane to feeding by canegrubs is being investigated. A large number of clones (n = 359) were tested for tolerance to feeding by canegrubs and antibiosis of canegrubs (generally *A. consanguineus*) in pot trials (Allsopp *et al.*, 1997). Tolerance was measured by determining the loss of stalk and leaf material, the loss of roots and the loss of the stubble (underground sections of stem providing buds to generate future crops). Antibiosis was determined by measuring survival and weight gain of canegrubs. Trials showed considerable variation in tolerance and anitbiosis effects amongst commercialised cultivars and other clones. Current commercial cultivars generally had poor tolerance and little antibiosis.

However, potential exists for tolerance, in the form of general plant vigour, to be selected for in breeding programs. Field trials were established in 1997 to test how well results from pot trials reflect those in the field. Antibiosis was correlated with four flavonoid compounds. Other plant chemicals may be responsible for antibiosis, including terpenoids, cell wall phenolics and polysaccharides. The association of plant chemicals with antibiosis is the subject of continuing research.

Four types of non-sugarcane gene were investigated for genetic engineering. They were genes for: *Bacillus thuringiensis* (Bt) proteins, plant proteinase inhibitors, plant lectins and avidin. The antimetabolic compounds were incorporated into an artificial diet to test for toxicity to canegrubs. The New Zealand Bt isolate DSIR517, which showed some toxicity to New Zealand grass grub, *Costelytra zealandica* (White), had no effect on canegrubs (Allsopp *et al.*, 1996). Proteinase inhibitors, identified after *in vitro* screening, and Snowdrop and wheatgerm lectins were toxic to canegrubs in the bioassays. Selected varieties of sugarcane have been transformed with three different genes coding for antimetabolites. These are: potato proteinase inhibitor II (*PinII*), *Nicotiana alata* proteinase inhibitor (*NaPI*), and the snowdrop lectin (*GNA*). Over 300 plants have been transformed successfully. In one of three pot trials, plants with the *PinII* or the *GNA* gene had significant effects on the growth and survival of canegrubs.

System redesign

Increased understanding of the biology of canegrubs in sugarcane is necessary to identify changes to farming practices that may limit damage by canegrubs. Research was initiated on the biology of Childers canegrub and negatoria canegrub, the major canegrub pests in south Queensland, and on greyback canegrub in north Queensland in the early 1990s. Computer models were constructed for Childers and negatoria canegrubs, and the effect of some current practices of sugarcane agriculture on their population dynamics was simulated. Maintaining short crop cycles (3-4 years) and treating each crop with suSCon Blue, was the best option for reducing numbers of both Childers and negatoria canegrubs. The effect of farming practices on the abundance of Childers canegrub is currently being studied. Initial surveys of farming practices found that the abundance of Childers canegrub tends to increase with crop age, and declines with increasing length of break and frequency of tillage between successive sugarcane crops (Fischer & Allsopp, 1997). A new *Adelina* sp. was recently found infecting Childers canegrubs in this study (D. Dall, unpubl.). The importance of disease in the population dynamics of Childers canegrub and other species in south Queensland is unknown.

Adults of greyback canegrub fly to favored feeding trees before returning to oviposit in the tallest crops of sugarcane. Growers in the Burdekin region have adopted the practice of avoiding infestation in the more valuable plant crops by delaying planting. In this way, plant crops are smaller than the surrounding ratoon cane (cane that has been harvested and regenerated to produce another crop), and less likely to attract egg-laden females. Fewer canegrubs have been found where cane trash was retained to form a mulch on the soil surface (trash-blanketing), than where the soil was left bare (Robertson & Walker, 1996). A higher incidence of disease under trash mulch may be partly responsible. Six primary pathogens have been isolated from greyback canegrub (Dall *et al.*, 1995). These are *Metarhizium anisopliae*, *Bacillus popillae*, two entomopox viruses and two protozoans, *Adelina* sp. n. and *Nosema* sp. n. *Metarhizium*

and *Adelina* were the most common pathogens found infecting greyback canegrubs. Other pathogens were relatively rare.

Discussion

Canegrub research has benefited from the establishment of the Sugar Research Council (SRC) by statute in 1985. The SRC, later to become the Sugar Research and Development Corporation (SRDC), funded the first project on canegrubs in the 1988/89 financial year. Since then, funding for canegrub research has increased substantially (Fig 2). Coincident with the increase in research funds from the SRDC, there has been an increase in the number of pest management staff employed by BSES, the principal provider of canegrub research, from 2-6 during period 2, to 14-15 in period 3. The BSES remains the major supplier of canegrub research. But, the introduction of the SRDC has resulted in the involvement of many new organisations in sugar research. Companies supplying insecticides to the sugarcane industry have also contributed significantly to canegrub research. Research funding provided by Crop Care Australasia in particular, led to the development of controlled-release insecticides.

The first significant research on canegrub biology since the 1930s was initiated in the 1990s. Research on the biology of greyback canegrub will continue, and new work will start on the canegrub *Rhopaea magnicornis* Blackburn. The development of biologically-based solutions to canegrub control can be complex and generally requires specialist expertise. This need has led to collaborative research, such as between CSIRO insect pathologists and applied entomologists from BSES. New research on pheromones of cane beetles will involve collaboration between biochemists at the University of Queensland and BSES. Within BSES, research on canegrub tolerant sugarcane and on transgenic sugarcane involves collaboration between plant breeders, biochemists and entomologists.

The ultimate goal of canegrub research is the successful control of canegrubs following adoption of research results by growers. The broad-based program of canegrub research on alternatives to insecticides has led to several promising options for canegrub management (e.g. BioCane™, late planting, trash-blanketing), and may lead to the development of other control measures in the future (e.g. transgenic cane, tolerant cultivars, other *Metarhizium*-based products). These options for canegrub management, together with insecticides, form components of an IPM program. Samson *et al.* (1998) consider that the development and implementation of IPM programs for canegrubs are more likely to be successful if growers are more closely involved in research than in the past. Research on the effect of farming practices, such as minimum tillage planting, trash blanketing and different fallow type and lengths, on canegrub abundance is particularly suited to close involvement of growers because it is often necessarily on-farm research. The development of options for inclusion in IPM still requires basic research at the laboratory and small-plot field trial level. This research includes the continuing development of transgenic sugarcane, and the investigation of the potential of the canegrub pathogens, *Adelina* sp. n. and *Nosema* sp. n. as new microbial insecticides.

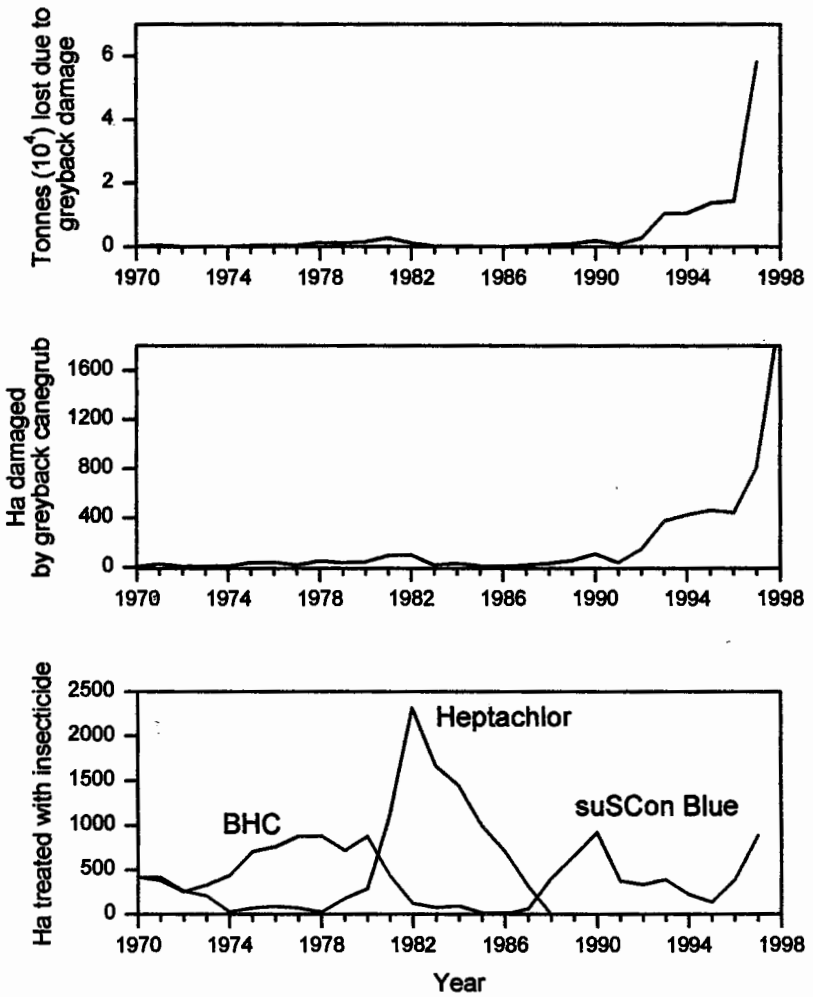

Figure 1. Damage to sugarcane (tonnes and area in hectares) by greyback canegrub and the area treated with insecticide to control greyback canegrub, for the Inkerman mill area, Burdekin district, in north Queensland. Data supplied by Don Williams, Inkerman Cane Pest and Productivity Board.

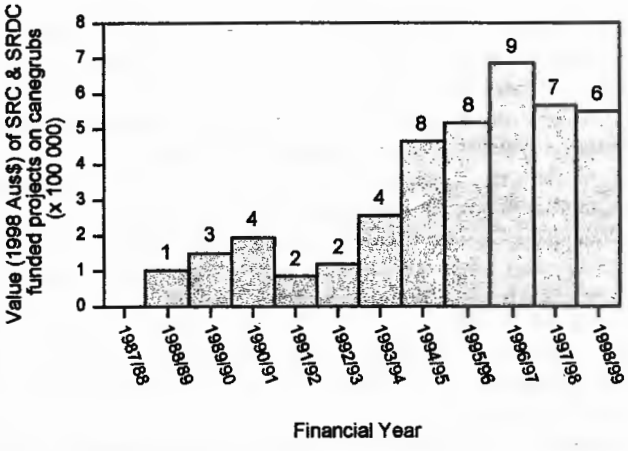

Figure 2. Funds contributed to canegrub research by the Sugar Research Council (SRC) / Sugar Research & Development Corporation (SRDC). Values for each financial year have been converted to 1998 Aus$. The number at the top of each bar is the number of projects that were funded in the financial year.

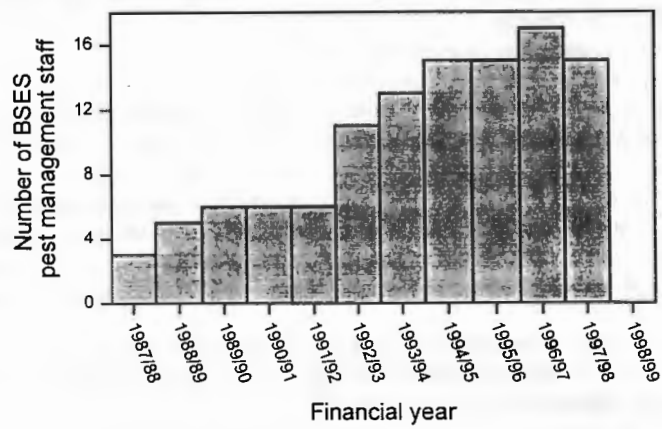

Figure 3. Numbers of pest management staff of the Bureau of Sugar Experiment Stations, the principal organisation conducting research into canegrubs.

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pp.131 - 137

Review of the development of *Metarhizium anisopliae* as a microbial insecticide, BioCane™, for the control of greyback canegrub *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae) in Queensland sugarcane

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Abstract: Greyback canegrub, *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae), is the major insect pest of sugarcane grown in north Queensland. Collaborative research between the Bureau of Sugar Experiment Stations, CSIRO, Bio-Care Technology Pty Ltd and the Sugar Research and Development Corporation began in 1994 to determine whether isolates of the fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin could control greyback canegrub in sugarcane. Rice grain-based formulations of the isolates FI-1045 and FI-147 were tested against greyback canegrub in field trials in north Queensland sugarcane. A commercially-viable application rate of 5 g/m (1 x 10^{10} conidia/m, 33 kg/ha) of *Metarhizium* FI-1045 gave 50-60% control of greyback canegrub after 6 months. The rice-based formulation of FI-1045 will shortly begin the process of registration as the microbial insecticide BioCane™ for the control of greyback canegrub.

Introduction

The major insect pests of sugarcane grown in Australia are canegrubs (Coleoptera: Scarabaeidae), the larvae of endemic melolonthini. Canegrubs damage sugarcane by feeding on the root and stool (underground portion of the plant producing shoots and new roots), reducing plant growth and sometimes killing plants. Eighteen species of canegrubs are recognised as pests of sugarcane in Australia, the most damaging species of which is greyback canegrub, *Dermolepida albohirtum* (Waterhouse) (Allsopp *et al.*, 1993). Greyback canegrub has a life-cycle of 1 year, and is currently in outbreak numbers in the Burdekin, a major canegrowing region in Australia. Lost production in the 1997/98 sugarcane growing season in the Burdekin has been estimated at A$8-15m based on aerial photographs.

The control of canegrubs in sugarcane has depended on the use of insecticides since 1947. Organochlorine insecticides (BHC and heptachlor) were successfully used to control greyback canegrubs and other canegrub species for about 40 years. The use of BHC and heptachlor were banned by the Australian Government in 1987 following reports of residues in some foodstuffs (Sturgess, 1987; Allsopp & Chandler, 1989). A controlled-release insecticide, suSCon® Blue (140 g/kg chlorpyrifos), is currently the major form of control for canegrubs in Australian sugarcane. Concerns about relying on a single insecticide to control canegrubs led to the expansion of research into other forms of control (Robertson *et al.*, 1995; Samson *et al.*, 1998). The potential of the fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin to control canegrubs was recognised in the 1910s (Tryon, 1914). *Metarhizium*, cultured on corn meal, was unsuccessfully trialed against greyback canegrub in 1920 (Illingworth, 1921). Further

work with *Metarhizium* was not continued until recently. Here we provide some background to and describe the most recent and successful trials (1994-1997) of *Metarhizium* against greyback canegrub.

Background

In the mid-1980s, a collaborative program of research began between the Bureau of Sugar Experiment Stations (BSES) and the Waite Institute, University of Adelaide, and between BSES and the Commonwealth Scientific and Industrial Research Organisation (CSIRO). Strains of *Metarhizium* highly infective to a number of canegrubs species were isolated (Milner, 1985). A batch process was used to mass produce *Metarhizium* by inoculating sterile wheat, rice or maize grain in 2 kg bags. Promising strains were tested against several different canegrub species in field trials in south Queensland (n=14) (P. Allsopp pers. comm.), and against greyback canegrub in field trials in north Queensland (n=16) (K. Chandler pers. comm.). These trials were largely inconclusive. Control of the canegrub *Antitrogus parvulus* Britton was achieved in one trial in south Queensland, with the strain WF2X (Samuels *et al.*, 1990). Further work to develop *Metarhizium* as a microbial insecticide was hindered by the lack of a suitable industrial scale production facility.

In the early 1990s, the BSES and Incitec Ltd collaborated in a study of the ecology and production of *Metarhizium* (Li & Holdom, 1993, 1994, 1995; Holdom & Li, 1996). A mycelial formulation and a partially-sporulated rice-based formulation failed to provide effective control of canegrubs in field trials in 1991 (n=8). However, a conidial suspension of *Metarhizium* (strain EF173), injected into soil at the base of sugarcane plants, reduced numbers of the canegrub *Antitrogus consanguineus* (Blackburn) in one of two field trials in 1993 (Allsopp *et al.*, 1994).

Current Work

In 1994, BSES, CSIRO and Bio-Care Technology Pty Ltd (Bio-Care) with some funding from the Sugar Research and Development Corporation (SRDC), collaborated to produce and trial a granular formulation of *Metarhizium*. Bio-Care has facilities to produce *Metarhizium*, grown on rice in special pouches, in commercial quantities. The microbial insecticide BioGreen®, a *Metarhizium*-based product developed for the control of the dynastine *Adoryphorus coulonii* (Burmeister), a pest of pastures in southern Australia, is produced commercially by Bio-Care.

Selection of strains for field trials

Metarhizium was isolated from infected canegrubs and from soil samples (Milner, 1992). Two strains, FI-1045 and FI-147, were identified as having potential for success in field trials against greyback canegrub. The strain FI-147 was isolated from a natural epizootic affecting the canegrub *Lepidiota consobrina* Girault (Milner, 1992) and FI 1045 was isolated from greyback canegrub (Robertson *et al.*, 1997). FI-1045 was selected as being the best isolate following initial screening trials using a high dose of conidia on third instars of greyback canegrub. FI-1045 gave 100% mortality after 14 days incubation at 25°C, while FI-147 was less pathogenic, causing 70% mortality in that time. Despite its lower virulence, FI-147 was included in trials as it is effective against a range of canegrubs (Milner, 1992), and it was hoped that it could be used for the control of a range of canegrub species. The two isolates are genetically very distinct, with FI-1045 belonging to Clade 9 (*M. anisopliae* var. *anisopliae*) and FI-147 belonging to Clade 8, which is thought to represent a new variety of *M. anisopliae* (Driver & Milner, 1998).

Field trials of Metarhizium 1994-1996

In the spring of 1994 and 1995, different rates of *Metarhizium* FI-1045 and FI-147 were applied to small-scale plots (5m long, 3 rows wide in 1994 or 9m long, 4 rows wide in 1995) in cane fields (Robertson *et al.*, 1997). *Metarhizium* was applied by hand to furrows which were later filled with soil. The plots were sampled in early autumn the following year, when greyback canegrubs were third instars. *Metarhizium* FI-1045 significantly reduced numbers of greyback larvae (Fig 1) in three of five trials. An application rate of 50 g/m reduced numbers of greyback larvae by 60-96% relative to untreated areas, while a rate of 5 g/m reduced numbers of greyback canegrubs by 45-60%. Both rates led to significant increases in yield. No effect of *Metarhizium* was found in two trials. Both of these trials were poorly infested with canegrubs. *Metarhizium* FI-147 was less effective than FI-1045 (Fig 1), significantly reducing numbers of greyback canegrubs in only one of three trials.

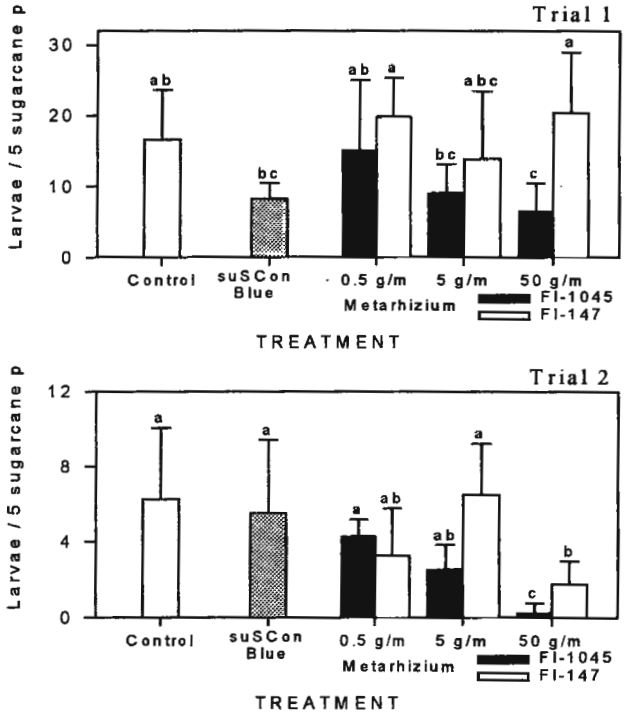

Figure 1. The effect of two strains of *Metarhizium* (FI-1045 and FI-147), applied at different rates, and the controlled-release insecticide suSCon® Blue (140g/kg chlorpyrifos) on numbers of greyback canegrubs approximately 6 months after application in small-plot trials. Error bars are one standard deviation. For each graph, columns with the same letter are not significantly different (LSD test, $P>0.05$) after ANOVA of $x^{0.4}$ - transformed data.

In 1996, *Metarhizium* FI-1045 was applied mechanically to large-scale plots (4 or 5 rows wide, 50 or 100m long) at five sites (L. Robertson & C. Kettle, unpubl.). There was no significant effect of *Metarhizium* on numbers of greyback canegrub in the four trials sampled

when transformed data ($x^{0.4}$) were analysed by ANOVA. Low replication (n = 2 or 3) of treatments, and poor or highly variable infestations of greyback canegrubs may have obscured differences between treatments. Numbers of canegrubs in treated plots were lower than numbers in untreated plots (Table 1).

Table 1. The reduction in numbers of greyback canegrub in areas treated with 5,6 or 10 g/m of FI-1045 relative to untreated areas, in 1996. n = no. of trials

Rate of application	Median reduction	Range	n
5 or 6 g/m	41.5%	31-75%	4
10 g/m	43.0%	17-57%	3

Commercial scale trials in 1997

In 1997, BSES, CSIRO, Bio-Care, and the Cane Protection and Productivity Boards of the Burdekin sugarcane-producing district, entered into an agreement with cane growers in the Burdekin district to conduct large-scale field trials as a means of testing the commercial-scale application of *Metarhizium* FI-1045 (BioCane™). Co-operating cane growers signed a Supply and Release agreement setting out the terms and conditions of co-operation, the possible risks of BioCane™ to human health, and directions for its use and application. BioCane™ was supplied in 2.5 kg bags with a yield of at least 2 x 10^9 spores/ g. In spring 1997, about 4 t of BioCane™ was applied by growers to 36 fields of plant cane on 35 different farms under the supervision of research staff (L. Robertson, C. Kettle & R. Cocco, unpubl.). BioCane™ was placed in the open furrows of young crops of sugarcane (4-6 months old) by a tractor-drawn applicator. Following its application, BioCane™ was covered with soil to a depth of approximately 100-150 mm. Many trials had 2-3 replicates of two rates of BioCane™ (5 g/m or 10 g/m) and a control. Rates were chosen to achieve a balance between cost and the level of control of canegrubs.

Approximately six months after application of BioCane™ (February-March 1998), ten fields had infestations of sufficient density to warrant sampling treated areas. Application rates of 5 g/m and 10 g/m produced similar reductions in the numbers of greyback canegrub, relative to the untreated area (Table 2) (L. Robertson, C. Kettle & R. Cocco, unpubl.).

Table 2. The reduction in numbers of greyback canegrub in areas treated with 5 or 10 g/m of BioCane™ relative to untreated areas. n = no. of trials

Rate of application	Median reduction	Range	n
5 g/m	59%	8-80%	10
10 g/m	60.5%	0-100%	10

Efficacy data summarised in our paper have been collated as part of a planned submission to the National Registration Authority (NRA) for the registration of the product BioCane™ as a microbial insecticide. The process of registration is expected to take at about 18 months to complete and BioCane™ may be on the market during the year 2000. As part of this

registration application, data were collected on the effect of BioCane™ on non-target organisms and on the persistence of BioCane™ in soils in north Queensland. BioCane™ did not affect non-target organisms such as earthworms, other scarabs and elaterids (L. Robertson, unpubl.). The persistence of BioCane™ and other formulations of FI-1045 (conidial suspensions in water and a wettable powder based partly on a clay carrier) is being measured in PVC rings buried in fields of sugarcane. After 12 months, *Metarhizium* in most formulations, including BioCane™, survived at 25-60% of the original level (R. Milner and L. Robertson, unpubl.).

This year, further commercial-scale application of BioCane™ will be carried out in the Burdekin sugar-cane growing area under an experimental permit from the NRA. The trials are likely to involve up to 60 cane growers and a total of 6 t of BioCane™. An experimental use permit will be sought for the application of approximately 20 t of BioCane™ to sugarcane in 1999.

Discussion

In the near future, BioCane™ is likely to become a commercially-available microbial insecticide for the control of greyback canegrub. The successful development of *Metarhizium* FI-1045 as BioCane™ has been based on collaborative research between CSIRO, BSES and Bio-Care since 1994, but was also built on the experience of earlier collaborative work. Key factors in the successful research program are the identification of a suitably pathogenic strain, a large-scale production process with high yields of conidia per unit weight, and a suitable application method.

Many sugarcane growers in the Burdekin district were willing to be involved in commercial-scale field trials of *Metarhizium*, and may be willing to use BioCane™ when it is commercially available. Greyback canegrub has been increasing in numbers in the Burdekin since 1994 and is currently at outbreak levels there. The cost of applying BioCane™ is currently equivalent to the cost of applying suSCon Blue. However, the chlorpyrifos in suSCon Blue is often subject to accelerated degradation in the high pH (>6) soils of the Burdekin (Robertson *et al.*, 1998). The premature degradation of chlorpyrifos can be ameliorated by the application of ammonium sulphate and sulphur (Chandler *et al.*, 1998). The addition of both fertilisers increases the cost of applying suSCon Blue by about 70%, and thus may make BioCane™ more attractive to canegrowers.

Sugarcane is a semi-perennial crop, harvested annually for around 3-6 years or more, and then ploughed out and re-planted. The effect of BioCane™ on the numbers of greyback canegrubs 6 months after application in plant cane is reasonably well known. However, further research is necessary to determine the level of control of greyback canegrub by BioCane™ after the initial 6 months. Increased levels of inoculum generated by infections within 6 months of application of BioCane™ may lead to high disease levels and good control for several years after application. Some farming practices in sugarcane may encourage the persistence and spread of *Metarhizium* (Robertson & Walker, 1996; Samson & Phillips, 1997). Understanding how such practices affect BioCane™ during the life of a sugarcane crop will enable better management of BioCane™ as a means of canegrub control. On a wider scale, the experience gained in the development of BioCane™ for greyback canegrubs may assist in the development of *Metarhizium*-based products for the control of other species of canegrub.

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