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Integrated Control of Soil Pests "*Melolontha*"

editor:

Siegfried Keller

IOBC wprs Bulletin
Bulletin OILB srop

Vol. 25 (7) 2002

International Organization for Biological and Integrated Control of Noxious Animals and Plants
Organisation Internationale de Lutte Biologique et Intégrée contre les Animaux et les Plantes Nuisibles
West Palaearctic Regional Section / Section Régionale Ouest Paléarctique

IOBC / WPRS

Working group „Integrated Control of Soil Pests"
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 REUNION

at / à

Aosta (Italie)
24-26 September 2001

Edited by Siegfried Keller

IOBC wprs Bulletin
Bulletin OILB srop **Vol. 25 (7) 2002**

The IOBC/WPRS Bulletin is published by the International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palearctic Regional Section (IOBC/WPRS)

Le Bulletin OILB/SROP est publié par l'organisation Internationale de Lutte Biologique et Intégrée contre les Animaux et les Plantes Nuisibles, section Régionale Ouest Paléarctique (OILB/SROP)

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ISBN 92-9067-144-9

web: http: // iobc-wprs.org

Preface

The working group "Integrated Control of Soil Pest" subgroup "*Melolontha*" held its third meeting within the frame of IOBC. 39 participants from 8 countries met from 24-26 September 2001 at Aosta, Italy. The meeting was co-organised with the EU-Project BIPESCO FAIR CT98-4105 which held its meeting the two preceding days. The local arrangements and a half-day excursion to *Melolontha* and historical sites in the environment of Aosta were organised by Frédéric Bondaz.

22 oral and 3 poster contributions were presented dealing with the following topics: Population development, biology, surveys, integrated and biological control, current situation and prospects. 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*. Due to the presence of the BIPESCO members emphasis was put on this fungus with topics like monitoring, dissemination, survival, interactions with soil, production and formulation. Attempts with other pathogens failed, but good control of *Amphimallon* larvae were obtained with nematodes.

A highlight of the meeting was the excursion in the environment of Aosta where white grubs cause severe damages in meadows and orchards. The problems were demonstrated and discussed and partly recorded by the Italian television.

Future work of the group will concentrate on improving the existing control measures and exploring new ones as well as including other soil pest insects. With respect to *B. brongniartii*, the quality of the material and of the application and the efficacy must be improved which will take place in close collaboration with partners from the BIPESCO-team. In this context soil-fungus interactions and new products need to be studied in more details. It was decided that the next meeting will be in Innsbruck, Austria, in 2004.

Siegfried Keller

Convenor of the subgroup

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Contents

Biology, ecology and production of *Beauveria brongniartii*

Population development, biology, integrated control

Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.3 - 5

Le hanneton en Vallée D'Aoste: Evolution de la situation depuis 1987

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Résumé : Le ver blanc (larve de *Melolontha melolontha*) demeure le principal ravageur des vergers et des prairies de la Vallée d'Aoste. Depuis 1987, des sondages sont réalisés chaque année, afin de suivre l'évolution du parasite. La situation demeure stable, avec 23,28 L2/ m² en 1999 dans une aire de presque 8.000 ha. Cette densité de vers blancs est insoutenable pour les vergers. La culture pluri-millénaire du pommier risque de disparaître. Les techniques de lutte proposées jusqu'à présent s'adaptent mal aux conditions de la Vallée d'Aoste.

Abstract : The white grub (larva of *Melolontha melolontha*) is the main pest insect of the orchards and meadows of Aosta valley. Detailed investigations have been done since 1987 in order to follow the evolution of the diffusion of this plant pest. The situation is stable with an average number of 23,3 larvae (L2) per square metre in 1999 in an area of about 8000 ha. This population density is intolerable for the orchards : the thousands of years old apple crop will run the risk of disappearing. None of the control techniques suggested so far have been satisfying in relation to the environmental conditions of the Aosta valley.

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

Introduction

Cette communication a pour objet de vous mettre au courant de l'évolution de la population du hanneton (*Melolontha melolontha*) en Vallée d'Aoste depuis notre précédente rencontre, à Sion (Suisse), il y a trois ans.

Rappelons les éléments essentiels du problème du hanneton dans notre région :

- en 1644, une procession est organisée pour libérer les campagnes de ce parasite.
  Le hannetonnage est financé d'abord par le « comice agricole », dès la fin du XIX^{e} siècle, puis par l'Assessorat régional de l'agriculture jusqu'en 1989 ;

- pendant les vols de 1962 et de 1965, 1 130 kg de Dimetoate sont pulvérisés par hélicoptère ;

- en 1989, 24 097 kg de blastopores de *Beauveria brongniartii* à 1,2 x 10^{9} sont pulvérisées par hélicoptère sur 50 ha de forêts.

Les buts de cette recherche était :

- Vérifier si l'aire de diffusion s'est modifiée ;

- Comparer la densité des différents cycles ;
- Contrôler l'évolution des auxiliaires.

Méthodes

- Creusage de tranchées de 1/6 de m² avec des pioches « Blaisinger » (lame de 16 cm) tous les 100 m sur l'axe Morgex-Montjovet ; au total, 386 sondages réalisés chaque année.

Résultats

- L'aire de diffusion est restée la même que lors des cycles précédents : environ 7 830 ha ;
- Le résultat du creusage de trachées est présenté sur la figure 1 et comparé avec les données dès 1987.
- Le nombre moyen de larves (L2) est presque identique à celui relevé lors du cycle précédent : 24 en 1996 et 23 en 1999.
- Les pourcentages de larves du deuxième stade trouvées momifiées par la moisissure *Beauveria brongniartii* demeurent très faibles : 0,84% en 1996 et 0,93 en 1999.

Conclusions

Le ver blanc demeure le principal ravageur des vergers et des prairies. Chaque année, il cause à l'agriculture valdôtaine des pertes de l'ordre de plusieurs millions d'euros. Des dommages commencent à être signalés dans les vignobles, avec l'expansion de la pratique de l'engazonnement. Le taux moyen de larves présentes dans les terrains plantés de vergers est insoutenable pour les porte-greffes faibles, et les arbres francs, eux aussi, supportent mal ces attaques. L'arboriculture valdôtaine est à bout de souffle. Le manque de remèdes efficaces et économiques décourage nos arboriculteurs :

- Les insecticides sont polluants et peu efficaces ;
- Les filets mobiles sont efficaces mais s'adaptent mal à nos terrains très morcelés et en forte pente ; les filets fixes n'ont pas connu d'applications importantes ;
- L'efficacité de la lutte biologique avec la moisissure *B. brongniartii* exige encore des contrôles.

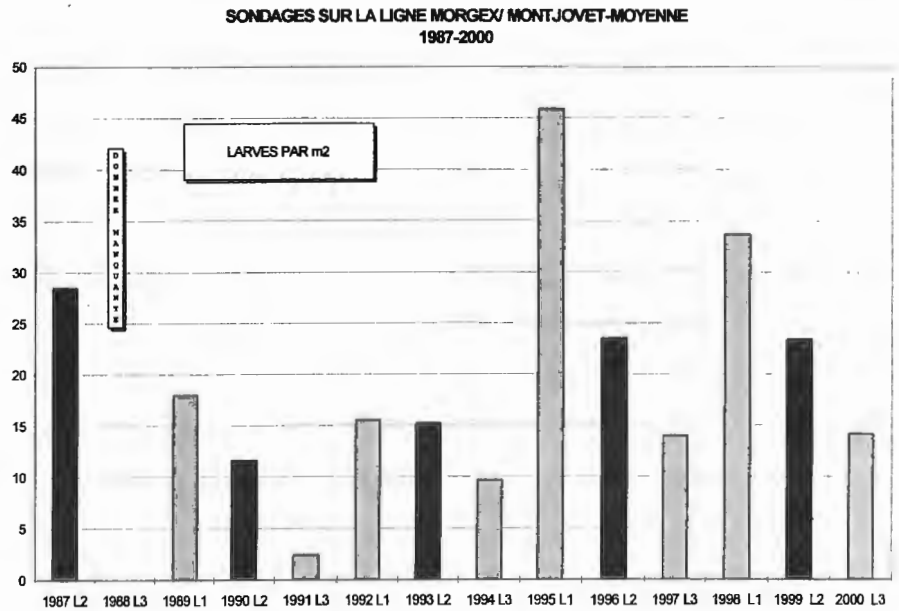

Figure 1. Densités (larves par m^2) moyen des vers blancs.

Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.7 - 13

Preliminary results of investigations on cockchafer flights in Austria from 1950-2000

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Abstract: Cockchafer (*Melolontha* spp.) flights in Austria have been monitored in Austria since 1949. FABER (1960) published a map of the cockchafer flight years in Austria. All three 3-year and all four 4-year cycles are represented in the country. All 12 years the same 3- and 4-year cycles coincide. One of these coincidental cycles is followed through the 50-year period, and maps for the years of this cycle show the development of the respective populations. Diagrams showing the strength of cockchafer flights for some regions of Austria affected by this cycle for the entire period demonstrate the fluctuations.

Key words: cockchafer, *Melolontha* spp., flight cycles, phenology, Austria

Introduction

Massive cockchafer flights have always fascinated both scientists and the general public. The media report on cockchafer flights every year, with variable tenor. Cockchafers have even become the theme of a popular song by Reinhard Mey, which is better remembered by most people than school biology lessons or reports by more serious news disseminators.

The reality is much more complex and diversified. In Austria cockchafer flights occur in both 3- and 4-year cycles, and all seven possible cycles (three 3-year and four 4-year cycles) occur in some part of the country. The flights have been investigated intensively by Zweigelt (1928) and Faber (1961). Data collected within the last 50 years show that flight intensity varies considerably, but that both cycle period and distribution remain remarkably consistent.

Material and methods

Zweigelt was the first to make detailed investigations on the intensity and distribution of cockchafer flight cycles in Austria and also in other parts of the former Austro–Hungarian Empire. In the first two decades after the Second World War massive cockchafer flights occurred in Austria, and Faber, working at the Federal Agency for Plant Protection in Vienna, used the data in Zweigelt's monograph to draw up a map of flight distribution in Austria. He soon noticed that in some areas Zweigelt's flight data did not correspond to actual cockchafer flights at that time.

He therefore decided to check this data and record the true distribution of cockchafer flights throughout Austria by enlisting the aid of schoolchildren across the country. He sent questionnaires to all approximately 3900 country elementary schools in the nation, asking that information on cockchafer flights, especially concerning flight intensity, be returned using a postcard-size return card detached from the questionnaire. He began his nation-wide inquiry in

1952, asking for data back to 1949. Considering that exact data could not be obtained, he asked for a estimate of flight intensity according to the criteria "no flight", "sporadic flight", "weak flight" and "strong flight".

Faber published the results of his inquiries in 1961, also drawing up a new map showing the changes in flight distribution since Zweigelt's time. He showed that all 3-year and all 4-year cycles are represented in Austria, some distributed over large areas, others only locally. He gave each cycle a number, combining a Roman numeral (III or IV for a 3- or 4-year cycle) with a Arabic numeral (0,1,2 or 3) to distinguish which of the 3- or 4-year cycles is indicated. Four-year cycles occur only in the higher parts of alpine valleys and in the mountainous region of NW Lower Austria.

Even after the publication of the first decade of results, he continued his inquiries until his death in 1979. As his successor at the Plant Protection Agency, the author also continued the inquires up to the present time, so that now more than fifty years of cockchafer flight data have accumulated. All these data have in the meantime been put into electronic form, thus enabling the mapping of flight intensity and distribution for single years and also the drawing up of flight intensity diagrams for various regions of Austria.

Results and discussion

All yearly maps show the flight intensity and distribution for one each of the three and four year cycles. Every twelve years the same three- and four year cycles coincide, so that a comparison of the maps in 12-year intervals show clearly any changes in distribution and intensity.

A comparison of the maps for the years 1952, 1964, 1976 and 1988 show the changes in intensity and distribution in the cycles III_2 and IV_0, for the years 1951, 1963, 1975, 1987 and 1999 the changes in cycles III_1 and IV_3, and for the years 1953, 1965, 1977 and 1989 the changes in the cycles III_0 and IV_1. There is generally a marked decrease in intensity, beginning in the early 1970s, in all areas except for some alpine valleys in Carinthia and the Tyrol as well as in a few very local areas in other provinces, notably Upper Austria, Salzburg, Carinthia and Styria. Some local areas have shown a slight recent increase in the last years, especially in Vorarlberg, Carinthia and Styria.

The diagrams of flight intensity over the 50-year period for different areas of Austria were compiled by giving the intensity criteria "no flight", "sporadic flight", "weak flight" and "strong flight" numbers from 1 to 4. This naturally does not mean that there is a linear relationship between the criteria, which is surely not the case. But as these highly subjective estimations cannot be correlated by any other relationship, this gives a certain rough idea of the fluctuations of flight intensity through time. For some regions schools at higher elevation were excluded, as cockchafer flights did not extend to their elevations. For each year a mean number was calculated from all schools returning the questionnaire cards.

As an example of data presentation, the maps for the years 1951 (fig. 1), 1963 (fig. 2), 1975 (fig. 3), 1987 (fig. 4) and 1999 (fig. 5), representing the cycles III_1 and IV_3, show intensity and distribution of cockchafer flights in Austria. On the map for 1951 the location of the counties discussed in more detail below are indicated.

The general decrease in flight intensity beginning in the first years of the 1970s is easily discernable in fig. 3, and fig. 4 represents the lowest population densities within the entire time period. An increased flight intensity for some local regions in western and southern Austria can be seen in fig. 5. These correspond to the counties marked "B" and "H" on the map for 1951.

Flight intensity diagrams are given for the counties Bludenz & Feldkirch ("B") in the state Vorarlberg Gänserndorf ("G") in the state Lower Austria and Hermagor ("H") in the state Carinthia. In the case of the counties Bludenz and Feldkirch (fig. 6) there is a steady decrease until 1963, a more or less stable condition until 1984, then a sharp renewed decrease in 1987 and since then a gradual increase. In fact, in the last few years many complaints of damage in grassland by white grubs have been reported from this area. The county Gänserndorf (fig. 7) is typical for the eastern part of Austria, where cockchafer flights have almost ceased to exist. The diagram shows a fairly stable flight intensity until 1969, then a gradual decrease until 1984, followed by only very sporadic flights up to the present day. Curiously enough, several reports of very local white grub damage to young vines were reported from neighboring regions in the year 2000, for the first time in decades, but they cannot be correlated with higher cockchafer flight intensity reports from the same areas. The county Hermagor (fig. 8) shows a very stable flight intensity at high level until 1984, a very marked decrease in the year 1987 followed by a gradual return to the previous high level, which coincides with reports of massive damage by white grubs in grassland and various fruit and vegetable cultures as well as of defoliation of trees by cockchafers.

These data give first general impressions on the temporal and spatial distribution of cockchafer populations in Austrian. A more detailed analysis of the data in presently being undertaken, especially with respect to the occurrence of weaker secondary flights in the years between the main flight cycles, local population fluctuations and possible changes in cycle stability in small areas outside the main distribution centers and correlation of flight intensity and distribution fluctuations with climatic and landscape changes.

Acknowledgements

I would like acknowledge the assistance by the children, teachers and directors of all the elementary schools taking part in our inquiries for sending back the questionnaires regularly mailed to them. The compilation of such an amount of data for so many years would not have otherwise been possible. I thank Dr. M. Wandl from the Institute of Soil Sciences, Vienna, for his expertise and patience in drawing the maps with the use of GIS software.

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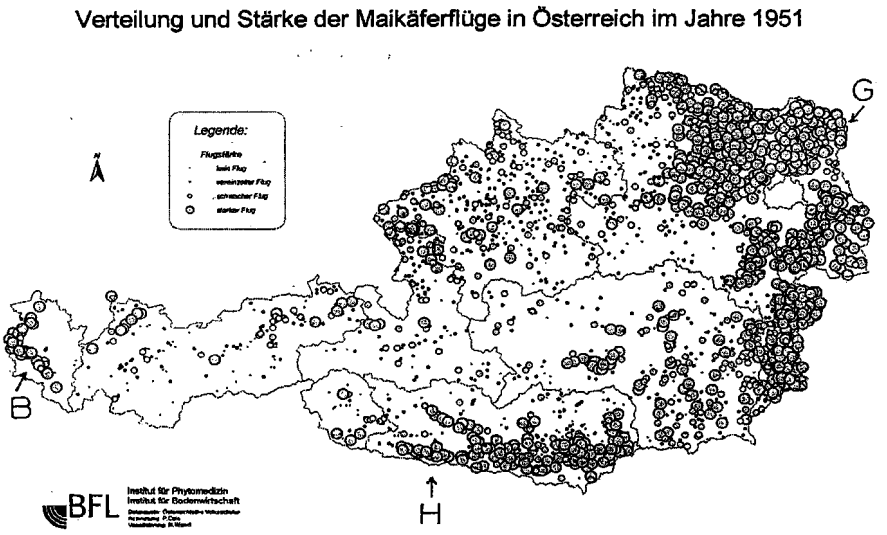

Figure 1. Intensity and distribution of cockchafer flights in Austria in the year 1951. B: counties Bludenz und Feldkirch; G: county Gänserndorf; H: county Hermagor. Legend: „kein Flug" = no flight; „vereinzelter Flug" = sporadic flight; „schwacher Flug" = weak flight; „starker Flug" = strong flight.

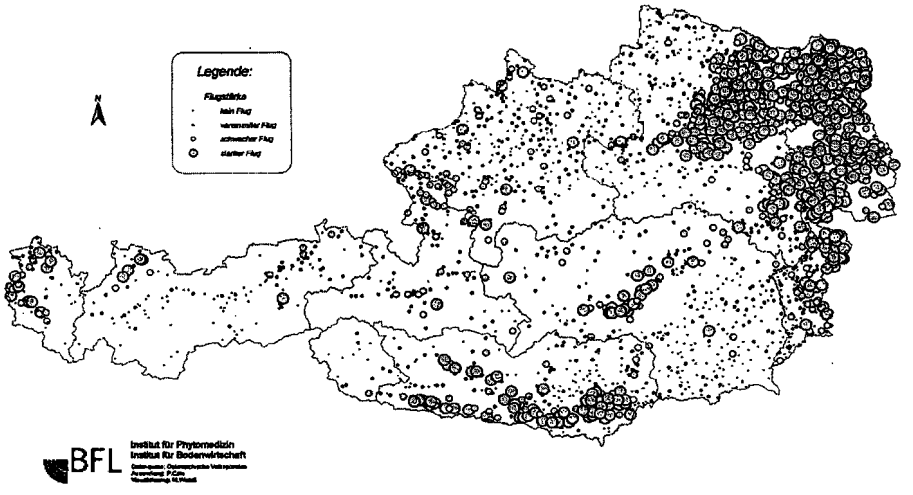

Figure 2. Intensity and distribution of cockchafer flights in Austria in the year 1963.

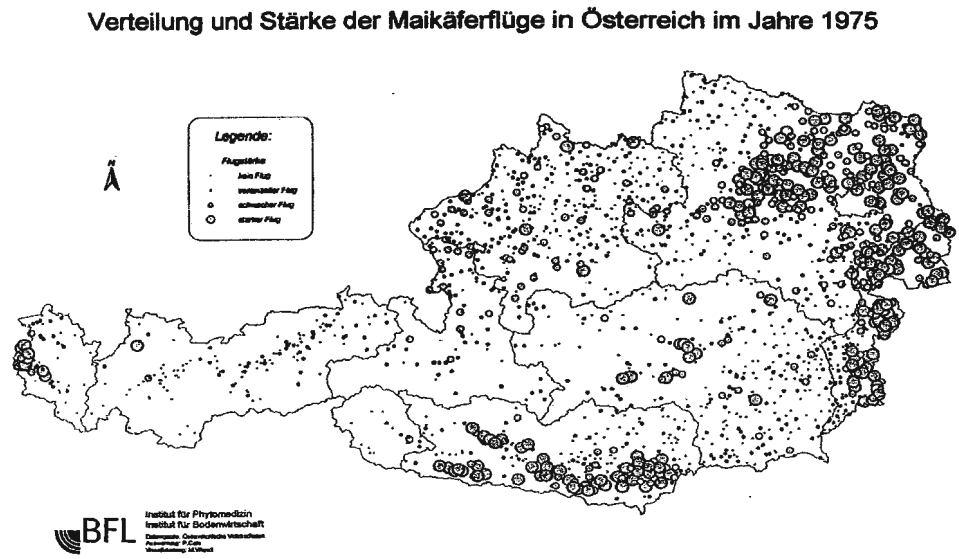

Figure 3. Intensity and distribution of cockchafer flights in Austria in the year 1975.

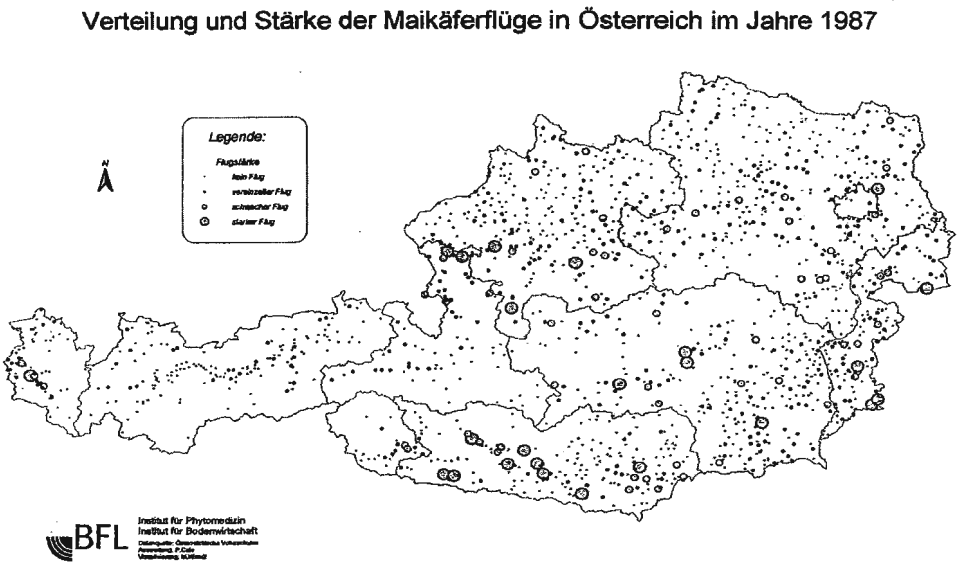

Figure 4. Intensity and distribution of cockchafer flights in Austria in the year 1987.

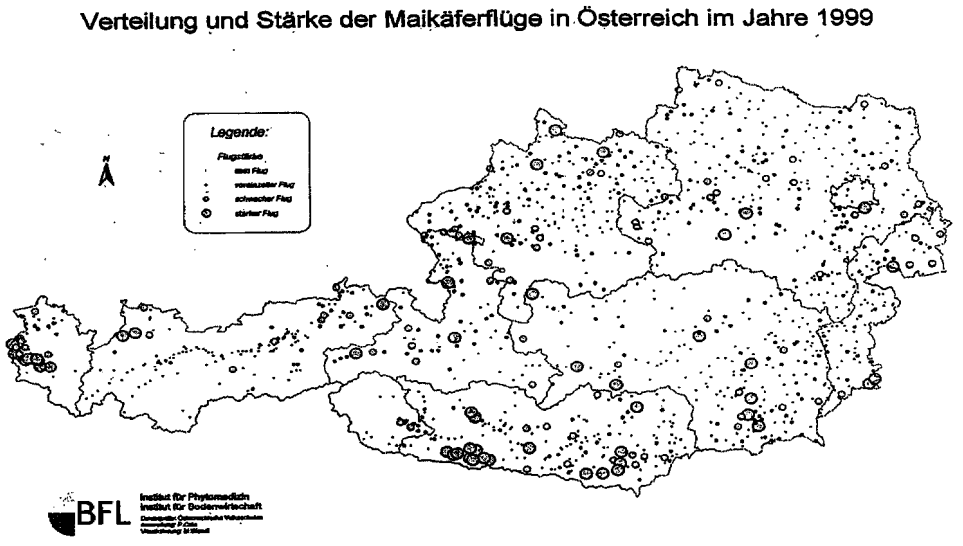

Figure 5. Intensity and distribution of cockchafer flights in Austria in the year 1999.

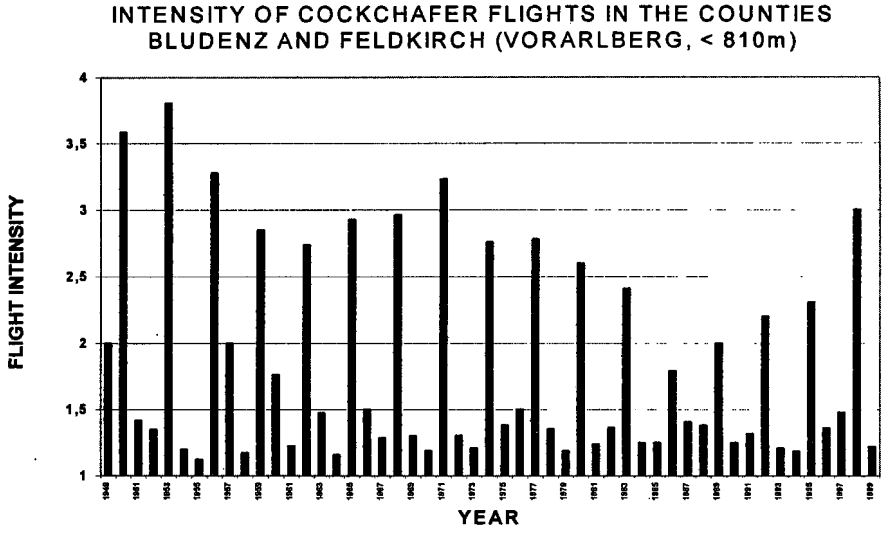

Figure 6: Cockchafer flight intensity in the counties Bludenz and Feldkirch (Vorarlberg, < 810m) in the years 1949 – 2000.

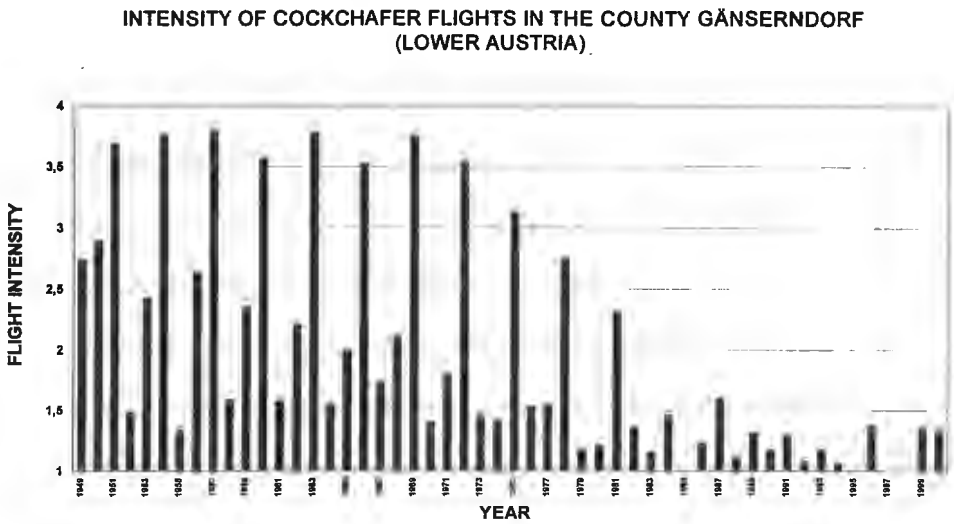

Figure 7: Cockchafer flight intensity in the county Gänserndorf (Lower Austria) in the years 1949-2000.

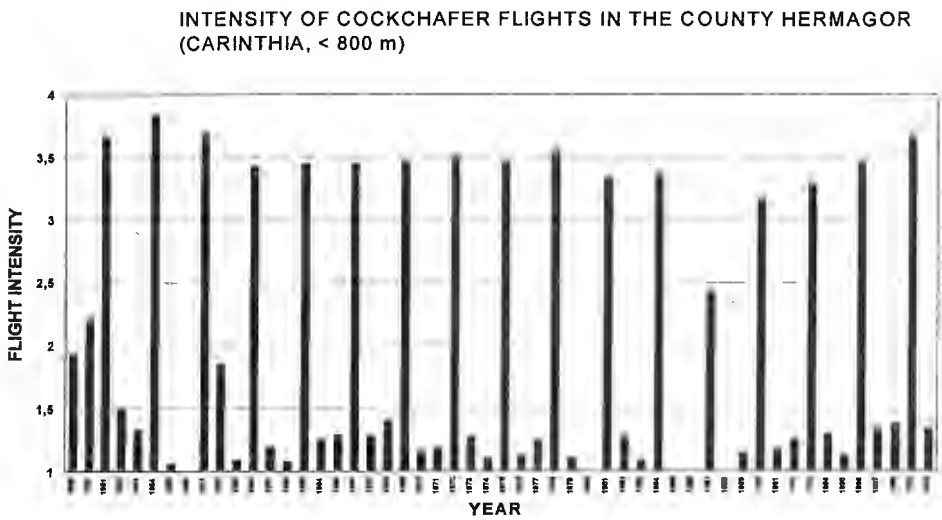

Figure 8: Cockchafer flight intensity in the county Hermagor (Carinthia, < 800m) in the years 1949 – 2000.

Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp. 15 - 20

Chemical orientation in *Melolontha* cockchafers

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Abstract: The results of a 4-year research project are summarised that aimed to investigate the role of plant volatiles and sex pheromones in the mate finding of cockchafers. Experiments investigating the forest cockchafer, *M. hippocastani* revealed that males of this species orientate during the swarming period at dusk towards the scent of damaged leaves originating from both host and non-host trees. This behaviour leads them to females that stay during this time on the host trees and continue to feed. The attractiveness of damaged leaves towards *M. hippocastani* is due to the so-called leaf-alcohol, (*Z*)-3-hexen-1-ol. In order to distinguish between feeding females and unspecific leaf damage, males use the beetle-derived compound 1,4-benzoquinone that synergistically enhances the attractiveness of the plant volatiles. First results on *M. melolontha* demonstrated that this species has a similar mate finding strategy as *M. hippocastani*.

Key words: *Melolontha*, kairomone, sex pheromone, field experiments, plant volatiles, 1,4-bezoquinone, (*Z*)-3-hexen-1-ol

Introduction

During the swarming period at dusk, increased mating activity of *Melolontha* cockchafers can be observed in the field. The clear sexual dimorphism of cockchafer antennae indicates that chemical cues are involved in the process of mate finding in this genus. Antennal clubs of males consist of seven large lamellae whereas female clubs contain only six significantly smaller ones. This suggests that olfactory abilities of both sexes of *Melolontha* cockchafers differ. Although the existence of sex pheromones in *Melolontha* cockchafers has been assumed (e.g. Keller, 1986), convincing evidence for their involvement was missing until recently.

During the past decades, sex pheromones of several phytophagous scarab beetles have been identified and some of them have already been established to control scarab pests (reviewed by Leal, 1997, 1998). The active compounds revealed to be chemically very diverse ranging from fatty acid derivatives, aromatics, terpinoids, and alkaloids to amino acid derivatives.

Plant volatiles attracting phytophagous scarab beetles have been identified in several studies (e.g., Ladd & McGovern, 1980; Ben-Yakir et al., 1995; Loughrin et al., 1996a,b, 1998). Furthermore, plant volatiles are known to interact with insect sex pheromones by synergising the response of the recipient or by stimulating the release in the emitter (Landolt & Phillips, 1997). This phenomenon has been used to improve the effectiveness of commercially available lures for scarab beetle control, e.g. in the Japanese beetle *Popillia japonica* (Klein et al., 1981).

The present paper summarises the results of a research project that aimed to investigate the role of pheromones and plant-derived volatiles involved in mate finding of *Melolontha* cockchafers, in order to develop synthetic lures that may be used in control measures against these polyphagous pests.

Role of plant volatiles

In preliminary laboratoray experiments using pitfall arenas, 83% of male *M. melolontha* were caught in pitfall traps baited with female conspecifics feeding upon twigs of *Fagus sylvatica* whereas only 17 % of the males responded towards the scent of feeding males. This male preference for females was absent when the conspecifics used as lures were not allowed to feed. In contrast, females discriminated in this bioassay neither between feeding males and females nor between conspecifics that were not allowed to feed (unpublished data). These results suggested that mate finding in *M. melolontha* is mediated chemically and that volatiles from host plants play a decisive role for males to find the females. However, corresponding experiments using *M. hippocastani* failed due to the missing activity of this species in the pitfall bioassay.

Field observations revealed that mate finding was performed by flying cockchafers during the swarming period. Therefore, the approach using laboratory bioassays was dropped and field studies were conducted instead. For this purpose, funnel traps were constructed that were similar to commercially available Japanese beelte traps (e.g. Trécé) but had to be adapted to the size of cockchafers (Ruther et al., 2000). These traps were arranged in randomised blocks of 3-5 traps per block and placed at equivalent positions of infested host trees. It turned out to be essential to hang the traps directly into the host trees because responding beetles hovered along the twigs very closely.

Experiments investigating the response of *M. hippocastani* towards plant volatiles revealed that exclusively males were attracted in high number by the scent of mechanically damaged leaves from the host plants *Carpinus betulus* (hornbeam) and *Quercus rubra* (red oak) as well as from the non-host plant *Prunus serotina* (black cherry). The mechanical damage of the leaves was necessary for their attractiveness. The activity of volatiles from host plants and the non-host plant was statistically not distinguishable (Ruther et al., 2000).

Chemical analyses of the volatiles emitted from intact and damaged leaves of the three plant species tested in the field were performed using a purge and cold trapping technique (Ruther & Hilker, 1998) and subsequent coupled gas chromatography-mass spectrometry (GC-MS). These analyses revealed a tremendous increase of the emission of so-called green leaf volatiles (GLV) after mechanical damage in all investigated species (Ruther et al., 2001 b). The term GLV describes a series of saturated and monounsaturated six-carbon aldehydes, alcohols, and corresponding esters (Ruther, 2000). Electrophysiological studies using electroantennography (EAG) and gas chromatography with electroantennographic detection (GC-EAD) demonstrated that antennae of male and female forest cockchafers are able to detect a multitude of typical plant volatiles including most of the GLV (Ruther et al., 2000, 2001b).

Funnel trap experiments in the field using synthetic chemicals instead of damaged foliage as lures demonstrated that a mixture consisting of electrophysiologically active GLV mimicking the bouquet of mechanically damaged leaves was highly attractive towards swarming *M. hippocastani* males (Ruther et al., 2001b). Experiments addressing the contribution of individual compounds to the attractiveness of damaged leaves revealed that only one compound, i.e. the so-called leaf alcohol (*Z*)-3-hexen-1-ol was attractive towards male forest cockchafers whereas the corresponding acetate and the (*E*)-2- and the saturated derivatives were behaviourally inactive (Ruther et al., 2001b).

Corresponding field experiments on *M. melolontha* showed that males of this species also responded towards damaged host leaves. In contrast to *M. hippocastani*, all tested green leaf alcohols, i.e. not only (*Z*)-3-hexen-1-ol, but also (*E*)-2-hexen-1-ol and 1-hexanol attracted *M. melolontha* males (Reinecke et al., submitted).

As mentioned above, only male cockchafers responded towards mechanically damaged plants irrespective of their suitability as host plants.This and the fact that males in most cases start to their swarming flights from trees having appropriate foliage in surplus suggested that the male response towards plant volatiles does not serve for the location of new food sources but is part of their mate finding strategy. In this context it was important to know if only the response towards plant volatiles is male specific or if there is a sexual dimorphism regarding the flight behaviour during the swarming period, i.e. only males perform the swarming flight and females remain on the host trees. Estimation of the sex ratio of flying and sitting *M. hippocastani* revealed that indeed 94% of the flying beetles were males, whereas the majority of those beetles that remained on the host trees and continued to feed were females (82%) (Ruther et al., 2001a). Thus, the reaction of *Melolontha* males towards damage-induced green leaf alcohols leads them to feeding females. However, the question arised how males are enabled to distinguish between leaf damage that is indeed due to feeding conspecifics and non-specific leaf damage caused by other herbivores or non-biotic processes. A possible tool is the use of a beetle-derived chemical that may provide information on the organism causing the leaf damage.

Role of sex pheromones

The pheromonal communication of *M. hippocastani* was shown in the field using a landing cage bioassay. Pairs of wire cages baited with living beetles or with extracts from beetles were mounted on twigs of infested host trees and the number of male landings was recorded during the swarming period. Significantly more males alighted on cages baited with living females that were allowed to feed than on comparable cages containing males. When the cages were mounted on twigs from which the foliage had been removed, thus preventing the beetles to feed, swarming males again preferred the females. In the second experiment the total number of male landings was clearly reduced suggesting a synergistic interaction between feeding-induced plant volatiles and a beetle-derived component. When both cages contained feeding males and were additionally baited with male or female dichloromethane extracts, the cages with female extracts were preferred by swarming males, definitely demonstrating the existence of a sex pheromone in *M. hippocastani* (Ruther et al. 2000).

Analysis of the dichloromethane extracts by GC-EAD and subsequent identification of the electrophysiologically active compounds by GC-MS revealed the presence of 1,4-benzoquinone and toluquinone (2-methyl-1,4-benzoquinone) in extracts from beetles of both sexes (Ruther et al., 2001a). A series of field experiments was performed to test for the attractiveness of these compounds towards swarming forest cockchafer males. Since our previous experiments already indicated a synergistic effect, benzoquinones were tested alone and in combination with the synthetic GLV mixture that had been shown before to attract male cockchafers. 1,4-Benzoquinone was only slightly attractive when tested alone but increased synergistically male catches in GLV-baited funnel traps. Toluquinone did neither alone nor in combination with GLV affect the behaviour of swarming *M. hippocastani* males (Ruther et al., 2001a). These results showed that 1,4-benzoquinone is the sex pheromone of *M. hippocastani* enabling swarming males to discriminate between feeding females and non-specific leaf damage.

The fact that both sexes of *M. hippocastani* contained 1,4-benzoquinone explains male-male copulation attempts that can be observed from time to time in the field (Schwencke, 1974; Keller, 1986) and seems at first glance to contradict the role of this compound as a sex pheromone. However, the chance for swarming males to meet male conspecifics instead of females when orientating towards feeding-induced plant volatiles and 1,4-benzoquinone is low because of the demonstrated sexual dimorphism regarding the flight behaviour at dusk when

mainly males exhibit flight activity. Furthermore, quantitative chemical analyses demonstrated that females contained significantly higher amounts of 1,4-benzoquinone than males (Ruther et al., 2001c) and that the release rate of this compound from females was significantly higher (8 ± 4 ng per 8 hrs) than from males (4 ± 1 ng per 8 hrs) (unpublished data). These results might explain the demonstrated preference of swarming *M. hippocastani* males for females and female extracts.

Secondary function hypothesis

Both 1,4-benzoquinone and toluquinone identified in whole body extracts from *M. hippocastani* are known since the very beginning of chemoecological research as defence compounds of numerous arthropod taxa among them the intriguing bombardier beetles (Eisner, 1958a; Schildknecht & Holoubek, 1961), tenebrionid beetles (Tschinkel, 1975), rove beetles (Steidle & Dettner, 1993), cockroaches (Eisner, 1958b), and millipeds (Eisner et al., 1978). Burger et al. (1995) identified 1,4-benzoquinone for the first time among the defence chemicals of a scarab beetle, i.e. the dung beetle *Oniticellus egregius* (Scarabaeinae).

Leal (1997) found pheromones of the subfamily Melolonthinae to have antimicrobial properties. Hence, he hypothesized that the evolution of sex pheromones from defence compounds might be a common feature in this taxon. The results of the *Melolontha* project revealed a very common defence compound to be active as a sex pheromone in *M. hippocastani* and thus, strengthen this secondary function hypothesis. Furthermore, chemical analyses of dichloromethane extracts from grubs (L-3) demonstrated that the behaviourally active 1,4-benzoquinone was present also in immature stages of *M. hippocastani* at even higher levels than in the adults (Ruther et al., 2001c). To further investigate a possible defence function of benzoquinones in *M. hippocastani*, a series of antimicrobial assays was performed using 1,4-benzoquinone and toluquinone. These experiments revealed inhibitory effects towards the model organisms *Escherichia coli* (gram-positive bacterium) and *Saccharomyces cerevisiae* (yeast) and the entomopathogenic fungi *Beauveria brongniartii* and *Metarhizium anisopliae* (Ruther et al., 2001c). However, the amounts necessary to inhibit the yeast and the entomopathogenic fungi were much higher than those found in the extracts from adults and grubs of *M. hippocastani*.

Practical aspects

The research project on the chemically mediated mate finding of *Melolontha* cockchafers allowed the development of a potent lure to attract swarming males. By using a combination of synthetic GLV mimicking the odour bouquet of mechanically damaged host leaves and the sex pheromone 1,4-benzoquinone, up to 300 male forest cockchafers were caught within 30 min in a single funnel trap. However, adult feeding is not the central problem in *Melolontha*-infested areas since the consumed biomass can normally be compensated during a secondary sprouting period in June (Schwencke, 1970). Since the major losses during cockchafer infestation are due to the root-feeding grubs, it has to be tested in future studies how lures attracting only males may be integrated in control measures aiming at the immature stages.

Mass trapping of cockchafers using the developed attractants seems not to be a realistic approach because of the extremely high population densities that can be observed after mass breeding in many areas of Europe. However, monitoring of cockchafer densities during progradation might be helpful to initiate conventional and/or biological control measures timely and thus, to prevent development of new areas of mass breeding.

Great efforts have been undertaken during the past decades to develop biological and integrated control methods against cockchafers. One of the most promising antagonists is probably the fungus *B. brongniartii.* The main problem is to establish the fungus in the soil where the target organisms, i.e. the larvae develop. Keller et al. (1997) treated the borders of deciduous forests infested by *M. melolontha* with a blastospore suspension and used the ovipositing females as vectors to establish the fungus in the soil. This technique required large amounts of blastospore suspension which was spread by helicopters. Investigating the Japanese beetle *P. japonica,* Klein & Lacey (1999) developed an autodessimination trap in which beetles were caught and successfully infected with conidia of *M. anisopliae.* The trap was designed to release the beetles after infection and thus, using them as vectors for the fungus. The infection caused high mortality rates, and it was shown that infected beetles passed the fungus on to untreated beetles that lived in the same feeding container. This "trap-and-infect" technique might be also a promising approach to use the developed *Melolontha* attractant for biological cockchafer control. The advantage could be a more economic use of fungal spore formulations and a more selective spreading of the fungus. However, future studies will have to demonstrate first, that the infection cascade is working, i.e. that the fungus is passed by the males on to the females during copulation and finally is established in the soil by ovipositing females.

Acknowledgements

The authors thank Dr. H. Gossenauer-Marohn and Dr. M. Rohde (Hessen-Forst, Hann.-Münden) for their help in financial and logistic organisation of this study, Prof. Dr. W. Francke and Till Tolasch (Institut für Organische Chemie, Universität Hamburg) for performing some of the electrophysiological experiments, and Dr. M. Fröschle (Landesanstalt für Pflanzenschutz, Stuttgart) for organisational support of the *M. melolontha* work. This project was financially supported by the Hessian state forest administration and in part by the Deutsche Forschungsgemeinschaft (Hi-416/13-1).

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General biological control

Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.23 - 28

Evaluation of a new *Steinernema* sp. of *Melolontha melolontha* and *Amphimallon solstitiale*

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Abstract: Although nematodes are now widely used to control the garden chafer (*Phyllopertha horticola*), we still lack an efficient nematode against the closely related grub species *Melolontha melolontha* and *Amphimallon solstitiale*. The most successful species tested so far was *Steinernema glaseri*, however, since this species originates from the USA its use in Europe is restricted. Recently entomopathogenic nematodes have been isolated from different locations in Europe resembling the "Steinernema glaseri group". These species are characterised by their size and most of them seem to be associated with scarabaeid larvae. An isolate of this group from Morocco found in larvae from *Rhizotrogus majalis* was evaluated for its pathogenicity against larvae of *M. melolontha* and *A. solstitiale*. Good laboratory efficacy encouraged the field evaluation against *M. melolontha*. Of 3 field trials against *M. melolontha*, only one with a high dose (2.5 Mill./m²) gave significant control. A preliminary trial against *A. solstitiale* could not be interpreted due to a low grub infestation level. The results encourage further field work with this and other nematode isolates from the "Steinernema glaseri group".

Key words : *Steinernema, Melolontha melolontha, Amphimallon solstitiale,* biological control

Introduction

Entomopathogenic nematodes are successfully used for biocontrol of the scarab species *Phyllopertha horticola*, *Aphodius* sp. and *Hoplia philanthus* Peters and Ehlers, 1999 In contrast, commercially available nematodes failed to control two important pest species, *Melolontha melolontha* and *Amphimallon solstitiale*. In laboratory studies, the best performing nematode against *M. melolontha* was *Steinernema glaseri* (Peters, 2000). This species has so far only been found in North America and on the Acores. However, a lot of isolates resembling this species (the so called "S. glaseri group") have recently been found in the Slovak Republic, the Czech Republic, Switzerland and Southern France (Sturhan and Mracek, 2001). Common to these nematodes is a large infective juvenile (> 1000µm) and many of these nematodes have a close association to scarabaeid hosts.

In 2000, a nematode was isolated from infected grubs (presumably *Rhizotrogus majalis*) in a vineyard in Morocco. It belongs to the genus *Steinernema* and is likely to be a new species. According to its long infective juveniles and the association to scarab hosts it can be grouped into the "S. glaseri group". This isolate (strain MD) was tested in the laboratory and in the field against the June beetle, *Amphimallon solstitiale,* and the European cockchafer, *Melolontha melolontha.*

Material and methods

Laboratory bioassays

Grubs from various locations in Germany were collected in the field and sent to us. The grubs were put one by one in 3cm diameter vials and covered with silver sand (10% moisture w/w,

grain size 0.25 to 0.4 mm to a depth of 1.5 cm). Nematodes (*Steinernema* sp. MD) were added on top of the sand and the vials were left at 22-24°C for 7 (*A. solstitale* and *Phyllopertha horticola*) or 14 days (*M. melolontha*). The number of grubs per treatment and the time before mortality was assessed is given in the results.

Field trials

Field trials against *M. melolontha* were performed on 2 sites. In an apple orchard in Mecklenburg Vorpommern (Dodow) L2/L3 larvae were treated (trial 1). Another trial against the L1/L2 was done near Münster in a nursery of 15 years old plum stocks for cutting production. (trial 2).

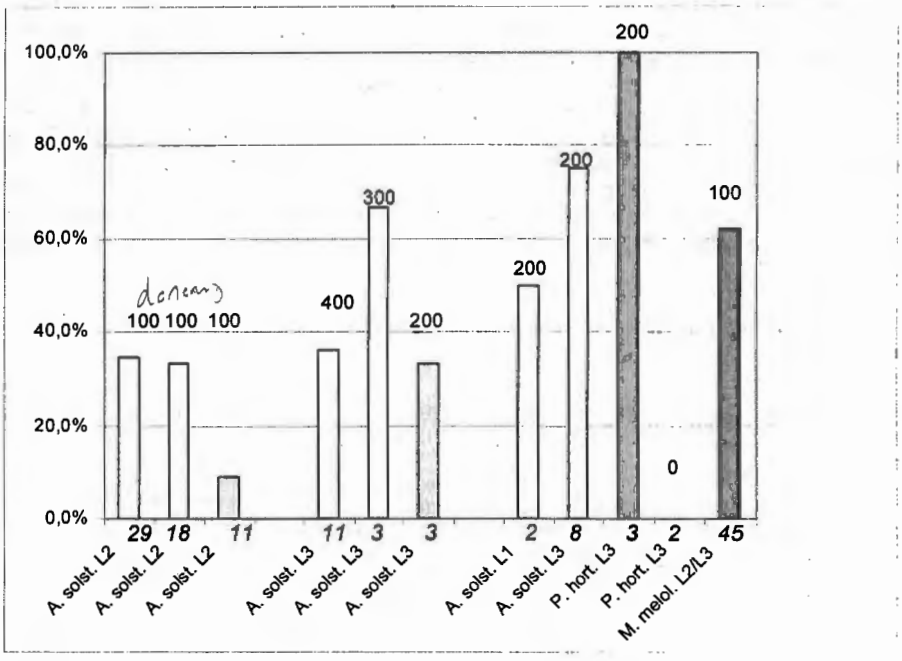

Figure 1. Mortality (%) of *Amphimallon solstitiale, Melolontha melolontha* and *Phyllopertha horticola* 14 days after treatment with *Steinernema* sp. MD in laboratory trials. The nematode dose per grub is written over the columns. Numbers of grubs tested are given below the columns.

In trial 1 two treatments were done. The first treatment done on 04.07.2001 consisted of 4 x 25 m treated plots with a dose of 500.000 *Steinernema sp.* MD per m of the row of apple trees. Nematodes were applied with a watering can with 10 l on 25 m². After treatment the rows were irrigated with 0.5 l/m². Intersects of 10 m length between the treated rows were kept as a control. The trial was evaluated 43 days after treatment by digging holes of 30cm x 30cm holes of 25 cm depth around randomly chosen trees and counting the number of grubs

per sample. Another evaluation was made 77 days after treatment with 2 samples per treated an 6 samples in total from the untreated plots. Four samples were taken from each treated plot and a total of 4 samples from the untreated controls.. The second treatment on the 16.08.2001 was done on 10 trees by applying clay-formulated nematodes directly into the soil at the tree roots (about 2 cm deep). A package of 25 million nematodes were distributed among 10 trees so the dose was approx. 2.5 million per m². Four trees were randomly chosen for evaluating the number of grubs 35 days after treatment and 5 trees were sampled from the untreated control.

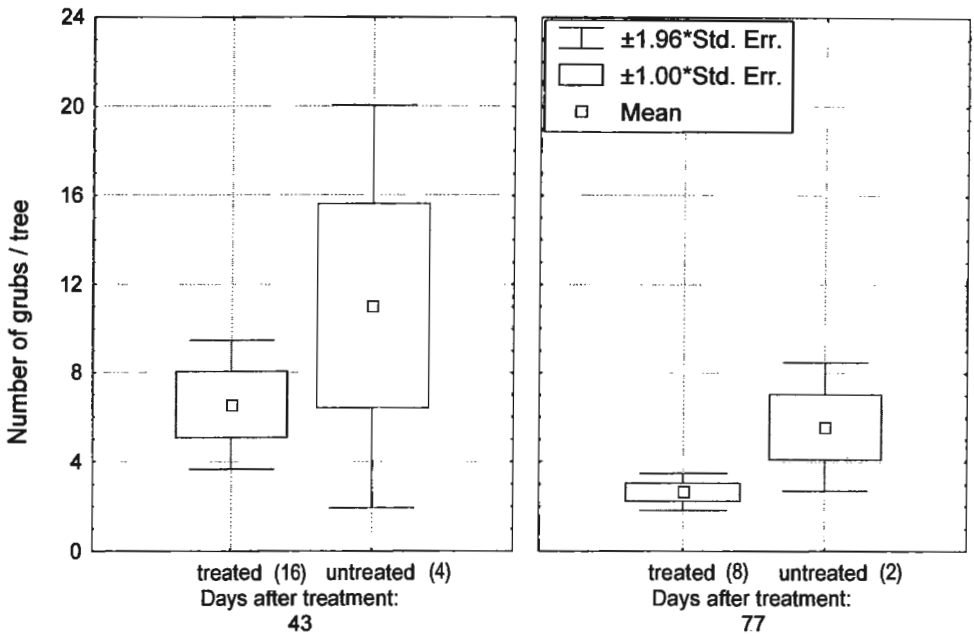

Figure 2. Number of grubs per apple tree 43 and 77 days after treatment with *Steinernema* sp. MD at a dose of 500,000 nematodes/m². Number of samples in brackets.

In the 2nd trial nematodes were applied at a dose of 500.000 nematodes per m² with a boom sprayer on 2 x 1000 m² plots. The amount of water applied was 0.8 l/m². Two lanes of 10 m covering 7 rows of plum-stocks were sprayed alternating with two lanes (= 7 rows) of untreated stocks. Application was done on August 23rd with approx. 30°C ambient temperature. The evaluation was done by digging out samples of 50 x 50 cm * 40 cm depth around randomly chosen trees.

Another 3 trials were performed on a golf course at Motzen (Berlin) against *A. solstitiale*. Each trials consisted of 5 nematode-treated and 5 untreated plots (each plot 4 x 4 m). Treatments were done with *Steinernema* sp. MD at a dose of 500,000 nematodes, *H. bacteriophora* (500,000 nematodes/m²) and a mixture of *H. bacteriophora* and *Steinernema*

sp. MD (each 500.000 nematodes/m²). Nematodes were applied with 0.75 l of water/m². The untreated controls were treated with water only. The field was irrigated for 5 minutes before and after treatment. Six weeks after treatment the number of grubs were counted in 5 samples (10 cm diameter and 5 cm depth) per plot.

Results and Discussion

Laboratory bioassays

Mortality of grubs was relatively high (Fig. 1). With a dose of 100 nematodes per grub, 35 % of *A. solstitiale* L2 grubs died of nematode infection within 7 days (1st column in Fig. 1). The surviving grubs were treated again and the same proportion was killed (2nd column in Fig. 1). When the surviving grubs of the second trial were treated again, only 9% died. In further independent experiments it could be shown, that first and third instar larvae of *A. solstitiale* are similarly susceptible to the MD strain.

Larvae of the garden chafer, *Phyllopertha horticola*, were highly susceptible to the nematodes. Larvae of *M. melolontha* all obtained from an apple orchard in Mecklenburg Vorpommern in the 2nd year from flight were also susceptible to the new *Steinernema* isolate with 62% of the L2/L3 larvae being killed within 14 days. This is high compared with the results of Peters (2000) with *S. glaseri* where 60% were killed at a dose of 2000 infective juveniles per larva. The good laboratory performance of the MD-strain encouraged us to do some field trials.

Field trials

No nematode infected grubs were found in any of the trials performed. In trial 1 a decline in the number of grubs was detectable after the first treatment with 500,000 nematodes/m² (Fig. 2). Analysed by itself none of the two evaluations yielded statistically significant less grub numbers in the treatment. Strictly speaking, the two observation are repeated measures of one treatment. Analysed accordingly reduction in grub numbers in the treated plots also was not significant (MANOVA, $p>0.1$). After adding grubs in a 5-fold concentration a significant decrease in the number of grubs per sample was observed (ANOVA, $p<0.07$; Fig. 3). The treatment against the L1/L2 larvae did not show any reduction in grub numbers (data not shown). In the field trial against *A. solstitiale* no firm conclusions could be drawn because of the low level of infestation on the trial spot. In the plots treated with *H. bacteriophora*, only 3 of 50 samples contained one *A. solstitiale* larvae in the treated plots. Only one positive sample was found in the mixed treatment (in a control plot). Further 7 positive samples (5 in the control and 2 in the treatment) were found in the treatment with *Steinernema* sp. MD.

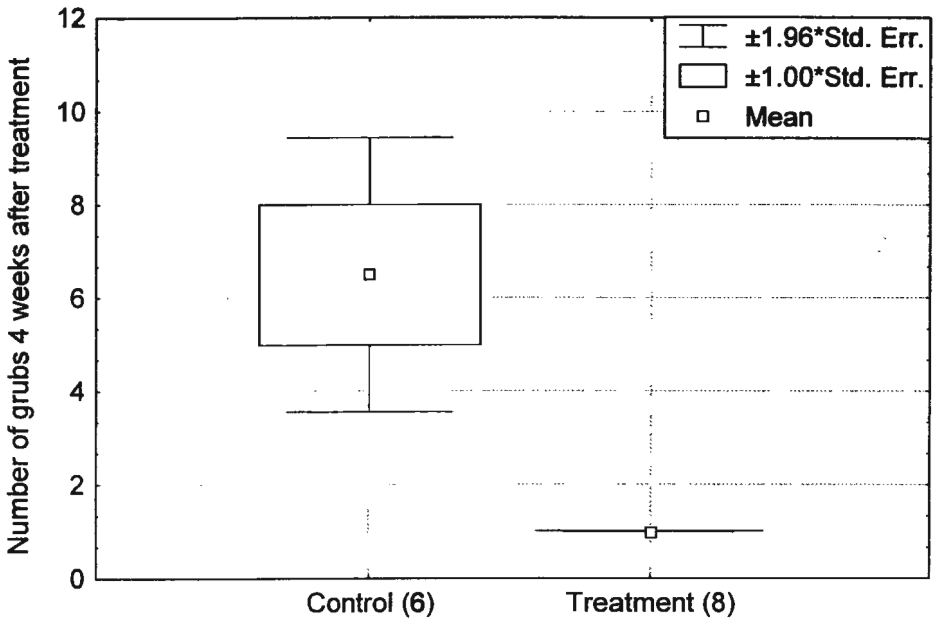

Figure 3. Number of grubs per apple tree 34 days after treatment with *Steinernema* sp. MD at a dose of 2,500,000 nematodes/m². Number of samples in brackets.

Despite the good laboratory control, field performance of the nematodes against *M. melolontha* was detectable but poor compared to the results achieved against the garden chafer (Sulistyanto and Ehlers, 1996). Similarly, Deseö *et al.* (1991) observed a high mortality of *M. melolontha* in the laboratory with *H. bacteriophora* strain I-127 and an Australian *S. glaseri* strain. In the field, however, numbers of older grubs were maximally reduced by 20% after application of 1 million nematodes per m². On the other hand, infections of *M. melolontha* with *Heterorhabditis* sp. have been reported from Italy (Deseö *et al.*, 1991) and *Steinernema* sp. species have been found in *Melolontha* species on several occasions (Poinar 1992).

No judgement can be made on the efficacy of the new nematode against *A. solstitiale*. Further trials must be done to assess the efficacy of *Steinernema* sp. MD against this species. Like from *M. melolontha, Heterorhabditis* and *Steinernema* species have also been found in *A. solstitiale* (Glare *et al.*, 1993; Peters, 1996). The possibility to control these two grub species with nematodes can not be rejected until such nematode strains have been assessed.

Acknowledgements

Thanks are due to Mrs. Rehm from the Landespflanzenschutzamt Mecklenburg Vorpommern, Schwerin and to Vinzenz Winter from Gartenbauzentrum Westfalen Lippe and Mr. Bredow, the greenkeeper of the Golf-Course Motzen for support during the field trials.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.29 - 34

Field trials with the entomopathogenic nematode *Heterorhabditis bacteriophora* against white grubs of the European cockchafer (*Melolontha melolontha*) in the southern part of Germany

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Abstract: The entomopathogenic nematode *Heterorhabditis bacteriophora* is successfully used to control white grubs of the garden chafer (*Phyllopertha horticola*) in meadows and golf courses. To test the efficiency of this nematode against white grubs of the European cockchafer (*Melolontha melolontha*) in the field we performed two trials in orchards and one in an vine yard in the southern part of Germany. In one orchard *Beauveria brongniartii* had been additionally applied. Only under optimal conditions and with high doses of nematodes 33 % (1 million nematodes per m^2 and *B. brongniartii*) and 35 % (2.5 million nematodes per m^2) control of grubs could be reached. We report about the results and discuss the methods and difficulties of this trials. For the application of nematodes we propose a new variant.

Key words: *Heterorhabditis bacteriophora, Beauveria brongniartii, Melolontha*, white grubs, biological control

Introduction

The entomopathogenic nematode *Heterorhabditis bacteriophora* is successfully used to control white grubs of the garden chafer *Phyllopertha horticola* on meadows and golf courses (Sulistyanto and Ehlers, 1996) or in special cultures like potted yew against grubs of the Japanese beetle *Popillia japonica* (Wright et al., 1988). To test the efficiency of this nematode against white grubs of the European cockchafer (*Melolontha melolontha*) in the field we performed two trials in orchards and one in an vine yard in the southern part of Germany. In one orchard the entomophagous fungus *Beauveria brongniartii* had been applied additionally before and during the trial.

Materials and Methods

Nematodes

The nematode *Heterorhabditis bacteriophora* were obtained from E-Nema GmbH, Raisdorf, Germany. The nematodes were mass reared in liquid culture and sold normally under the product name 'nema-green®'. The following concentrations of nematodes were tested: **c0** = 0 (control plain, only water), **c1** = 0.5 million DL/m^2, **c2** = 1.0 million DL/m^2, **c3** = 2.0 million DL/m^2, **c4** = 2.5 million DL/m^2 (DL/m^2 = Dauer Larvae of the nematode per square meter).

Fungi

Beauveria brongniartii were obtained from Samen-Schweizer AG, Thun, Switzerland. The application were conducted with an modified sowing machine laying the granules in three rows (30 cm distance) and 10 cm depth into the soil.

A new variant of application for nematodes

In the Kaiserstuhl field trial we tested a new variant of application for nematodes. A standard pesticide sprayer for vine yards had been modified. Two grooves of approximately 8 cm depth were dug into the soil and the nematode suspension was poured directly into these grooves. The grooves were covered again through the tyres of the pesticide sprayer. The space between the two grooves was 1 m and the distance of two rows of vine was 1.70 m. In the two apple orchards the nematode suspension had been poured on the soil surface with a watering can.

Characterisation of the field trials

	Orchard in Nieder-Beerbach, near to Darmstadt	**Orchard in Dielheim-Horrenberg, near to Heidelberg**	**Vine yard in the area of the Kaiserstuhl**
date of application	4.9.98	14.8.99	19.7.01
date of control	26.5.99	19.5.00	12.9.01
Plots	5 x 4 = 20 m^2	5 x 3.5 = 17.5 m^2	1800 m^2
c0	8 plots	32 plots	1 plot
c1	8 plots	32 plots	-
c2	6 plots	30 plots	-
c3	-	29 plots	-
c4	-	-	1 plot
soil	clay soil	clay soil	clay soil
kind of application	watering can on the soil surface	watering can on the soil surface	modified pesticide sprayer into the soil (8 cm deep)
volume	0.8 l/m^2	0.5 l/m^2	0.2 l/m^2
Beauveria brongniartii	had been applied before and during the trial	had been applied before the trial	no prior application
date of application	20.4.99 (3 g/m^2)	-	-
weather	rain before, during, and after application	rain before and during application, afterward very hot and dry	rain before application, afterward very hot and dry

Evaluation of nematode efficiency

The grub density of the treatments and the control plots were determined by counting the number of grubs from 9 or 10 soil samples of 20 x 20 cm (Nieder-Beerbach, Dielheim-Horrenberg) or 50 x 100 cm (Kaiserstuhl) and 30 cm depth from each plot. In Dielheim-Horrenberg only 10 plots from each treatment were evaluated. The mean number of larvae in treated and untreated plots at each sampling date were compared with Tukey's test (Biosoft Stat100, Version 1.28). The reduction rate was calculated according to Abbott (1925).

Occurrence of infections in the grub populations

While the sampling dates we registered all founded dead or infected larvae and determined the responsible pathogen so far as possible.

Results and discussion

Table 1. Overall occurrence of pathogens in white grubs of the European cockchafer while the control dates.

pathogen / area	Nieder-Beerbach	Dielheim-Horrenberg	Kaiserstuhl
Beauveria brongniartii	4.0 %	9.9 %	2.0 %
Metarhizium anisopliae	0	0	0.1 %
Heterorhabditis bacteriophora	0	0	0.2 %
not defined	1.6 %	3.7 %	0.5 %

Table 2. Abbott corrected reduction rates of grubs. No significance could be recorded using Tukey's test ($p < 0.05$).

concentration / area	Nieder-Beerbach	Dielheim-Horrenberg	Kaiserstuhl
c1	9.5 %	0	-
c2	33.1 %	0	-
c3	-	13.3 %	-
c4	-	-	35 %

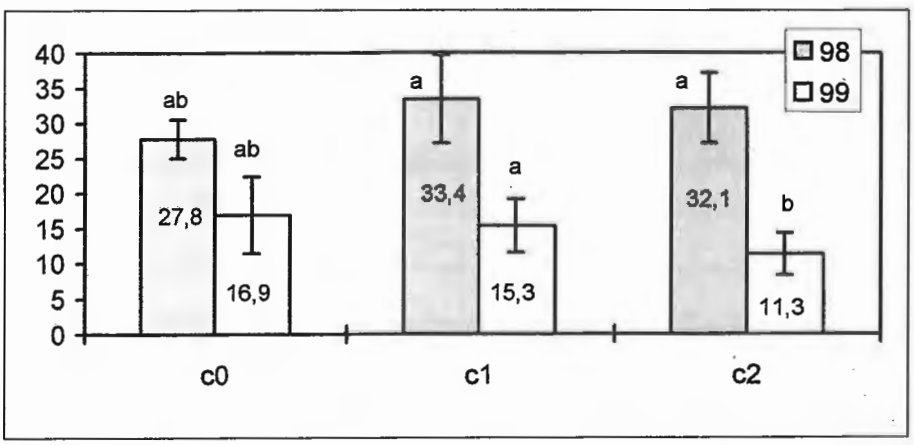

Figure 1. Field trial in Nieder-Beerbach, near to Darmstadt. Average numbers of grubs per square meter for each nematode concentration (c0, c1, c2). The left columns (grey) show the numbers before the treatment with nematodes and the right columns (white) show the numbers of the first control. Means followed by the same letter are not significantly different (Tukey's test, $p<0.05$).

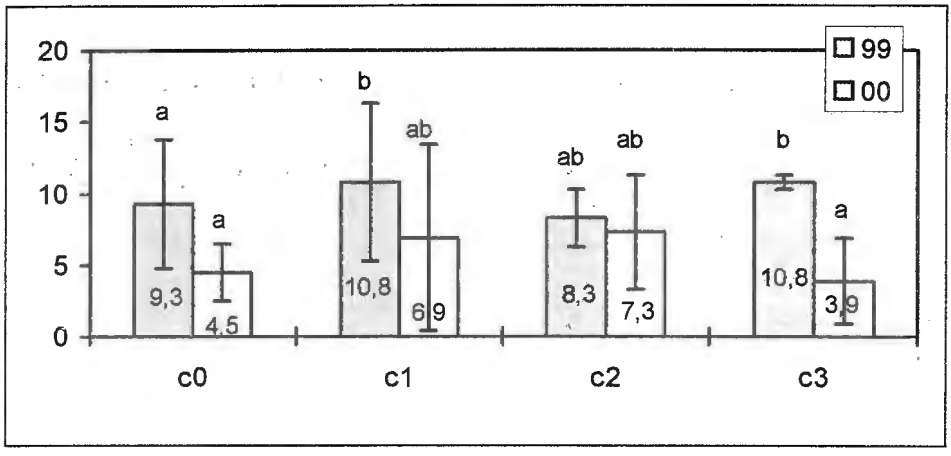

Figure 2. Field trial in Dielheim-Horrenberg, near to Heidelberg. Average numbers of grubs per square meter for each nematode concentration (c0, c1, c2, c3). The left columns (grey) show the numbers before the treatment with nematodes and the right columns (white) show the numbers of the first control. Means followed by the same letter are not significantly different (Tukey's test, $p<0.05$).

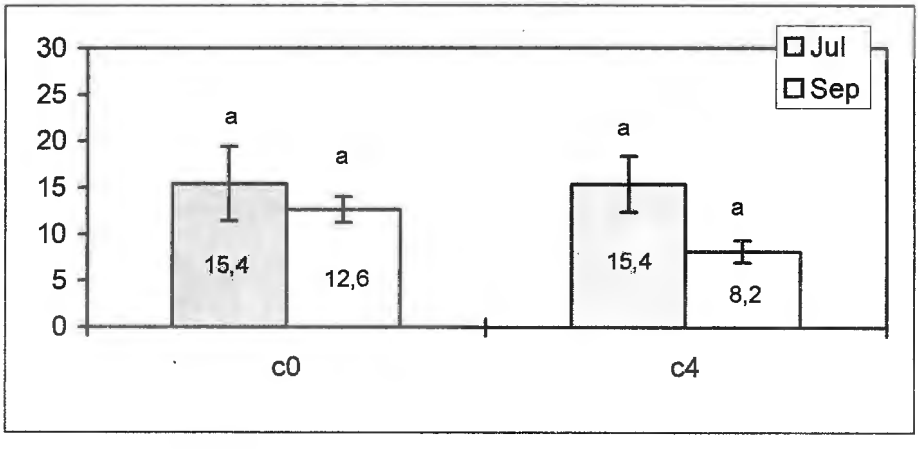

Figure 3. Field trial in the Kaiserstuhl area. Average numbers of grubs per square meter for each nematode concentration (c0, c4). The left columns (grey) show the numbers before the treatment with nematodes and the right columns (white) show the numbers of the first control. Means followed by the same letter are not significantly different (Tukey's test, $p<0.05$).

These results showed that only with optimal weather and application conditions, a high grub density, a high dose of nematodes (1 million DL/m^2 or more) and an additional application of *Beauveria brongniartii* a grub reduction of up to 33 % (Nieder-Beerbach, figure 1 and table 2) could be reached. The additional application of the entomophagous

fungus *B. brongniartii* seems to have a synergistic effect on the efficiency of the nematodes. In all three areas we could record a relatively high infestation with this fungus, even in the Kaiserstuhl trial where no prior application of this fungus had been performed (table 1). The reason for this high rate in the Kaiserstuhl might be that some vine yards in the neighbourhood of the trial yard had been treated with *Beauveria*. The application of *Beauveria* in the two apple orchards during the last few years explains the relatively high infestation by this fungus in these areas (table 1). The overall reduction of the grub density in all plots of the two orchards at the control date has to be explained by the long time period between nematode treatment and the control.

A general problem while performing field trials to control white grubs of the cockchafer is the extremely inhomogeneous distribution of the grubs in the soil, particularly if the density is not very high. This case we had especially in Dielheim-Horrenberg, were we found a relatively low density of grubs and a high fluctuation of the number of grubs between and within plots (figure 3). In this trial as well as in the Kaiserstuhl trial the weather conditions after the application were very detrimental for nematodes (very hot and dry for some weeks). Therefore only 13% reduction with 2 million DL/m^2 and no effect with the lower doses could be recorded in Dielheim-Horrenberg. In the Kaiserstuhl trial we found a high density and a relatively homogenous distribution of grubs about a large area (figure 3). Despite bad weather conditions the reduction reached 35 % (2.5 million DL/m^2). This relatively good result might be due to the new application variant and the high dose of nematodes. The new variant combines several advantages like an effective protection of the nematodes against UV radiation and desiccation and no loss of nematodes on the leaves of the grass leading to a possible reduction of the total quantity of nematodes. In none of the three trials we ever found a nematode infected grub. Therefore it was very surprisingly and interestingly for us to find two nematode-infected, red-brown coloured grubs in the Kaiserstuhl area (one we found before nematode application and one after application on the control plot far away from the nematode-treated plot and close to the position where the first had been found). In the lab we were able to isolate these nematodes and to propagate them in *Galleria* larvae. We proved via RAPD-PCR clearly that this isolate is a new *H. bacteriophora*-isolate (Berner, 2001, not published). This was the fourth proof of *H. bacteriophora* from Germany and the first proof of this nematode species from grubs of *M. melolontha* under natural conditions. Natural infections with *H. bacteriophora* in grub populations are pretty rare. Glare et al. (1993) could prove *H. bacteriophora* infections in grubs of the June beetle *Amphimallon solstitialis* near to Darmstadt in the southern part of Germany. Considering that the carcasses of nematode-infected insects in the soil are dissolved quite fast (in comparison with *Beauveria* infected ones) we assume that the rate of infection with this nematode is much higher than we could recognise it (table 1). Investigations about the putative control potential for grubs of this new isolate are currently in work.

Compared to the results of other authors reaching up to 83 % control against the garden chafer *Phyllopertha horticola* (Sulistyanto and Ehlers, 1996) and more than 60 % against the Japanese beetle *Popillia japonica* (Villani & Wright, 1988), respectively, our results are not satisfying. Further studies should be done to find new isolates and to increase the infectivity of the commercially produced nematodes. Berner & Schnetter (2001a) started a selection program to increase the infectivity for *H. bacteriophora* and *Steinernema glaseri* against grubs of the European cockchafer via host passages which was successful only with the latter species. *S. glaseri* and closely related nematode species are known to be the most effective species against many scarab larvae but most of them are not indigenous and their use in Europe will be restricted (Peters, 2000; Berner & Schnetter, 2001b). Some new European

nematode isolates probably belonging to the *'S. glaseri*-group' has been found recently and should be tested.

Currently the commercially produced entomopathogenic nematode *H. bacteriophora* can only be recommended as an additional factor in combination with the entomophagous fungus *B. brongniartii* to control white grubs of the European cockchafer. Further studies to improve the application techniques (e.g. combined application of *Beauveria* and nematodes with one machine at the same time) should be done.

Acknowledgements

We thank the growers Willi G. Muth, Manfred Epp and Volker Buchmüller for their support in practising the nematode application. Special thanks to E-Nema for supplying their nematode product.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.35 - 40

Application of ISSR-PCR to characterise entomopathogenic nematodes within a selection program for higher efficiency against white grubs of the cockchafer (*Melolontha* spp.)

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Abstract: In order to find genetic markers to identify entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* we evaluate the method of ISSR-PCR (Inter Simple Sequence Repeats-PCR). Our aim is to discriminate isolates, inbred lines and via selection improved lines and to prove the crossbreeding between isolates (hybrids) of nematodes. These investigations are enclosed within an program that consists of screening nematode-isolates and of selection for higher efficiency against white grubs of the cockchafer (*Melolontha* spp.). From the nematode *Heterorhabditis bacteriophora* we investigated 11 geographical isolates, 3 re-isolates of field released nematodes, 2 hybrids which are arose from 7 original isolates by crossbreeding (E-Nema, Raisdorf, Germany) and one line which was selected via host passages in white grubs and 20 inbred lines which are derived from one of the hybrids. From the nematode *Steinernema glaseri* we investigated 3 geographical isolates and one in white grubs selected line. Our data show that the method is useful to discriminate isolates and even some of the inbred lines.

Key words: *Heterorhabditis, Steinernema*, genetic fingerprinting, SSRs, ISSR

Introduction

Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are widely used in biological control of insect pests. In the last few years great efforts have been undertaken to improve the technique of mass rearing and thereby the quality of the nematode product (Ehlers, 2001). In order to improve beneficial traits of the nematodes and to investigate the heritability of these traits systematic breeding programs have been started (Johnigk et al., 2001). Despite these progresses little is known about the genetic structure of entomopathogenic nematodes and reliable genetic markers to monitor these processes are not available yet. In order to find such specific markers we evaluate the genetic fingerprinting technique of Inter Simple Sequence Repeats Polymerase Chain Reaction (ISSR-PCR). This technique based on Simple Sequence Repeats (SSRs), also known as microsatellites or Simple Tandem Repeats (STRs). SSRs consist of short tandem repeats out of two to five base pair motifs and are highly informative and widely distributed throughout eukaryotic genomes (Tautz and Renz, 1984). The detected polymorphism is due to the highly variable number of tandem repeats. For the classical use of SSRs as a genetic fingerprinting technique knowledge of the flanking sequence is required. This precondition makes the development of SSRs relatively labour-intensive. On the other hand, with ISSRs no prior information on the sequence is required, and reproducible polymorphism are produced in high number. ISSR-PCR analysis employs oligonucleodites based on an SSR anchored at either the 5′ or 3′ end with two to four purine or pyrimidine residues, to initiate PCR amplification of genomic segments flanked by inversely orientated, closely spaced SSRs (Zietkiewicz et al., 1994). The detected polymorphism is based on the number of tandem repeats of the SSR, the length of

the segment between two inversely orientated SSRs, and on sequence variations of the flanking side of the SSR which correspond to the anchored side of the primer.

This paper reports on the use of ISSR-PCR to assess the feasibility of discriminating isolates, inbred lines and via selection improved lines and of the parental analysis of entomopathogenic nematodes. These investigations are enclosed within an program that consists of screening nematode-isolates and of selection for higher efficiency against white grubs especially of the cockchafer (*Melolontha* spp.).

Material and Methods

Nematode culture

The nematode isolates used in our investigations are listed in table 1. Each isolate was passaged through *Galleria mellonella*, and emerging infective juveniles were collected, washed, centrifuged, and stored frozen at -20°C until used for DNA extraction.

DNA extraction and ISSR-PCR

DNA extraction of nematodes was performed with the SIGMA GenElute™ Mammalian Genomic DNA Kit according to the manufacturer's instructions. The PCR was performed with reagents and buffers from MBI Fermentas. The reaction mixture (25 µl) contained 2 mM $MgCl_2$, 200 µM dNTPs, 2.5 µl of 10x PCR Buffer with $(NH_4)_2SO_4$, 1 u Taq DNA polymerase, 2 µM primer, and 10 ng of template DNA. The primers we screened are listed in table 2. From all positive primers we used 3´- or 5´-anchored primers for our further investigations. The primers were obtained from Interactiva Biotechnologie GmbH. The amplifications were performed in an Biometra™ TGradient Thermoblock with cover heating. The PCR started with 5 min denaturing at 95°C followed by 34 cycles consisting of 45 s at 95°C, 60 s at 45°C (or 40°C), 120 s at 72°C and a final extension of 5 min at 72°C.

Electrophoresis

2 µl aliquots of the PCR mixture were mixed with formamide loading buffer (80% formamide, 10 mM EDTA, pH 8, 1mg/ml of xylene cyanol FF, 1 mg/ml bromphenol blue), denatured 5 min at 95°C, chilled on ice and then loaded on a 45 x 20 cm sequencing gel (6% polyacrylamide, 8 M urea, 1x TBE). Electrophoresis was carried out at 1500 V for 2 h. Silver staining was performed according to the ETCWebForum (www.etcelpho.com/html/body_silver.htm). Reproducibility of DNA profiles was determined by replicating all ISSR reactions twice.

Data analysis

Banding patterns of all primer/isolate combinations were transformed into a 1/0 matrix (1 = presence, 0 = absence of a band). Cluster analysis were performed with the neighbour joining method using the PAUP software (Version 4.0b8 for Microsoft Windows). Pairwise similarity between isolates was calculated using the method described by Nei and Li (1979).

Table 1. Designation and source of nematodes which are involved in ISSR analysis. A commercially produced hybrid strain (Hy) of *H. bacteriophora* and *S. glaseri* strain NC were used in the selection program for higher infectivity (Berner & Schnetter, 2001). Re-isolates are field released nematodes which could be isolated again from the soil. All inbred lines are derived from the hybrid PS7. PS7 arose of 7 parental isolates by pairwise crossbreeding. For the hybrid strain Hy see Johnigk et al. (2001).

Isolate	**Source**	**Remarks**
H. bacteriophora		
hybrids and derived lines		
Hy	Peters, E-Nema, Germany	hybrid of EU and US isolates
RW	Ehlers, Germany	re-isolate of Hy
Re02	Ehlers, Germany	re-isolate of Hy
Re08	Ehlers, Germany	re-isolate of Hy
H10	Berner, Germany	Hy, 10 x propagated in grubs
H14	Berner, Germany	Hy, 14 x propagated in grubs
PS7	Ehlers, Germany	hybrid of 7 isolates
inbred lines (20)	Ehlers, Germany	inbred lines of PS7
Steinernema glaseri		
NC	Koppenhöfer, USA	
RS92	Ehlers, Germany	
NJH	Ehlers, Germany	
S10	Berner, Germany	NC, 10 x propagated in grubs

Table 2. SSRs primers which were tested using genomic DNA from *H. bacteriophora* and *S. glaseri*, respectively, and the optimal annealing temperatures for these primers.

Primer	**Annealing Temperature [°C]**	**PCR Amplification**
$(GACA)_4$	45	+
$(GGAT)_4$	45	
$(TGTC)_4$	45	+
$(GACAC)_3$	45	+
$(AAT)_5$	45	
$(TCC)_5$	45	
$(CAG)_5$	45	+
$(GATA)_4$	45	
$(ACTG)_4$	45	+
$(ACGA)_4$	45	+
$(GAA)_5$	45	
$(TGT)_5$	45	
$(GTG)_5$	45	+
$(CA)_{10}$	40	
$(GA)_8$	40	+
$(CT)_8$	40	
$(GC)_8$	40	

Results and discussion

Our preliminary results using ISSR-PCR for genetic fingerprinting of entomopathogenic nematodes showed that this method is well suited to discriminate between isolates within a species and even between inbred lines within an isolate. Since there was no prior knowledge about SSRs sequences in the genome of these nematodes we screened primers and tested different annealing temperatures (table 2). From all positive primers we created 3'- or 5'-anchored primers for our further investigations which are currently in work. The polymorphism in the banding patterns depend of the primer used in the PCR. For instance primer '01' ($(GA)_8YG$) resulted in highly conserved banding patterns whereas primer '06' ($(GACAC)_3YC$) amplified polymorphic markers even among the inbred lines of *H. bacteriophora* (figure 1). In this manner we were able to designate subgroups among the inbred lines. In combination with a systematic analysis of the variability and heritability of beneficial traits of the nematodes which is currently in work in the group of Dr. Ehlers (Johnigk et al., 2001) we hope to get a powerful tool of monitoring genetic changes while selecting nematodes. Leroy et al. (2001) were even able to detect an in vitro culture-induced instability in tissue cultures of rape (*Brassica oleracea*) via ISSR-PCR. As altered bands provide valuable information on where genomic events occurred it is possible to detect involved genes via sequencing of the amplification product. This might be interestingly if a correlation between a special trait and a defined fingerprint/marker could be detected.

All geographic isolates of the genera *Heterorhabditis* and *Steinernema* were easily to distinguish and banding profiles were reproducible. Reproducibility in ISSR analysis is much higher than in RAPD analysis due to longer primers and higher annealing temperatures used in the PCR (Zietkiewicz et al., 1994).

Table 3. Nematodes tested against third instar larvae of the European cockchafer (*Melolontha melolontha*). Hybrid 1, 2, 3 are different batches of the commercially produced hybrid strain.

Nematode	Designation	Origin	Mortality in %
Steinernema glaseri	NC	USA	40
S. glaseri	RS92	USA	60
S. spec.	Marocco	Marocco	60
S. arenaria	-	Russia	10
S. feltiae	Neud.	Neudorff	0
S. feltiae	Ehl	Germany	3
Heterorhabditis bacteriophora	HK3	Germany	20
H. bacteriophora	HDO	Germany	10
H. bacteriophora	H06	China	0
H. bacteriophora	Hybrid 1	E-Nema	5
H. bacteriophora	Hybrid 2	E-Nema	5
H. bacteriophora	Hybrid 3	E-Nema	20
H. bacteriophora	PS7	E-Nema	0
H. marelatus	-	USA	20

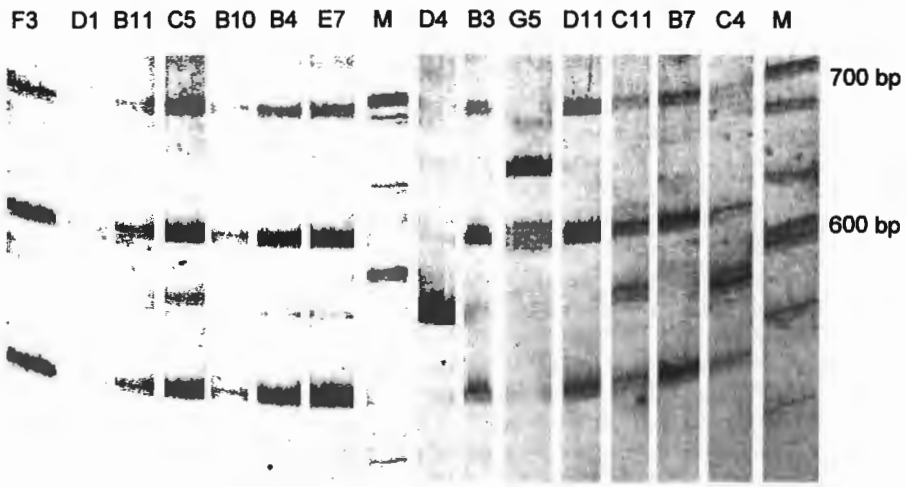

Figure 1. Banding profiles from 14 inbred lines of *H. bacteriophora* on a PA gel. Amplification products from the ISSR-PCR were obtained using primer $(GACAC)_3YC$. M = marker, F3-C4 = inbred lines.

The results of screening entomopathogenic nematodes with high infectivity against white grubs are shown in table 3. We could confirm that *S. glaseri* and closely related nematode species are the most effective species against grubs of the cockchafer. HK3 (isolate from Dr. Bathon, Germany) was the best isolate of *H. bacteriophora*. The commercially produced hybrid showed high variability between different batches (table 3).

Our selection program showed that the infectivity of *S. glaseri* (NC) against grubs could be increased by selection via host passages whereas no success could be reached with *H. bacteriophora* (Hy) (Berner & Schnetter, 2001). This result might be due to different live cycles of these two nematodes: in *H. bacteriophora* the first generation in an insect host is a self fertilising hermaphrodite whereas *S. glaseri* has only males and females. Some authors consider *H. bacteriophora* therefore as a nearly clonal organism with a low genetic variability (Shapiro et al., 1997). The via ISSR-PCR detected variability among the inbred lines of *H. bacteriophora* might be an effect of crossbreeding and the subsequent segregation of specific bands. Damodar Reddy et al. (1999) showed that the strain-specific ISSR pattern was inherited and segregated in a Mendelian fashion in the silk worm *Bombyx mori*. The increased infectivity of *S. glaseri* was reproducible and seems to be genetically stable (Berner, 2001, not published) suggesting to look for ISSR markers of the selected line. These investigations are currently in work.

ISSR analysis were proved to be a powerful tool in genetic characterising entomopathogenic nematodes. The method seems to be well suited to monitor long-term artificial culture of nematodes and selection programs as well as the crossbreeding of isolates and the field release of nematodes.

Acknowledgements

We thank Dr. R.-U. Ehlers for supplying the inbred lines.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.41 - 47

Proteolytic activation and inactivation of Cry8C from *Bacillus thuringiensis japonensis* Buibui by proteolytic enzymes in the midgut juice of *M. melolontha*

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Abstract: The successive degradation of the scarab-specific insecticidal Cry8C toxin of *Bacillus thuringiensis japonensis* Buibui (BTJ) in the midgut juice of the BTJ-resistant European Cockchafer (*Melolontha melolontha*, Scarabaeidae) was investigated in vitro: In an initial step the crystalline 130 kDa protoxin was dissolved in a chaotropic-reducing environment and subsequently activated to a toxic 65 kDa fragment by endogenous proteinases of the bacterium. Proteolytic assays with partially purified midgut juice proteinases of *M. melolontha* revealed that mainly trypsin-like enzymes are responsible for the inactivation of Cry8C by degrading it to fragments < 10 kDa.

Key words: *Bacillus thuringiensis*, Cry toxins, *Melolontha melolontha*, proteinases

Introduction

The efficacy of the Cry toxins of *Bacillus thuringiensis* (BT) depends on the solubilization and the proteolytic activation of the parasporal protoxin crystals in the gut system of susceptible insects before the cytolytic toxin specifically binds to surface receptors of midgut epithelium cells (Oppert, 1999).

There are only few BT strains which are active against coleopterans (Schnetter et al., 2001). Whereas the scarab specific Cry8C toxin of *Bacillus thuringiensis japonensis* strain Buibui (BTJ) is active against the Japanese Beetle (*Popillia japonica*) and *Anomala* species it is ineffective against the grubs and adults of the European Cockchafers (*Melolontha melolontha* and *M. hippocastani*, Scarabaeidae).

Whereas the activated 65 kDa Cry8C fragment is toxic in bioassays with *Anomala cuprea* (Hori et al., 1994) the 130 kDa Cry8C protoxin is proteolytically degraded to fragments < 10 kDa in the midgut juice of *M. melolontha* (Wagner et al., 2000).

Franken (1994) demonstrated that the coleopteran-specific 67 kDa Cry3A toxin of *Bacillus thuringiensis tenebrionis* is degraded to a 55 kDa fragment by midgut proteinases of *M. melolontha* which caused a loss of toxicity in bioassays with the Colorado potato beetle (*Leptinotarsa decemlineata*).

Paenibacillus popilliae which is an obligate pathogen of *Melolontha* spec., *Amphimallon* spec., and other scarabs produces a crystal protein (Cry18A) which is closely related to the BT toxins (Zhang et al., 1997). In contrast to Cry8C this 79 kDa toxin is stable in the midgut juice of *M. melolontha*.

On the basis of these findings it can be assumed that the conditions in the gut system lead to activation or inactivation of different Cry toxins with varying biochemical properties. Among the mechanisms of resistance to BT proteinase-mediated inactivation of the Cry toxins are frequently discussed (Oppert et al., 1997). The proteinases in the midgut of *M. melolontha* were identified and characterized (Wagner et al., 2001a, Fig. 1). In this study we

present the proteinases which are critical in the destruction of the Cry8C toxin using partly purified midgut proteinases. Moreover, successive degradation steps could be identified.

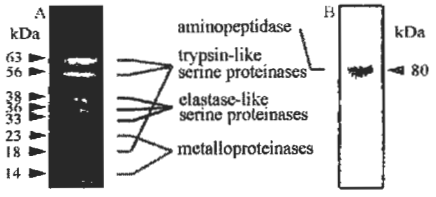

Figure 1. Proteinases of the midgut juice of *M. melolontha* (Wagner et al., 2001a). In addition to the endoproteinases presented in (A) which were identified by a different zymogram technique there are also endoproteinases > 100 kDa (see Fig. 3B, # D6). (B) Activity blot.

Materials and methods

Culture of bacteria; preparation and purification of crystals

BTJ was obtained from J. S. Feitelson, Molecular Biology, Mycogen Corporation. Bacteria were cultured according to Faloci et al. (1990). Bacteria-autolysed spores and crystals were washed 3 times with 50 mM Tris/HCl pH 8.0, 5 mM $CaCl_2$ (buffer A) and subsequently applied to a continuous 34-56 % (weight/vol) NaBr gradient and centrifuged at 80000g for 15 h at 5 °C. The bottom band was collected and washed 3 times with buffer A.

Solubilization of crystals and preparation of toxin

Crystals were solubilized in chaotropic-reducing conditions (5 % crystals, 250 mM DTT, 250 mM Na_2CO_3 pH 8.0) for 90 minutes at room temperature with a magnetic stirrer. After centrifugation (15000g, 5 min) the supernatant was concentrated 10 fold with a vivaspin concentrator (Vivascience) and subsequently dialysed against 50 mM Tris/HCl pH 8.0 (buffer A*) for 16 h at 4 °C to remove DTT and Na_2CO_3. After another centrifugation (15000g, 5 min) the toxin-containing supernatant was aliquoted and stored at –20 °C until use.

Insect cultures

Third instar larvae of *M. melolontha* were collected near Obergrombach, Germany. Larvae were fed with carrot slices and kept in the dark at 14 °C from March to October and at 7 °C from November to February. Insects which refused food were not used for experiments.

Preparation of midgut juice

Larvae were rinsed with water and chilled on ice for at least 10 minutes. Most of the hemolymph were carefully pressed out of the larval bodies through openings made by cutting off the legs. The head capsules were removed. The anterior end of the midgut was prepared by carefully squeezing the posterior end of the larvae. Residual hemolymph was sucked up with absorbent tissue. The luminal contents of the midgut were collected by penetrating the midgut epithelium with a pipette and subsequently centrifuged (150,000 g at 4 °C for 2 h) to remove insoluble material. The supernatant, henceforth called midgut juice, was aliquoted and kept at -70 °C until use. Dilutions were carried out with distilled water.

Ammonium sulfate precipitation

The midgut juice was diluted 1:10 with distilled water, completed with 40 % ammonium sulfate and stirred for 4 hours. After centrifugation (15000g, 5 min) the pellet was solubilized in 1/10 volume of buffer A and subsequent dialysed against buffer A for 16 h at 4 °C. After

another centrifugation (15000g, 5 min) the supernatant was subjected to column chromatography.

DMAE- and Benzamidine Sepharose column chromatography

The midgut juice proteinases were fractionated by chromatography on Fractogel DMAE Sepharose (Böhringer) equilibrated with buffer A. The column size was 0.5 cm in diameter and 2 cm long. Chromatography was performed with buffer A at 4 °C with a gradient of 0 – 2 M NaCl. Fractions of 0.5 ml/ 1 min were collected and either used for proteolytic assays or pooled for dialysis (buffer A, 16 h, 4 °C, supernatant) and subsequent Benzamidine Sepharose 6B (Pharmacia) column chromatography using the same chromatography conditions.

Protein concentration

The concentration of protein was measured according to the method of Bradford (1976). Bovine serum albumin solutions of 0-2 mg/ml were used as standard.

Proteolytic assays

Proteolytic reactions were carried out with buffer A*, a final toxin concentration of 1 mg/ml, and ½ Vol of column chromatography fractions for 16 hours at room temperature.

Analysis of proteolytic activity with zymograms

Zymograms were prepared according to Heussen and Dowdle (1980): Samples were prepared with 5x non-reducing sample buffer (0.4 M Tris pH 6.8, 5 % SDS, 20 % glycerol, and 0.03 % bromophenol blue) without heating, incubated at room temperature for at least 10 minutes, centrifuged at 15000 g for 5 minutes and loaded onto the gel (12.5µl/lane). Casein was copolimerized into a 15% SDS-gel to a final concentration of 0.1 %. After electrophoresis, SDS is removed by washing the gel in 2.5 % Triton X-100 with gentle agitation at room temperature for one hour, followed by an incubation of 20 minutes at room temperature in buffer A. Gels were stained overnight in 30 % ethanol, 10 % acetic acid, 0.5 % Coomassie brilliant blue G-250 and destained with 20 % ethanol, 7 % acetic acid.

Sample preparation and SDS-PAGE

Samples were incubated for 2 minutes at 95 °C to stop proteolytic reactions. Samples were completed with 4x sample buffer (Roti-Load1, Roth) and subjected to the sample preparation method and SDS-PAGE described by Laemmli (1970). 15 µg protein/lane was loaded onto 15 % minigels. Gels were stained with 0.1 % Coomassie Brillant Blue R, 45 % EtOH, 10 % acetic acid for 1 h and destained with 20 % EtOH, 7 % acetic acid. Molecular mass determinations which were based on the protein standards 69810 and 69811 (Fluka) were carried out using the BioDocAnalyze program (Biometra).

Results and discussion

Solubilization and activation of Cry8C

In earlier studies we showed that the 130 kDa Cry8C protoxin of BTJ is proteolytically degraded to fragments < 10 kDa without intermediates in the midgut juice of *M. melolontha* (Wagner et al., 2000). This process implies a solubilization mechanism, because proteinases usually need soluble protein substrates. As the pH of the midgut juice is about 8.0 an alkaline solubilization of the crystals which can be found in heteropterans (Reeck et al., 1999) can be excluded. Instead, chaotropic-reducing conditions were successfully used to solubilize the Cry8C crystals in vitro. It can be assumed that such an environment might also be present in vivo.

SDS-PAGE revealed that the 130 kDa protoxin was degraded to a 65 kDa fragment in every solubilization technique used (Fig. 2). Hori et al. (1994) demonstrated that this fragment is active against *Anomala cuprea*. The activation is due to endogenous proteinases of BTJ which could not be seperated from the crystals during the purification steps (see Materials and

Methods). These proteinases are known to be tightly bound or integral factors of BT crystals and they are responsible for the proteolytic activation of protoxins in some other BT strains (Oppert, 1999). Inhibitor studies with zymograms revealed that the endogenous proteinases of BTJ are high molecular metalloproteinases (Wagner, unpublished).

The chaotropic-reducing environment also enables the trypsin-like enzymes of the midgut juice to degrade the 130 kDa protoxin (Wagner, 2000). Thus, two parallel processes might occur in vivo, whereupon the trypsin-operated degradation of Cry8C to fragments < 10 kDa superposes the endogenous proteinase-generated 65 kDa toxin stability.

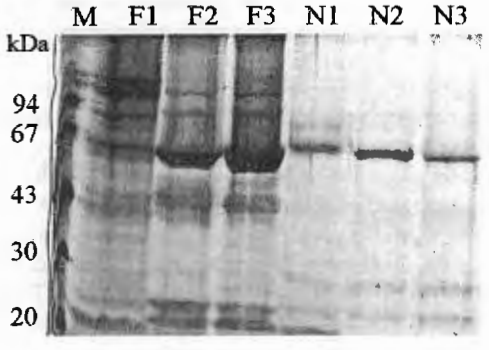

Figure 2. SDS-PAGE analyzing the solubilization and degradation of the 130 kDa protoxin crystals to a 65 kDa toxin. Various alkaline (F1-F3) and chaotropic-reducing (N1-N3) methods were tested (Wagner, methods not shown). In F1 intermediate bands > 65 kDa are also visible. This is due to an incomplete degradation at shortened incubation periods, i. e. 30 min. instead of 90 min. (see Materials and Methods).

Degradation of the 65 kDa toxin to a 55 kDa fragment

Two methods were used to investigate the further degradation of the 65 kDa toxin which is stable in the absence of the midgut juice proteinases. (1) Proteinase inhibitor studies with the midgut juice revealed that exclusively trypsin-like proteinases are responsible for the degradation to a 55 kDa fragment (Wagner, 2001b). (2) In this study, discrete partially purified midgut juice proteinases (Fig. 3) were tested. SDS-PAGE revealed that column chromatography fractions containing trypsin-like proteinases (Fig. 3A, # D1,# 6-11; Fig. 3B, # P) caused a degradation of the 65 kDa toxin to a 55 kDa fragment (Fig. 4A, # D1,# 6-11; Fig. 4B, # P). In contrast, the 14 kDa metalloproteinase (Fig. 3B, # 52) and the endoproteinases >100 kDa (Fig. 3B, # D6) had no effect (Fig. 4B, # 52, D6). Proteolytic assays with fractions containing elastase-like proteinases (Fig. 3B, # 56, 60) resulted in slight decreases of the 65 kDa band intensities (Fig. 4B, # 56, 60), but a 55 kDa fragment could not be demonstrated. As this effect was only observed after an incubation period of 16 hours, it is unlikey that these proteinases play a major role in this single degradation step. The same reason can be given for the 23 kDa metalloproteinase (Fig. 3B, # 56; Fig. 4B, # 56).

Bioassays with BTJ-sensitive scarabs should provide information about the toxicity of the 55 kDa fragment. If there is none, this degradation step could be the reason for the resistance of *M. melolontha* to BTJ.

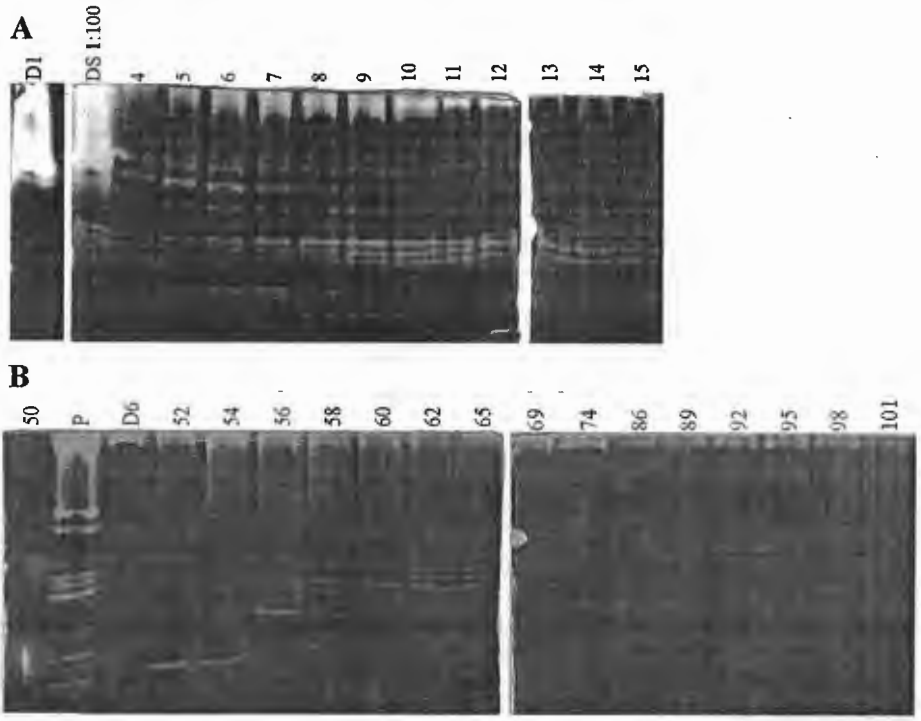

Figure 3. Zymograms analyzing proteolytic activity of fractions obtained by (A) DMAE column chromatography, (B) Benzamidine Sepharose column chromatography. Abbreviations: DS 1:100, 1:100 diluted midgut juice; D1, flow through of (A); # 4-15, elution fractions of (A); P, pool of fractions # 4-12 of (A) used as starting material for (B); D6, flow through of (B); # 50-101, elution fractions of (B).

Degradation to fragments < 10 kDa

The artificial in vitro conditions which include partial purified proteinases and dialysed toxin might explain the paradox that proteolytic assays result in a stable 55 kDa fragment instead of degrading it to fragments < 10 kDa. Restoring the putative chaotropic-reducing midgut environment using Na_2CO_3 and DTT this final proteolytic process could be simulated (data not shown). It can be assumed that structural changes of Cry8C occur under these conditions which make new proteolytic cleavage sites available leading to the final fragmentation of the protein.

Studies on the identification of the proteinases which are critical in this process are presently done. Most interestingly, a comparison with the processes occurring in a BTJ-sensitive scarab should finally explain the ineffectiveness of BTJ to *M. melolontha*.

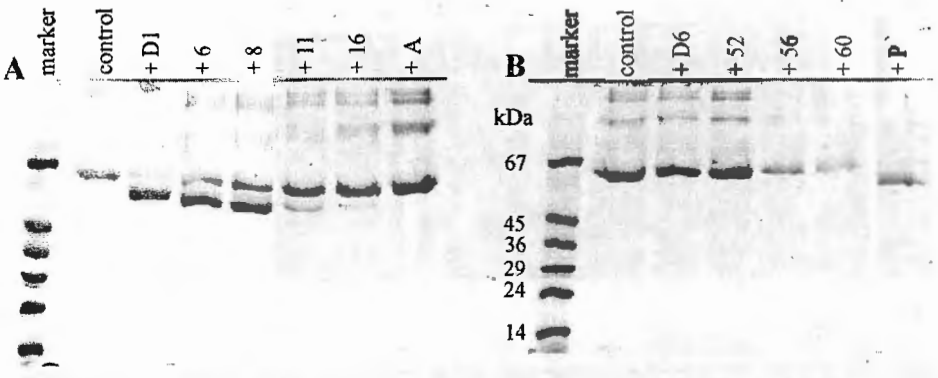

Figure 4. SDS-PAGE analyzing the degradation of the active Cry8C toxin (65 kDa) with fractions of (A) DMAE column chromatography, (B) Benzamidine Sepharose column chromatography. Abbreviations: control, 65 kDa toxin; + D1/D6, toxin + flow through of (A/B); + "number", toxin + elution fraction of (A/B); + P, toxin + pool of fractions # 4-12 of (A); + A, toxin + fraction A. This fraction exclusively contained the 14 kDa metalloproteinase. This was obtained using 40-50 % ammonium precipitate of the midgut juice followed by DMAE column chromatography (data not shown).

Acknowledgements

We thank Dr. Ralf Bischof (German Cancer Research Center, Heidelberg) for the technical HPLC support and Oliver Abel for the preparation and purification of BTJ crystals..

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Biological control with *Beauveria brongniartii*

Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.51 - 58

First field trials to control *Melolontha melolontha* with *Beauveria brongniartii* in Christmas trees in Denmark

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Abstract: Two Christmas tree plantations of *Abies nordmanniana* in Denmark (Vallø and Simmersholm) were treated with barley kernels colonised by *Beauveria brongniartii* (produced by Agrifutura, Italy) in spring for control of *Melolontha melolontha*. The barley kernels were either applied in holes around the trees at 10 cm depth or mixed into soil from the planting hole during replantation of trees. In addition, some trees were covered with net during the flight period of *M. melolontha*. At both locations soil samples were taken before application of the fungus and then 1, 6 and 12 months after application. For comparison of the persistence, *B. brongniartii* was also applied as conidia suspension in holes around the trees at 10 cm depth.

Tree damages were estimated from general tree health and colour scored in autumn 2001. Most of the trees were healthy and without damage, probably due to low populations of white grubs and good ability for tree recovering, however in the trials where the soil was mixed with the barley kernels, significantly more trees were damaged in the control than in the *B. brongniartii* treatment.

The fungus persisted or grew well at 5-25 cm depths, in all experiments. However, the densities of cfu's in 5 –15 cm's depth varied a lot between the treatments and production batch used. The lowest concentration found was $10^3 - 10^4$ cfu's per g of soil, while the highest concentration found was $10^5 - 10^6$ cfu's per g of soil. This was in accordance with results obtained by *Galleria* bait experiments where a much higher percentage of larvae were infected with the fungus in the soil with the high level of cfu's compared to the low concentrations.

Key word: European cockchafer, *Melolontha melolontha*, *Abies nordmanniana*, Nordmann fir, *Beauveria brongniartii*, biological control, entomopathogenic fungi.

Introduction

The European cockchafer, *Melolontha melolontha* L. (Coleoptera: Scarabaeidae) is a serious pest in the production of Christmas trees in Denmark (Harding, 1994). The larvae cause extensive and lethal damage on the roots of especially young Nordmann fir Christmas trees (*Abies nordmanniana*) (Harding, 1994). Chemical control of the soil-dwelling larvae of *Melolontha* spp. is not allowed in Danish forestry and mechanical control is not possible in these perennial crops. The growers have therefore no options to control the scarab larvae and thus prevent attacks. The fungus *Beauveria brongniartii* (Sacc.) Petch (Deuteromycetes: Moniliales) is considered to be the most important natural enemy of *M. melolontha* and promising results of biological control have been obtained in orchards and pastures using barley kernels colonised by this fungus (Keller, 1992; Zimmermann, 1992; Zelger, 1996; Keller *et al.*, 1997).

The potential for microbial control with *B. brongniartii* against *M. melolontha* larvae in Christmas trees was explored. Results of application of *B. brongniartii* were examined by determining the persistence in soil and scoring health/damage of Christmas trees caused by *M. melolontha*.

Materials and methods

Field experiments
Two field sites of *Abies nordmanniana* were selected based on earlier records of root damage caused by *M. melolontha*. Both plantations were around 0.5 ha and established in 1997 with 10.000 trees per ha. Since 1998 trees have been replanted each spring due to damage caused by *M. melolontha*. *B. brongniartii* was applied either as barely kernels colonies with the fungus or as conidial suspension (a detailed plan is shown below).

Barley kernels produced by Agrifutur srl in Italy were applied with 300 kg / ha (10 times the recommended doses), except in one treatment where 500 kg/ha was applied. Conidia were applied in a concentration of $1x10^{13}$ per ha. Extra trees were treated for the persistence studies and the exact placements of barley kernels or conidial suspension were marked with a small plastic stick.

The five experiments:

Vallø 1 (3 x 10 trees per treatment) [application date 2 May 2000]:
1. Control – untreated
2. 30 g barley kernels with BIPESCO 1 (batch a) were placed evenly in 10 holes in 10 cm's depth
3. Net (P-17, fibre net) covered the trees to prevent egg laying of adult *M. melolontha*
4. Combined treatment of 2 and 3

Vallø 2 (3 x 20 trees per treatment) [application date 14 April 2000]:
1. Control - untreated
2. 30 g barley kernels colonised with BIPESCO 1 (batch b) were mixed into approx. 2 l soil from the planting hole during replantation of trees

Vallø 3 (20 trees per treatment) [application date 11 July 2000]:
1. Control – untreated
2. 30 g barley kernels with BIPESCO 1 (batch a) were placed evenly in 10 holes in 10 cm's depth
3. 30 g barley kernels with BIPESCO 1 and 2 were placed evenly in 10 holes in 10 cm's depth
4. Conidial suspension (100 ml of 10^7 conidia/ml) of BIPESCO 1 produced on SDA were placed evenly in 10 holes in 10 cm's depth

Vallø 4 (10 and 15 trees per treatment) [application date 20 April 2001], one 3rd instar *M. melolontha* larva was added to each tree one month after application of barley kernels:
1. Control – untreated.
2. 50g barley kernels colonised with BIPESCO 2 were mixed into approx. 5 l soil from the planting hole during replantation of trees

Simmersholm (4 x 10 trees per treatment) [application date 9 May 2000]:
1. Control –untreated
2. 30 g barley kernels with BIPESCO 1 (batch a) were placed evenly in 10 holes in 10 cm's depth

Health score

From the treated plots at Vallø (1, 2 and 4) and Simmersholm tree health was scored based on viability and colour of the trees at 25 Oct. 2001 and 15 Nov. 2001, respectively. Vallø 3 was only used for persistence studies. A scale from 0 to 5 was used, category 0 was a dead tree, 1 – 3 was very damaged, light yellow to light green, 4 was moderately damaged and 5 was a dark green healthy tree. On some of the trees the root was also examined and a good correlation was found between damage and scored categories.

For statistical analysis of data the Mantel-Haenszel estimate of the weighed common Odds-ratio ($\square^*_{MH}$) between healthy (category 5) and damaged (categories 0 - 4) trees was calculated (Mantel & Haenszel, 1959) and a Chi-square test was used to test the null hypothesis that the weighed common Odds-ratio is equal to 1 (no difference between treated and control plots).

Persistence of *B. brongniartii* in soil

From the field sites at Vallø and Simmersholm soil samples were taken before application of the fungus and then after 1, 6 and 12 months. Samples were taken with a cylindrical soil sampler with an inner diameter of 3 cm at three depths (0-5, 5-15 and 15-25 cm), three samples were taken per plot and each sample consisted of a mix of three samples taken from the same tree. The samples were stored at 5°C at constant darkness until analysed. The persistence of *B. brongniartii* was determined both by the *Galleria* bait method (Zimmermann, 1998) and by soil dilution plate method (Goettel & Inglis, 1997). For bait experiments, approximately 60 g soil per sample was filled in a cylindrical plastic beaker with a diameter of 6 cm and a height of 6 cm and six *Galleria mellonella* larvae were added. The soil was incubated for 14 – 18 days at 20 – 22°C at constant darkness. During the first 5 days the samples were turned upside down every day. After incubation the larvae were observed for the occurrence of entomopathogenic fungi, which were determined to species. The samples were divided into four categories; 0= no larvae infected with *B. brongniartii*, 1= 1 – 32%, 2= 33 – 66% and 3= 67 – 100% infected with *B. brongniartii*.

The density of *B. brongniartii* in the samples was determined using a semi-selective medium (Strasser *et al.*, 1997). Two g from each soil sample was whirlmixed for 35 sec. in test tubes with 18 ml 0.05% Triton X-100 and eventually further diluted before 0.1 ml was spread onto three plates with the semi-selective medium (10 g peptone, 20 g glucose, 18, agar, 0.6 g streptomycin, 0.05 g tetracycline, 0.05 g cyclohexamide and 0.1 ml dodine/Radspor FL. Three replicates per soil sample were prepared and after 8-10 days at 20°C cfu=s (colony forming units) were counted.

Results

Health score

The scores of tree health are shown in figure 1. Most of the trees were healthy and without damage and mainly trees from categories 4 and 5 occurred. However, significantly more healthy trees were found in the treated plots in Vallø 2 ($p = 0.04$) and Vallø 4 ($p=0.001$) than in the control plots.

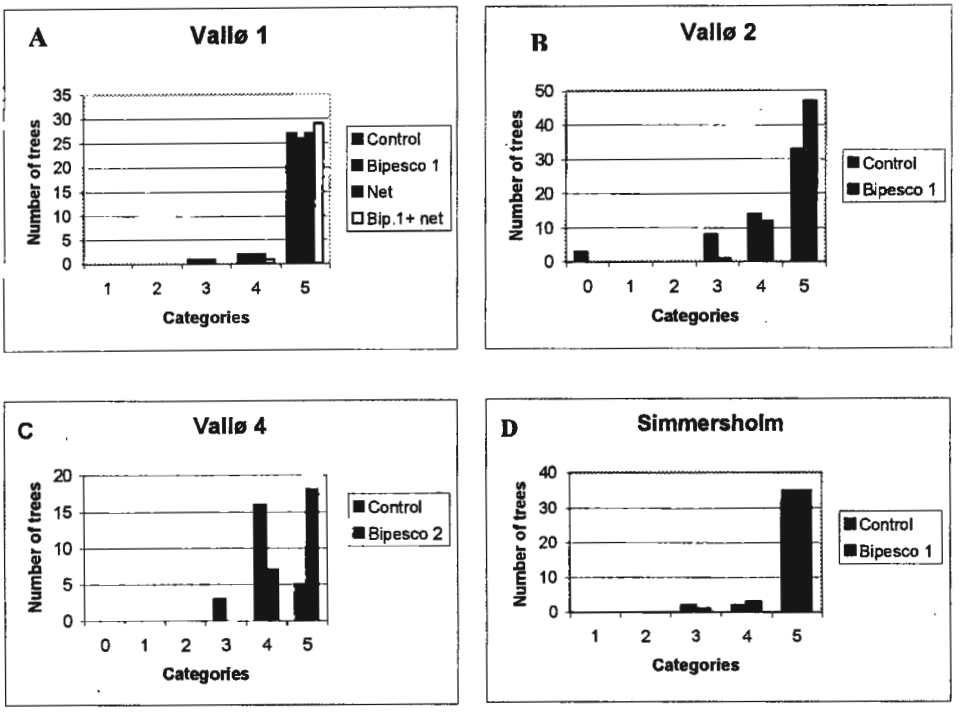

Figure 1. Health score of Christmas trees (*A. nordmanniana*) from 4 experiments evaluated on a scale from 0 to 5. Category 0: dead tree, 1 – 3: very damaged, 4: moderately damaged and 5: dark green, healthy tree.

Persistence of* B. brongniartii *in soil

The density of *B. brongniartii* cfu's in the soil was in all experiments approximately the same one year after application, as the density immediately after application (figure 2 and 3). The density of cfu's varied a lot between the production batches. The lowest density found was $10^3 - 10^4$ cfu's per g soil (figure 2 A and C), while the highest density was $10^5 - 10^6$ cfu's per g soil (figure 2 B and figure 3). At the high concentration more *Galleria* larvae were infected with the fungus when the soil was baited compared to the low concentration. The concentration of cfu's in 0 – 5 cm was normally lower compared to the soil at 5 – 25 cm depth. This was mainly caused by the fungus being applied at 10 cm depths; however in Vallø 2 where the barley kernels were mixed into the soil the concentration was still considerably lower at 0 – 5 cm compared to 5 – 15 cm depths. In Vallø 3 where different production batches of BIPESCO no. 1 and 2 were compared, the cfu's varied by 100 times between the lowest and the highest concentration in the soil (figure 3).

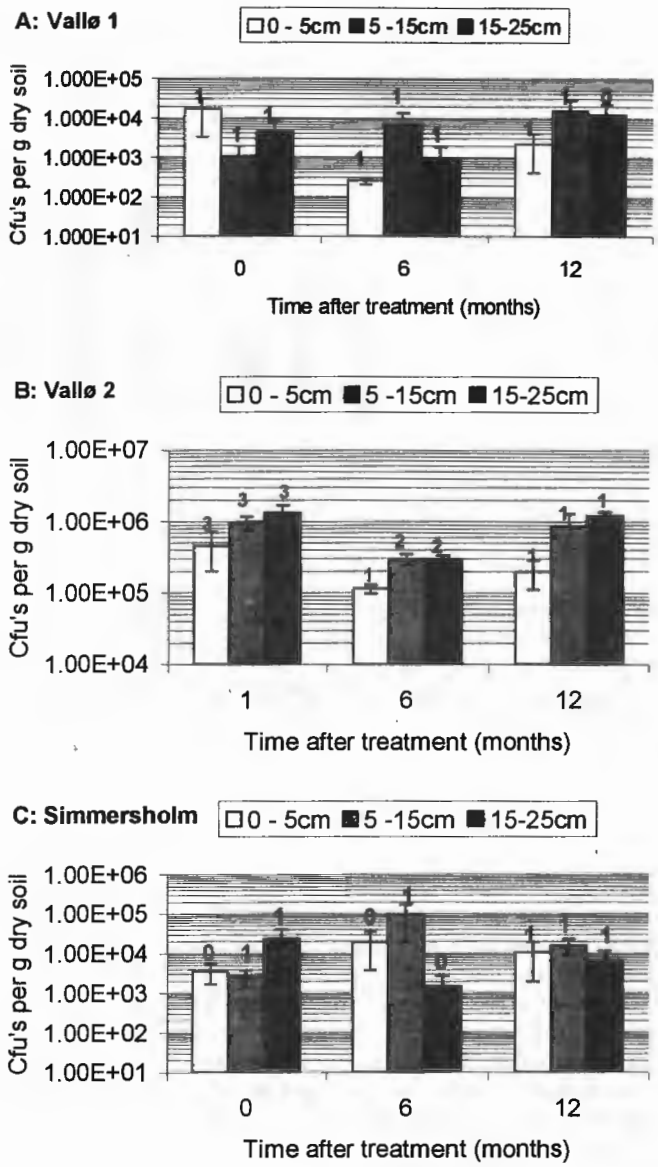

Figure 2. Density of *Beauveria brongniartii* at Vallø 1, 2 and at Simmersholm (Cfu's/g dry soil). Error bars indicate S.E of mean. Numbers above bars; 0= no *Galleria* larvae infected with *B. brongniartii* from this soil, 1= 1 – 32%, 2= 33 – 66% and 3= 67 – 100% infected with *B. brongniartii*.

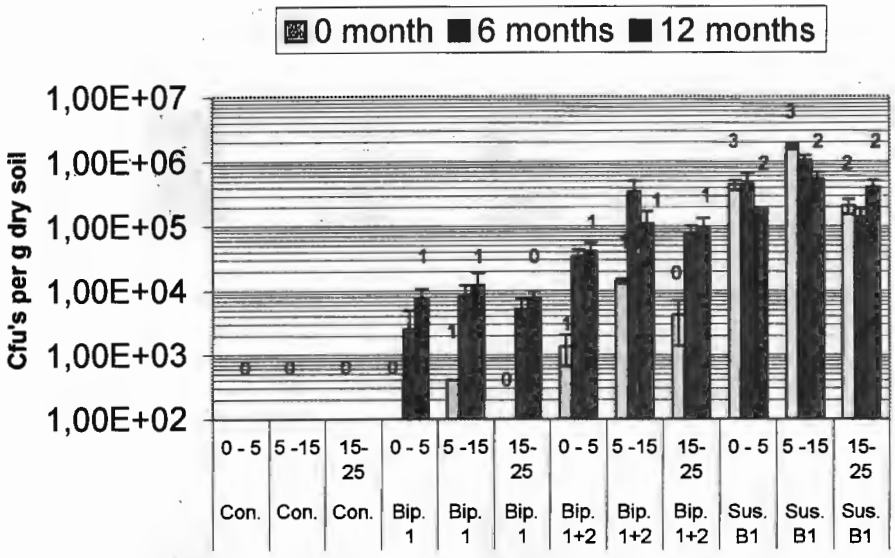

Figure 3. Density of *Beauveria brongniartii* at Vallø 3 (Cfu's/g dry soil). Error bars indicate S.E of mean. Numbers above bars; 0= no *Galleria* larvae infected with *B. brongniartii* from this soil, 1= 1 – 32%, 2= 33 – 66% and 3= 67 – 100% infected with *B. brongniartii*. Con = control, Bip. 1= Barley kernel colonised with BIPESCO strain no 1, Bip. 1+2= Barley kernel colonised with BIPESCO strain no 1 and 2, Sus. B1= Suspension of BIPESCO strain no 1.

Discussion

The damage caused by *M. melolontha* can be lethal to Christmas trees (*A. nordmanniana*) especially the first years after planting, but in many cases is it possible for the trees to recover even though almost all roots have been eaten (Harding, 1994). Recovery may happen when the larvae pupate, the larvae die, move to another tree or seek down to 30 to 50 cm depth where it over winter. Tree recovering is furthermore favoured by the fact that the trees especially in spring are able to grow at lower temperatures than the activity of larvae occurs. In our plantations in Denmark, *M. melolontha* has a four-year life cycle (Jørgensen, 1982), and year 2000 was a flight year on the field sites studied. This means that pupae and first instars larvae of *M. melolontha* dominated the field sites in 2000 and in 2001 field sites were dominated by first and second instar larvae.

For experiment Vallø 1 and Simmersholm the population of *M. melolontha* was not dense enough to kill the trees. In the experiment Vallø 2 the population of *M. melolontha* must have been higher and eventually several strains of *M. melolontha* were present compared to Vallø 1 and Simmersholm, since more damage occurred in the control plots. Also the good persistence or growth of *B. brongniartii* gave good control of the treated trees. In Vallø 4 where larvae were added to all trees good control was obtained for the treated trees.

The trend for causing high infection of *Galleria* was mainly when the concentration in the soil was above 10^5 cfu's g^{-1}. A correlation has also been found between the concentrations

of *B. brongniartii* cfu's in soil and infection of *Galleria* as well as control of *M. melolontha* larvae (Keller, pers. com.).
The persistence study of different batches of *B. brongniartii* also shows the importance of starting with a good product or a high number of conidia, because in most cases the number of cfu's did not increase much after application but the fungus persisted well at least for one year.

Conclusion

Economic damage was seen in the untreated plots. The treatment with *B. brongniartii* resulted in significant better health status of the trees, thus demonstrating that the fungus may assist in protection of the small trees during a very sensitive period of their life. The persistence of *B. brongniartii* was sufficient to ensure a long-term control.

We believe that *B. brongniartii* has a high potential for controlling *M. melolontha* larvae in Christmas trees. Several obstacles must be passed before commercial usage, including:

- *Timing of application.* It seems that the best way to apply is to mix into soil during plantation or replantation.
- *Dosage.* The dosages used were relatively high and lower dosages should be tested.

Acknowledgements

We thank J. Martin, C. Wolsted, R. Eliasen and C. Fisher for technical help and support and EU for funding by FAIR6-CT98-4105.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.59 - 63

How many spores of *Beauveria brongniartii* are needed to control *Melolontha melolontha*?

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Abstract: The larvae of the European cockchafer can be controlled with the entomopathogenic fungus *Beauveria brongniartii*. The fungus is produced on peeled barley kernels (fungus kernels). On a kernel it produces an average of 3.3 x 10^8 conidia on the surface of native soil (corresponding to 9.2 x 10^9 conidia g^{-1} product) or 2.33 x 10^7 CFU g^{-1} in native soil. The application of 40 kg fungus kernels theoretically corresponds to a dose of 3.7 x 10^{14} conidia ha^{-1} or to about 3 x 10^5 conidia g^{-1} soil. Calculating with colony forming units (CFU) the same dose results in 1.6 x 10^{13} ha^{-1} or 2 x 10^4 CFU g^{-1} soil. This value corresponds to or is slightly higher than the observed fungus density in fields treated with *B. brongniartii* for control purposes. It is assumed that the control success is the result of an induced epizootic and not the result of a high initial mortality. To improve or enhance the control process, a repeated application is recommended.

Key words: *Melolontha melolontha, Beauveria brongniartii,* microbial control, application rate, fungus density, ecology

Introduction

Beauveria brongniartii (Sacc.) Petch (Deuteromycota, Hyphomycetes) is an important natural enemy of the European cockchafer *Melolontha melolontha* L. (Coleoptera, Scarabaeidae). Epizootics caused by this fungus have frequently been observed and inspired researchers to use it for the control of this pest insect for more than a century. The question about the spore density necessary for the control of *Melolontha* larvae was first raised by French scientists (e.g. Ferron, 1978). They assumed that about 15 x 10^9 conidia m^{-2} would be needed, which was three times the amount produced on a single third instar white grub. However, better estimations can be done when spore densities are calculated based on dose-mortality-relations or determined either in areas where natural epizootics occur or where successful field trials were carried out. Data from the literature and from own investigations are compiled and discussed and put in relation to currently running control measures.

Material and methods

Conidia production on barley kernels

Commercial material "Beauveria Schweizer" produced by E. Schweizer Samen AG, Thun, Switzerland, was used. The product consists of peeled barley kernels colonised with *Beauveria brongniartii* (fungus kernels). In the first trial 20 kernels were spread individually on the surface of native soil, sterile soil and wet peat in five replicates each and incubated at 22^0C. After a month 10 kernels per method and replicate with optimal fungus growth were mixed in 100 ml water with 0.5% Tween 80 with a Vortex blender. After appropriate dilution the conidia were counted in a Thoma haemocytometer.

In the second trial three kernels were placed in 60 ml plastic boxes filled with 20 g native soil with 15 replicates. The soil, previously investigated and found to be free of spores of *B. brongniartii*, completely surrounded the kernels. After incubation at 22^0C for one month and three months respectively, the content of the plastic boxes was transferred to 200 ml Erlenmeyer flasks filled with 100 ml tap water with 0.1% Tween 80 to favour disaggregation. The flasks were shaken for three hours on a longitudinal shaker at 120 rpm. After 15 seconds of sedimentation 0.1 ml of the suspension was distributed with a Trigalsky spatula on a Petri dish (9 cm diameter) with selective media adapted from Strasser et al. (1997) (Keller et al., 2000).

Determination of the fungus density in soils

Soil samples were taken with a cylindrical soil sampler with an inner diameter of 5.5 cm. They were taken from a soil depth between 5 and 15-18 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 sieved through a metal sieve with 5 mm meshes. 20 g per sample of fresh soil were shaken for 3 hours at 120 rpm on a longitudinal shaker in 200 ml Erlenmeyer flasks with 100 ml tap water containing 1.8 g/l tetra-Sodiumdiphosphate-Decahydrat (= tetra-Sodiumpyrophosphate; $Na_4P_2O_7*10H_2O$) 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 per soil sample were prepared. After 8-10 days at 20°C the colonies of *B. brongniartii*, which have a typical appearance and stain the medium red-brownish, were counted. The soil suspension was not diluted, even if there were very high fungal densities exceeding 100 colonies/Petri dish. The fungul densities were expressed colony forming units (CFU) g^{-1} dry soil.

We use a semi-selective medium adapted from Strasser et al. (1996) 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.

Weight and number of fungus kernels

The weight of 1000 fungus kernels was 36.07 g. Therefore, a kilogram of the product contains 2.77×10^4 fungus kernels and the amount recommended for white grub control, which is 40 kg ha^{-1} contains 1.11×10^6 fungus kernels. A gram of the product corresponds to 27.7 fungus kernels.

Results

Trial 1: After four weeks the fungus on a single barley kernel produced on average 3.31×10^8 conidia on the surface of native soil, 2.42×10^8 on sterile soil and 2.14×10^8 on peat. The differences between native and sterile soil were statistically significant (t-test, p=0.0018, F = 20.88). The number of conidia produced on peat tended to be lower than on sterile soil and native soil, but differed significantly only from native soil (ANOVA, Tukey's HSD for different N, p= 0.0045, F=14.60). On sterile soil the fungus colonised a larger portion of the soil than on native soil, which could have resulted in the loss of some fungal parts when the kernels were picked off. Taken the amount of conidia produced on native soil, one gram of the product produced 9.2×10^9 conidia.

Trial 2: Fungus kernels produced on average 5.71×10^8 CFU per kernel in sterile soil after one month and 3.60×10^8 after three months. In native soil they produced 1.44×10^7 CFU per kernel after one month and 2.33×10^7 CFU per kernel after three months.

Fungus densities in fields colonised with *Melolontha* but without a treatment did not exceed 10^5 CFU g^{-1} soil (Table 1) . Recent investigations in Switzerland revealed maximum densities between 1.4-6.6 x 10^3 CFU g^{-1} soil (Keller et al., in prep.; Kessler, in prep.).

Table 1: Natural densities of *Beauveria brongniartii* in meadows at sites with *Melolontha melolontha*.

Author	Country	CFU/g soil (field average)		*M. melolontha*
		range	maximum	L / m^2
Ferron, 1983	France	10^2, 0 - 10^5	10^{5*}	76 L2, ca. 40 L3
Fornallaz, 1992	Switzerland	6.1 x 10^2		1.8 L3 + 2.2 with *B.brongniartii*
Strasser, 2000	Austria	0		
Keller et al. (in prep.)	Switzerland	0 - 1.4 x 10^3	1.4 x 10^3	Several fields with differnt densities
Kessler (in prep.)	Switzerland	8 – 6.6 x 10^3	6.6 x 10^3	

*assumed to be a spot with a diseased white grub

Soils treated with the commercial product contained on average between 73 and 7.1 x 10^3 CFU g^{-1} soil three months after application (Table 2). In presence of high densities of white grubs fungus densities increased up to 2 x 10^5 CFU g^{-1} soil, which is obviously the result of induced epizootics (Table 2).

Table 2: Densities of *Beauveria brongniartii* after application in meadows.

Author	Country	CFU/g soil (field average)		*M. melolontha*
		range	maximum	L / m^2
Ferron, 1983	France	10^4 - 10^5	10^5	76 L2, ca. 40 L3
Fornallaz, 1992	Switzerland	35 – 2.8 x 10^3	4 x 10^{4*}	53-82 L2
Strasser, 2000	Austria	10^4 – 2 x 10^5	2 x 10^5	70 L3
Keller et al.	Switzerland	11 - 3.9 x 10^3	1.9 x 10^{5**}	Ca. 40 L3, 80% mycosed larvae
Kessler***	Switzerland	73 - 7.1 x 10^3	7.1 x 10^3	

* single soil sample with a diseased white grub after 10 months

** two soil samples from an area with induced epizootic

*** 3 months after spring applications

Discussion

The recommended dose for the control of *Melolontha* larvae with fungus kernels in meadows is 40 kg (Keller, 2001). The number of conidia produced on this amount of kernels can be calculated either starting with the production of conidia on the soil surface or within the soil. We found that 3.31x 10^8 conidia were produced on a fungus kernel on native soil and calculated, that 1.11 x 10^6 fungus kernels ha^{-1} are applied. This results in a amount of 3.67 x 10^{14} conidia ha^{-1}. Supposed this amount is distributed in a 10 cm thick soil layer and the specific weight of the soil is 1.3 kg we can calculate that the conidia are distributed in 10^6 liter

soil with a weight of 1.3.x 10^9 g. The number of conidia g^{-1} soil can be calculated by dividing 3.67 x 10^{14} conidia by 1.3 x 10^9 g, which gives 2.8 x 10^5 conidia g^{-1} soil.

Starting with the production of CFU in native soil (1.44 x 10^7 CFU per kernel after one month) and using the same assumptions we get 1.60 x 10^{13} CFU ha^{-1} corresponding to 1.23 x 10^4 CFU g^{-1} soil. Taking the CFU production after three months this amount increases to 1.99 x 10^4 CFU g^{-1} soil. The relation between conidia and CFU per g native soil is 22.7 (28 x 10^4 / 1.23 x 10^4) when considering the values after one month. Neglecting some methodological differences the two values differ by a factor of about 20. However, these two values cannot be compared directly. The higher value represents the theoretical production potential of the fungus kernels while the lower value represents the number of fungal propagules that have developed in the soil and grow on selective media. But we don't know how many propagules are actually produced in the soil and how the proportion of successful re-isolation is. This proportion can be estimated to be between 1 and about 20.

Under natural conditions *B. brongniartii* could be found only at sites colonised by *Melolontha* spp. Maximum densities reached about 7 x 10^3 (Kessler, unpubl.) or 10^5 CFU g^{-1} soil at places with epizootics (Ferron, 1983) (Table 2). After the application of the commercial product fungus densities between about 10^2 and 10^4 CFU g^{-1} soil are usual with maximum densities of about 10^5 CFU g^{-1} (Table 2). It must be added that the data compiled in the tables 1 and 2 were achieved with two different selective media. Ferron and Fornallaz used that developed by Joussier (19977) while Strasser, Keller and Kessler used the one developed by Strasser & al. (1997).

LD50 values for this fungus-host combination range between 10^4 and 10^6 conidia g^{-1} peat depending on the isolate (Ferron, 1967). With virulent isolates nearly 100% mortality can be achieved with 10^7 conidia g^{-1} soil (Kessler & al., in prep.). The application of 40 kg fungus kernels ha^{-1} results in about 10^3-10^4 CFU g^{-1} soil which corresponds to a maximum of about 10^5 conidia g^{-1} soil. This values correspond to about the number of conidia needed to achieve an LD50. However, this amount is in most cases not sufficient to induce direct control (inundation strategy) but to induce epizootics supposed the density of white grubs is above a critical level (inoculation strategy). Such densities can not be reached by treating swarming adults with spores. Keller (unpubl.) observed that this method resulted in only about 0.5 mycosed females m^{-2}. The subsequent spread of the disease is strongly dependent on an high density of white grubs and needs 1-2 generations to induce substantial mortality (Keller, 2001; Keller & al., 1997).

The data and the calculations show that the application of 40 kg fungus kernel ha^{-1} is sufficient to cause infections of white grubs, which is also confirmed by numerous control measures. However, the control effect does not seem to be the result of a high initial mortality but of the induction of epizootics. Further it implies that the soil conditions must be favourable for the development of the fungus (Kessler & Keller, 2002). Low densities and unfavourable soil conditions may be the reasons for failures of this control method. Due to the shallow slope of the dose-mortality curves (Ferron, 1967) increased application doses are not considered as a mean to improve the control success and, therefore, are not recommended. However, a repeated application with the recommended or a reduced dose increases the number of inoculation spots in the soil. This approach is considered to favour the probability for infections and to enhance the control process.

Acknowledgements

The work is part of the EU-project BIPESCO (FAIR6-CT98-4105) and supported by grants from the Swiss Federal Office for Education and Science.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.65 – 71

Were control measures responsible for the decline of *Melolontha* populations in South Tyrol?

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Abstract: After a period of relatively low population densities of *Melolontha melolontha L.*, the cockchafer flight of 1986 marked the beginning of a pronounced gradation in several apple plantation areas in South Tyrol (Italy). Up to 100 larvae/m² and more were counted in the sandy soils close to the Adige River ("Unterland"), which means that the damage threshold due to root-feeding by the white grubs was surpassed several fold. Control strategies included chemical, biological and mechanical treatments. The use of insecticides against cockchafers during the maturation feeding in the woods was never considered in the "Unterland-Region" for reasons of water protection. Soil insecticides used in the orchards to control white grubs were mainly based on chlorphyrifos-ethyl- and ethoprophos-formulations and did not result in sufficient control. Use of the entomopathogenic fungus *Beauveria brongniartii* started 1989. The biological control agent (BCA) was applied as bio-mass produced on barley kernels directly into the planting hole for new plantations (ca. 10 g/hole) or using a adapted seed drill constructed by the Research Centre Laimburg (25-30 kg/ha). During spring 1989 a liquid formulation of *B. brongniartii* was applied using a helicopter to control the cockchafers during the flight. Due to strict dependence on weather conditions and little long-time efficacy the liquid application was not repeated. Starting with 1992 apple plantations were covered with hail nets during spring to prevent the cockchafers from the "ripening flight", reducing therefore the numbers of eggs deposed in the apple orchards. Mechanical treatments, e.g. rotary grinding during the summer following the flight etc., were recommended as well. During the summer of 2000 the density of *Melolontha* spp. white grubs (L3) was determined in the different apple plantation areas in South Tyrol. Whereas in the "Unterland" the density was reduced to levels of ca. 0-4 L3/m² with little damage for the agriculture, this value reached ca. 20 L3/m² in the Central Vinschgau Valley and up to more than 30 L3/m² in the Upper Vinschgau Valley causing considerable economic losses. Correspondingly, the cockchafer flight in 2001 was heterogeneous in different parts of South Tyrol. We will discuss possible explanations for these locally different gradations of the *Melolontha* populations in South Tyrol in respect to the control treatments used and the different agricultural systems.

Key words: *Melolontha spp.*, *Beauveria brongniartii*, population density, biocontrol

Evolution of control strategies since the mid '80ies

A massive increase of the cockchafer population occurred in several areas in South Tyrol (Northern Italy) in the 1980'ies, causing considerable economic losses mainly in apple plantations due to the root-feeding by the white grubs. The gradation was described in detail by Zelger (1993, 1995, 1996) with the main focus on the most heavily attacked Unterland Region. The following article gives a short overview on the type and success of the control measures from 1980 to date in different areas within South Tyrol and the present population densities.

Cockchafer control in the Unterland Region can be divided into the following phases:

1980-85: hardly any control.

1986-88 chemical phase: mainly products with the active components chlorpyriphos-ethyl and ethoprophos were used to control soil dwelling white grubs; quantity of insecticides used, time and number of application and amount of water used to pour the insecticides into the soil varied widely. Treatments against adult cockchafers in apple plantations were done rarely. Besides the Adige river also a narrow net of water canals runs through the Unterland Region, therefore the use of insecticides against cockchafers during the maturation feeding in the woods was never considered for reasons of water protection.

1989-92 biological phase: spraying of blastospores of *Beauveria brongniartii* onto the swarming cockchafers (spring 1989) at the margins of the wood showed only limited effects with no long-lasting establishment of *B. brongniartii* propagules in the soil (Zelger 1993). Twelve years after the spraying we could not re-isolate any *B. brongniartii* from treated areas (data not shown). The mass application of fungal biomass produced on barley kernels was started in 1989. About 80% of the affected area in the Unterland was treated in that year and again in 1990 using ca. 30 kg/ha's. From 1992 onwards the amount of applied barley kernels decreased rapidly, but remains stable now since the mid-nineties with ca. 4000 kg of the product applied annually.

1992-96 mechanical phase: first tests using plastic nets to prevent females from ovipositing were conducted already during the swarming of spring 1989. The positive results lead to a large scale action in spring 1992 when 65% of the cultivated area in the flight zone was covered. As shown by Keller et al. (1995) and Zelger (1993) female cockchafers may still deposit fertile eggs below the nets also without "egg maturation feeding", but only at much lower rates. The strong decrease of the cockchafer population after the swarming 1992 due to this treatment seems to be the most important single event responsible for the relatively low population density in the Unterland Region since these days (for details see Zelger 1993, 1995 and 1996).

1996-2001: a combination of biological and mechanical treatments is used to stabilise the low population densities in the Unterland Region. Very few farmers bought new plastic nets for soil covering, but the nets used already in 1992 were re-used on several dozen ha's during the swarming periods in spring 1995, 1998 and 2001. When used and stored properly, the quality of the plastic nets did not decreased significantly during this period. The initial high costs for the plastic nets (ca. 3500000 ITL/ha) therefore paid off rapidly in case of high value crops such as orchards. For the protection of newly planted apple trees many farmers add small quantities of barley kernels into the plantation hole (ca. 10 g/hole).

Present situation of cockchafer control

Since 1996 in the Unterland Region treatments were reduced due to the decreased density of white grubs in the soil. However, many farmers still use *B. brongniartii* barley kernels annually to prevent a renewed build-up of the population. On an average, 4000 to 5000 kg of barley kernels were applied mainly in spring, covering an area of ca. 200-300 ha's. Application rates of fungal material in autumn are very low, mainly because of the narrow time lapse in between the end of harvest and the first winter frosts. Circa 90 % of the barley kernels were applied using a seed drill, the resting 10 % by hand directly into the planting hole. An exact estimation of the infection rates of *M. melolontha* larvae is difficult due to the actually low density rate of white grubs in the Unterland region, but never exceeded 10–15 % in the last three years (1999-2001).

Long-term effect of *B. brongniartii* on adult cockchafers

During the "cockchafer-flight" in spring 2001 adult insects were collected from several areas in the Province of Bolzano and kept isolated in plastic boxes at 20 °C and 70 % RH. Infection rates with *B. brongniartii* were measured after 2 months.

Sample area 1: "Unterland", the zone close to the Research Center Laimburg with severe white grub damage in the period 1986-92. (number of cockchafers: 106)
Sample area 2: "Unterrain", ca. 10-20 km north-west of "Unterland" with lesser white grub problems and less *B. brongniartii* used. (number of cockchafers: 66)
Sample area 3: Central Vinschgau Valley, village of Schlanders: high occurrence of white grubs since the mid 80' ies. (number of cockchafers: 244)
Sample area 4: Upper Vinschgau Valley, village of Prad. High occurrence of white grubs, but few *B. brongniartii* was used. (number of cockchafers: 79)
Sample area 5: Upper Vinschgau Valley, village of Prad. Extensive apple orchard, treated with fungal barley kernels (40 kg/ha) in the year 1999. (number of cockchafers: 54)

Results

The percentage of cockchafers infected with *B. brongniartii* is shown in Fig. 1. Although this was not a strictly designed experiment and the amount of *B. brongniartii*-kernels used in the different sample areas is known just roughly (with the exception of sample area 5), a positive correlation between infection rate and long-term usage of *B. brongniartii* is evident. Interestingly, the observed infection rate in adult cockchafers in sample areas 1, 2 and 3 is significantly higher than that of the white grubs of the same areas, which were sampled during the years 1999 and 2000. Even in the "Unterland" (sample area 1) the observed infection rate of white grubs (2^{nd} and 3^{rd} instar larvae) was rarely above 10%. A high infection rate of adult cockchafers in the Unterland Region was observed already in the "flight years" 1995 and 1998 (data not shown).

Possible explanations for this effect might be:
- Underestimation of the infection rates of white grubs due to decomposition in the soil.
- High infection rate of adult cockchafers during soil dwelling before the flight.
- Latent infection of the larvae with sub-lethal doses of *B. brongniartii* with out-break of the symptoms in the late stage of development within the weakened chafers.
- At the moment it's not yet clear, if these high infection rates of adult cockchafers play a major role for the population density since death occurs after ovipositing in most cases.

Larval density in South Tyrol 2000

The development of the cockchafer infestation from 1980 to 1996 in South Tyrol was documented by Zelger (1996). For the year 2000 density rates of 3^{rd} instar white grubs are listed in Table 2. In spring 2001 cockchafers with both the 3-year-cycle and the 4-year-cycle flew in all infested areas. Only a very small percentage of the whole cockchafer population in South Tyrol follows a shifted cycle

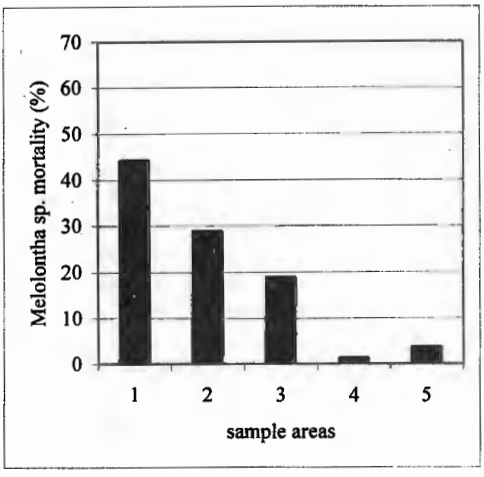

Fig. 1. Infection rates of adult cockchafers (*Melolontha* sp.) from different areas within South Tyrol.

Table 2. Density of *Melolontha* larvae (L3) in different areas in South Tyrol (Italy) in the year 2000.

Area	Density (No. white grubs/m²)
Unterland (Auer)	0-2
Unterland (Pfatten/Laimburg)	0-2
Unterland (Branzoll/Leifers)	0-4
Eisacktal (Albeins/Feldthurns)	0-2
Etschtal (Unterrain)	0-6
Etschtal (Nals)	0-4
Lower Vinschgau	0-4
Central Vinschgau (Schlanders)	ca. 20 (max 39/tree)
Upper Vinschgau (treated mechanically and biologically)	0-6
Upper Vinschgau (un-treated)	20-30

Compared to the periods with the highest *Melolontha*-densities (1986-91), the decrease of white grubs was remarkable mainly in the Unterland-Region. Low densities were found in the year 2000 also in the Eisacktal and the Etschtal, but these areas traditionally never had as many white grubs as the Unterland-Region.

The number of fertile eggs produced per female is believed to be related directly to the ecological fitness of the cockchafer populations. Increasing populations are characterised by high egg numbers (ca. 25-30; with 36 being the highest value attainable). In the Unterland Region the egg numbers of ca. 200 to 300 females were counted from every maturation flight since the late '80ies. A steady decrease of egg production was observed till 1998 with ca. 14

to 18 eggs counted on average. Egg numbers from the flight 2001 remained stable on this low level.

Experiences in the Vinschgau Valley

In the Central Vinschgau Valley close to the village of Schlanders, an area with intensive apple production, strong infection with cockchafers was also observed since 1986. The core zone with economic damages by root feeding covered an area of ca. 50 ha's at this time and enlarged since to ca. 200 ha's in the year 2001. In this area, biological control using *B. brongniartii* was carried out only to a low extent. Application with the seed drill is hindered partly by the stony soils in this zone, which make it very difficult to add the kernels in the right depth. Also the quality of the fungal barley kernels was not always satisfactory due to improper storage conditions. Control treatments in this area consisted mainly in the application of different soil chemicals with chlorpyriphos-ethyl as the active ingredient. Similar to results obtained by Zelger (1987, 1996) in the Unterland Region in the late '80ies, also in the Vinschgau Valley chemical control of the larvae turned out to be labour-intensive, expensive and of limited success. In some orchards damages to trees were limited using high doses of chemicals repeatedly, whereas in neighbouring plantations with very similar soil structure and larval density only low efficacy was observed. Of crucial importance –besides the quantity of chemicals used- is the amount of water used for dispersal of the insecticides (depending on the depth of the soil layer the larvae are dwelling) and the moment of application. If chemical treatments occur during a period with the white grubs being in relatively deep layers (e. g. during the casting of the skin) the chemicals may only act as a repellent for a limited time. The population density of cockchafers in these area was not reduced substantially, heavy damages in the orchards in next few years have to be expected.

Hail nets for preventing the "maturation flight" were used only rarely in the Central Vinschgau Valley during the '90ies. Farmers often complained a limited effect of this treatment with only small decrease of larval densities. In spring 2001 ca. 50 ha's were covered with nets. To decrease the number of cockchafers crawling below the nets additionally, insecticides (mostly based on chlorphyrifos-ethyl) were sprayed on the nets during dusk. Herbicides were used by some farmers to prevent growth of grass below the nets, which otherwise could serve as "hiding-place" for the chafers. First checks in summer 2001 indicate a high efficacy for this combination of mechanical and chemical treatments. Similar results were reported recently also from Southern Germany (Meinert et al., 2001). Organic growers in Italy are allowed to use only a very limited number of insecticides. Among the most important ones are the different forms of phyretrum, based on a plant extract from tropical compositae (Chrysanthenum = Pyrethrum) and rotenone, a root extract from the tropical plant *Derris elliptica* (Leguminosae). Tests in the lab against adult cockchafers resulted in low mortality rates (ca. 20%) for both insecticides when field doses were applied (data not shown). Field applications of these pesticides on the plastic nets by organic growers in the Vinschgau Valley also resulted in low cockchafer control, even when doses were increased up to 3 fold.

The Upper Vinschgau Valley differs in some important points from other areas in South Tyrol with cockchafer damages:

1) The four-year-cycle of the wood cockchafers (*Melolontha hippocastani F.)* – prevailing in this cooler area- seems to stress the apple trees less than the three-year-cycle of *M. melolontha.*
2) A considerable portion of the adult cockchafers remains in the apple plantation for the maturation feeding and can be treated easily with insecticides.

3) Hail nets were never used in significant amounts.
4) The area is characterised by the presence of large green meadows where no plant defence occur, serving as "retreat areas" for the cockchafers.

Recommendations for integrated cockchafer control

Based on the experiences made in South Tyrol and other countries the Research Centre Laimburg recommends the following strategy for cockchafer control:

Table 1: Strategy of cockchafer control in South Tyrol

PERIOD	BIOLOGICAL TREATMENT USING *BEAUVERIA BRONGNIARTII*	MECHANICAL TREATMENT	CHEMICAL TREATMENT
Every year (spring, fall)	"fungal barley" 25-30 kg/ha application using a seed drill	soil treatment: rotary-grinding, circular spike harrow, ...	none
Cockchafer-flight	as described above	covering the soil with hail nets	spraying on the nets (in limited cases only!)
Plantation	application of "fungal barley" into the planting hole (10 g/plant)	ploughing deeply after stubbing	None

Conclusions

The experiences made in South Tyrol during the last 15 years clearly show that there is no simple "one-way-strategy" for cockchafer control. Depending on the crops, climatic conditions, soil type, swarming behaviour of the beetles etc. the gradation may take different courses even in relatively small areas. However, a combination of mainly mechanical and biological methods was highly effective in most areas.

Acknowledgements

This project is funded partly by the European RTD programme FAIR6-CT98-4105 BIPESCO.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.73 - 77

Résultats des contrôles des épandages à la volée de graines enrobées de *Beauveria brongniartii*

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Résumé: Les techniques d'inoculation de la moisissure *Beauveria brongniartii* par semoir, fraisage, hersage ou labour ne sont pas adaptées aux conditions de l'agriculture valdôtaine. Les résultats des essais d'épandage à la volée sur neige ou sur herbe haute effectués sur des petites parcelles en 1995 étaient suffisamment encourageants pour que l'expérimentation soit poursuivie sur des surfaces plus étendues. Cent quarante ha ont ainsi été traités en 1996, 1997 et 1998. Les contrôles réalisés en 1980 ont donné pour résultat 6,06 larves vivantes par m² sur les surfaces traitées, contre 12,01 ailleurs. Sur ces mêmes surfaces, le pourcentage de larves momifiées par la *B. brongniartii* est de 42,11%, contre 0,00% ailleurs.

Abstract: None of the inoculation techniques of insect pathogenic fungus *Beauveria brongniartii* developed on the surface of rye kernels with seeder, milling or ploughing is satisfying considering the characteristics of Aosta valley agriculture. The results of trials of rye kernels applied on snow or on grass in little lots in 1995 encouraged to carry on the experimentation on wider surfaces. Thus 140 ha were treated in 1996, 1997, 1998. The controls carried out during the year 2000 gave the results of 6,06 grubs living on square metre in the treated surfaces while the average density in non treated surfaces was of 12,01. Further, the rate of grubs succumbed to *B. brongniartii* was 42,1% in the treated plots compared to 0,0 % outside the treatment.

Key words: *Beauveria brongniartii*, microbial control, application method, mortality.

Introduction

D'après Ferron (1983) « il est démontré qu'un traitement du sol à la dose de $10x10^9$ conidies par m² induit le développement aigu de la mycose... ». La dotation naturelle de conidies de *Beauveria brongniartii* dans les terrains de la Vallée d'Aoste est très faible. Pour 18 sites considérés, 80% des 193 échantillons prélevés ont donné un résultat négatif ; dans 58% des échantillons restants, la concentration est inférieure à $1x10^3$ CFU/g d.s. (Cravanzola & al., 1996), soit environ $1x10^8$ conidies par m².

Pour atteindre les valeurs préconisées de conidies dans le terrain, différentes techniques d'application de graines enrobées de moisissure *B. brongniartii* ont été proposées : (piochage, fraisage, hersage, labour léger, etc.). Mais toutes prévoient le remuage de la couche superficielle du terrain, ou l'emploi de semoirs particuliers. Ces techniques ne sont pas appliquées dans notre région, en raison de la pente des terrains, notamment.

Les résultats obtenus en 1995 après des essais d'épandages à la volée sur neige ou sur herbe haute (Bondaz & al., 1996) nous ont incités à élargir les superficies traitées à l'essai. En même temps, nous devons aussi vérifier l'efficacité de la souche introduite artificiellement.

Le but des ces recherches était :

- Vérifier l'efficacité de l'épandage à la volée de graines enrobées de moisissure *Beauveria brongniartii* souche F contre les larves de *Melolontha melolontha* ;
- Vérifier si les vers blancs ont effectivement été tués par la souche F.

Méthodes

- En plein air :

- graines de blé enrobées de la moisissure *B. brongniartii* souche F répandues à la volée par les arboriculteurs qui participent à l'essai:
- quantité: 1996: 70 q ; 1997: 70 q ; 1998: 70 q;
- dose: 50 kg/ha, $\approx 1x10^9$ conidies/m² chaque application ;
- époques: hiver, sur neige, ou avril, sur herbe;
- années des applications: 1996,1997,1998;
- cultures: vergers et prés-vergers;
- contrôles: 5 sondages de 1/6 de m² à l'intérieur des parcelles traitées et 5 sondages de 1/6 de m² à l'extérieur des parcelles traitées;
- surface des parcelles: > 1000 m² ; 140 ha au total.

- En laboratoire :

- isolement de *B. brongniartii*: après avoir placé des petites portions de mycélium de chacune des larves mycosées en suspension dans une solution de Ringer stérile, des dilutions successives ont été préparées, puis mêlées avec le milieu Sabouraud rendu sélectif par l'addition de 0,5 g/L de chloramphénicol et de 0,25 g/L d'actidion (Veen & Ferron, 1966).
- extraction de l'ADN des moisissures isolées en pureté en suivant le protocole décrit par Rogers & Bendich (1989) et modifié de manière suivante: avant tout le mycélium broyé est mis à incuber à 65°C dans un tampon avec 2 % de CTAB ; puis les protéines sont éliminées par extraction avec d'abord du phénol: chloroforme: alcool isoamylique (25:24:1) puis du chloroforme, la suspension de l'ADN est réalisée, précipitée avec de l'isopropanol , en TE (Tris-HCl, EDTA).L'on obtient ainsi deux échantillons d'ADN par extraction de deux cultures indépendantes pour chaque moisissure.
- sur chaque extrait d'ADN, une analyse RAPD-PCR est effectuée avec l'amorce OPM-03 (5'-GGGGGATGAG-3'). Il est ainsi possible de reconnaître la souche F : après la course sur gel d'agarose 1,5 % des différents morceaux amplifiés, l'on peut voir si la bande de 1300 bp caractéristique de la souche F est présente (Figure 1).

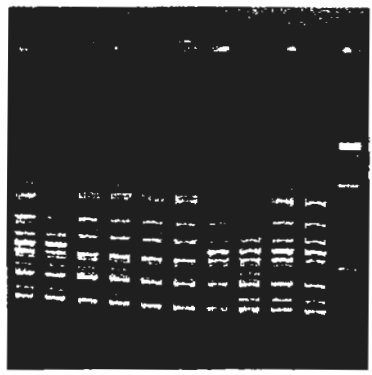

Figure 1: Course des fragments d'ADN après l'amplification avec l'amorce OPM – 03. On peut noter le fragment 1300 bp caractéristique de la souche F dans les échantillons 1, 2, 6, 7 , 8 et 9.

Résultats

En plein air

Sondages sur les parcelles traitées

	PROPRIETAIRE	LIEU-DIT	LARVES TOTAL	VIVANTES	n/m^2	MORTES AVEC B.br	n/m^2	% AVEC B.br.
1	GROSJEAN	QUART	14	9	10,8	5	6.00	35,71
2	CHAMONIN	GRESSAN	18	12	14,4	6	7,2	33,33
3	JOCALLAZ/FENOIL	ST.PIERRE	8	6	7,2	2	2,4	25,00
4	BORNEY/EMPERE	AYMAVILLES	5	3	3,6	2	2,4	40,00
5	EMPEREUR	AYMAVILLES	4	1	1,2	3	3,6	75,00
6	CURTAZ	GRESSAN	8	5	6.00	3	3,6	37,50
7	PRAZ	JOVENCAN	16	6	7,2	10	12.00	62,50
8	CHENEY	ST-CHRIST.	3	2	2,4	1	1,2	33,33
		TOTAL	76	44	6.06	32	4.08	42,11

Sondages hors des parcelles traitées

	PROPRIETAIRE	LIEU-DIT	LARVES TOTAL	VIVANTES	n/m^2	MORTES AVEC B.br	n/m^2	% AVEC B.br.
1	GROSJEAN	QUART	9	9	10,8	0	0	0
2	CHAMONIN	GRESSAN	12	12	14,4	0	0	0
3	JOCALLAZ/FENOIL	ST.PIERRE	4	4	4,8	0	0	0
4	BORNEY/EMPERE	AYMAVILLES	8	8	9,6	0	0	0
5	EMPEREUR	AYMAVILLES	18	18	21,6	0	0	0
6	CURTAZ	GRESSAN	6	6	7,2	0	0	0
7	PRAZ	JOVENCAN	9	9	10,8	0	0	0
8	CHENEY	ST.CHRISTOP.	15	15	18.00	0	0	0
		TOTAL	81	81	12.01	0	0	0

- En laboratoire

Sur 28 des 32 larves mycosées, il a été possible d'isoler le champignon et l'on a ainsi obtenu 56 extraits d'ADN. Les résultats sont illustrés dans le tableau ci-dessous (F=souche répandue).

Parcelle d'origine des larves mycosées	Nombre de souches F sur le total des souches analysées	% de souche F
1(Grosjean)	1 sur 5 (2/10)[1]	20,0%
2(Chamonin)	1 sur 5 (2/10)	20,0%
3(Jocallaz-Fenoil)	1 sur 2 (2/4)	50,0%
4(Borney-Emp.)	1 sur 2 (2/4)	50,0%
5(Empereur)	1 sur 3 (2/6)	33,3%
6(Curtaz)	3 sur 3 (6/6)	100,0%
7(Praz)	2 sur 7 (4/14)	28,6%
8(Cheney)	1 sur 1 (2/2)	100,0%
Total	11 sur 28 (22/56)	39,3%

[1]= les chiffres entre parenthèses indiquent le nombre total de souches analysées (deux extraits par moisissure). Cette donnée nous a toujours confirmé que les 2 *B. brongniartii* isolées sur la même larve appartiennent à la même souche. Le résultat indiqué hors parenthèses ne tient pas compte de l'analyse en double.

Discussion

Pour une même surface sondée (5/6 de m²), le nombre de larves vivantes repérées à l'intérieur des parcelles inoculées est de 44 contre 81 à l'extérieur; c'est à dire 6,06 au m² contre 12,01 au m². A l'intérieur des parcelles, 42,11% des larves mortes ont été momifiées par *B. brongniartii* contre 0,00% àl'extérieur.

L'analyse en laboratoire des moisissures prélevées sur les larves mortes a mis en évidence le fait que la souche F (celle qui a été inoculée) est présente, avec des pourcentages variables (de 20% à 100%) selon la parcelle considérée. Au total, le pourcentage de souche F est plutôt faible (39,3%), puisque dans les parcelles non traitées, aucune larve mycosée n'a été trouvée et qu'il n'y a donc pas de souches autochtones suffisamment virulentes. Ces données pourraient aussi porter à conclure que la souche inoculée ne se borne pas à agir directement mais qu'elle favorise aussi l'action de souches autochtones normalement peu virulentes.

Les résultats obtenus sont encourageants et nous engagent à continuer nos essais, pour confirmer l'efficacité de la technique d'inoculation et l'interaction éventuelle de la souche F avec les souches autochtones.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.79 – 84

Preliminary results on the efficacy of *Beauveria*-Schweizer in the Kaiserstuhl area after a three year period

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Abstract: In Baden-Württemberg/Germany, the highest damage caused by white grubs (*Melolontha melolontha*) was found in the area of the Kaiserstuhl followed by the western Kraichgau and the Schwäbische Alb. The aim of this project was to examine the efficacy of *Beauveria* Schweizer against the larvae of *M. melolontha,* to study the natural occurrence of he fungus and the persistence of *Beauveria brongniartii* in the soil and the quality of the product under various storage conditions. The fungus *B. brongniartii* was applied as *Beauveria* Schweizer on more than 8 ha vineyards and orchards in the Kaiserstuhl, on 2,5 ha in Hessia and on ca. 3 ha in Mecklenburg-Vorpommern. After three years of observation, preliminary results demonstrate the beginning of the efficacy of *B. brongniartii* against the white grubs. Studies on the number of conidiospores in soil showed a close relationship between the density of white grubs at the beginning of the project and the increase of the conidiospores. The method of tilling is closely related to the concentration of *B. brongniartii.* Assays on the storability demonstrate the importance of low temperature and darkness for the activity of the fungus.

Keywords: *Beauveria brongniartii*, biological control, M*elolontha melolontha*, soil

Introduction

After a cycle of 30 years, the population of the Maybeetle (*Melolontha melolontha*) is increasing since the late eighties of the last century (Fröschle 1994, Zimmermann 1998). In Baden-Württemberg, the main infested areas are the Schwäbische Alb, the Kraichgau and the Kaiserstuhl. The severest damage was observed in nurseries and newly planted vineyards and orchards. The infested area is spreading over the northern part of the Kaiserstuhl and reached now the eastern part next to the village of Bötzingen. Some parts of the central Kaiserstuhl are also infested.

The application of the entomopathogenic fungus *Beauveria brongniartii* (Sacc.) Petch. is an interesting possibility for the control of *Melolontha* larvae in soil. The natural occurring fungus attacks all instars of the maybeetle and is harmless from an ecological point of view.

In contrast to the positive results in Switzerland (Keller 1993, Fornallaz 1992, Keller et al. 1992) and South Tyrol (Zelger 1996), former experiments with *Beauveria* products in Germany were not satisfying (Albert & Fröschle 1988).

Within this project the reasons for the varying results should be investigated. The efficacy of the *Beauveria* product should be tested in the Kaiserstuhl area. The climate of the Kaiserstuhl is characterised by high average temperatures and low annual precipitation with long dry periods in summer, which is not favourable for fungi in the soil. Therefore, the aim of the project was to test whether the fungus can be established in the soil by controlling the white grubs under these conditions. The fungus was applied on 8 ha in the Kaiserstuhl area, 2,5 ha in Hessia and 3 ha in Mecklenburg-Vorpommern. Here, only the results of the Kaiserstuhl area are reported. The densities of white grubs ranged from 5 larvae (L)/m² to 45

L/m² at the beginning of the project. Another difficulty was the application of the fungus into the soil. A new machine was bought which is normally used for loosening the mulch soil and which was reconstructed for seeding.

The university of Heidelberg (working group of Dr. Schnetter) did the diagnosis of white grubs and maybeetles and the Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute for Biological Control in Darmstadt (working group of Dr. Zimmermann) was involved in the identification of *B. brongniartii* on infected grubs, the quality of the product and the persistence of the fungus in soil during the experiments.

Material and methods

Field studies

To get results about the needed and optimal concentration of *B.brongniartii* in soil in the Kaiserstuhl area, the number of applications was varying from 3 to 5 times in the trial over the three years. The application rate of *Beauveria*-Schweizer was 30 kg/ha/application. The following table shows the different variants, with the time of application and the number of applications (Tab.1).

Table 1. Number of *Beauveria* applications on the sampling fields

Variant	Samplingfields	Time of application					Number of application
		Summer 1998	Autumn 1998	Spring 1999	Summer 1999	Spring 2000	
1	4-10; 15; 16; 19; 20	x	x	x			3
2	6a	x	x	x	x		4
2a	1;1a	x	x	x	x	x	5
3	2, 3; 11; 12; 13; 14	x	x	x		x	4
4	17;18		x	x		x	3

All providing grounds were divided in a part, which was treated with *Beauveria* and a part, which remained untreated. The maybeetles were dug every year in spring and in autumn. Around 24 holes/ha were made, but 5 holes at the minimum in small fields. The surfaces of the holes were 0,25 m² and the holes were 20 to 50cm deep. The collected maybeetles were sent to the University of Heidelberg for diagnosis.

Density of spores

The samples of the soil for the analysis of the spores were taken from a dept from 5-20 cm, afterwards the soil was mixed. The working group Dr. Zimmermann tested the persistence of the fungus in soil.

Storability of Beauveria Schweizer

In the Kaiserstuhl area the quality of the *Beauveria* product was tested at about 4°C in a cool chamber for fruits and in a barn at ambient temperature. Furthermore, one sample of the

product was stored in closed bags the other in bags opened at the beginning of the trial. At the BBA, the quality was examined in an incubator under standardized conditions at 4°C, 20°C and 30°C. Every month a sample was collected and the kernels were incubated on humid turf. (Maass & Meinert, in prep.) The activity of the *B. brongniartii*-kernels was examined.

Results and discussion

Development of white grubs in soil

In the Kaiserstuhl area two different populations of the maybeetle exist, the main population with flights in 1997 and 2000 and a smaller population with flights in 1999 and 2002. The two populations were kept separately. The development of the white grubs, which were collected two times a year, was observed over the whole period of the project. In the main population, the density of the white grubs decreased between summer 1998 and spring 2000 on nearly the same level in the untreated as well as in the treated fields. There is only a non-significant change between the relative densities in the fields. First, during the sampling period in autumn 1998 the density in the treated fields is higher and one year later the density in the untreated fields was higher than in the treated ones. After the flight 2000 the differences between the untreated and treated fields are greater and the decline of the number of white grubs in the untreated fields is slower than in the treated fields (Fig. 1). We suggest, that the greater reduction of white grubs per m² in the treated fields was due to *B.brongniartii.* As already mentioned by other authors, the efficacy of *B. brongniartii* can be shown after the second generation of the maybeetle after fungus treatment (Strasser et al., 1999). It seems that *Melolontha* larvae can be controlled even under these difficult climatic conditions.

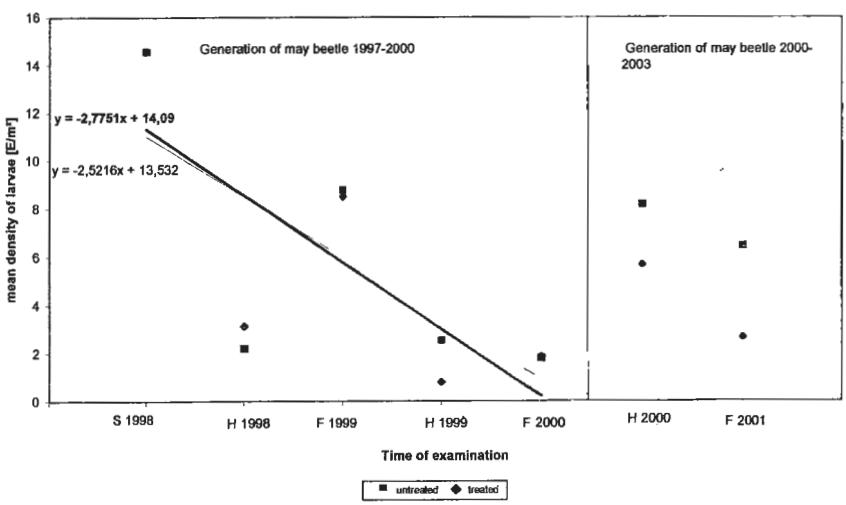

Figure 1. Development of white grub populations (Maybeetle generation 1997-2000-2003) in soil

Development of the spore density in relation to tilling and to the number of white grubs in soil

Within this project also the number of spores per gram of soil and the development of the fungus was examined over the last years. Fluctuations in the spore concentration were observed but also a slow rise of the concentration over the years.

Besides the number of applications, also the method of tilling and the density of the white grubs at the beginning of the project had a very high influence on the development of the spore concentration. A comparison of the number of spores in soil between two successive years was undertaken.

On sampling fields were the grass was only mulched, an increase in the number of spores of *B. brongniartii* depends on the number of applications and the density of white grubs per m^2. In one field the primary tilling was mulching then the vine grower decided to plant new vine plants. The soil was deeply ploughed, i.e. the fungus spores were distributed up to 40 cm of depth. Afterwards the density was lower than in the fields without *Beauveria*-application. (Fig. 2 left side).

The fungus proliferates particularly well on his host. So we found, that in fields with a low density of white grubs at the beginning of the project the concentration of *B. brongniartii* did not increase in relation to the number of applications, i.e. after five applications there was no further increase. But in a field with a high larval density at the beginning of the project, the concentration of spores in soil increased after three applications more than compared to those fields with five applications. (Fig. 2 right side)

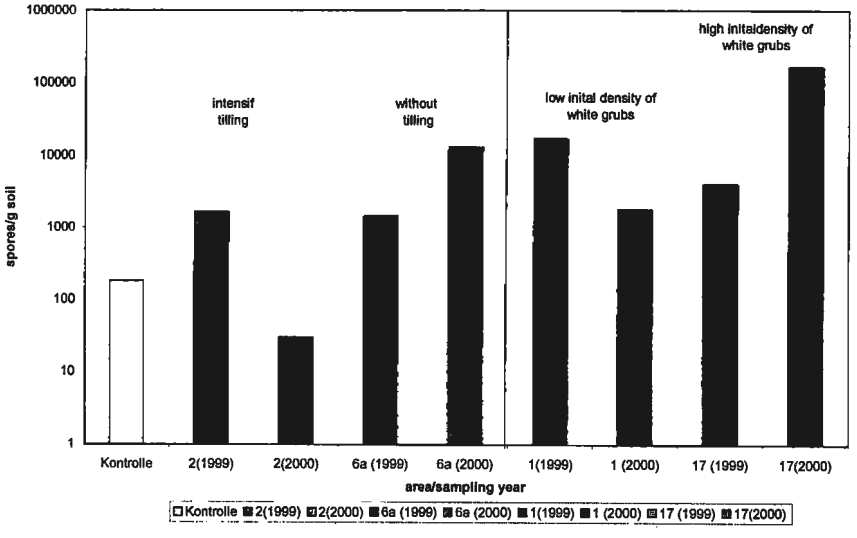

Figure 2: The development of the number of spores per 1 gr soil in relation to tilling and the number of grubs per m^2.

Studies on the storability of Beauveria Schweizer under different conditions

Normally, the fungus product is delivered, and than the application of the product is made within a few days after delivery. Sometimes, however, a storage for some days or weeks is necessary, for instance because of weather conditions.

To save the activity and to obtain a high efficacy of the fungus, the product has to be stored under dark, dry and cold conditions. However, the vine grower and the fruit grower often are not using optimal storage conditions. Therefore, we tested the stability of *Beauveria* Schweizer under different temperature regimes

In our experiment, the product could be stored in closed bags at 4°C in the cool chamber for more than two months without lost of activity, afterward the growth decreased and the kernels were overgrown with moulds. The kernels of *Beauveria*-Schweizer stored in open bags began to loose their activity after one month. Therefore, the product stored in the barn has to be applied within a month after delivery, otherwise the quality decreased. (Fig. 3)

The results of the BBA were worse. After a month storage at 4°C only 45 to 58 % of kernels showed growth of *B. brongniartii*, and 35-38 % after two months, the conditions during the delivery and transport from the Kaiserstuhl to Darmstadt were responsible for these results. The bags were stored outside for some days in the sunshine only covered with blankets. These results show the difficulties, which may occur before application and the importance to take care on the storage conditions.

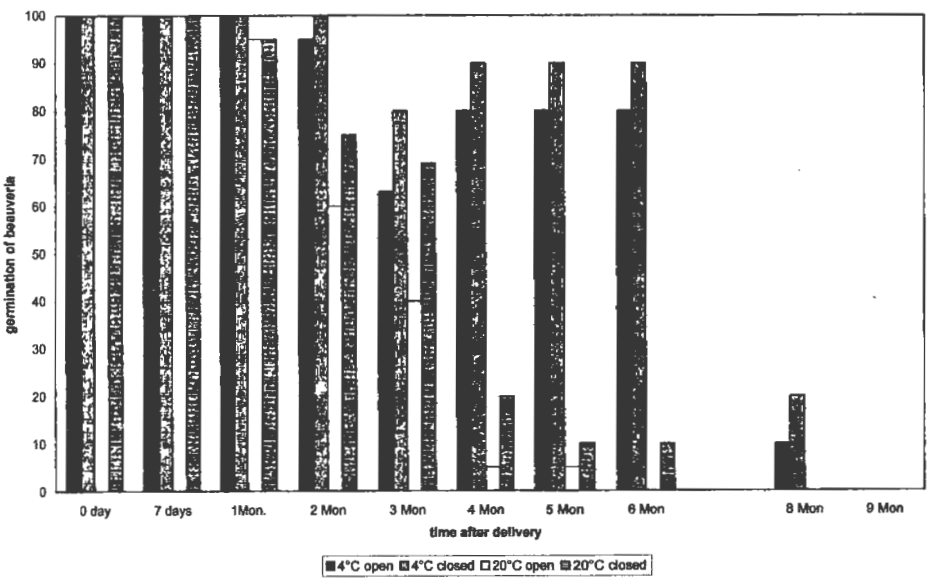

Fig. 3: Quality of *Beauveria brongniartii* (*Beauveria* Schweizer) under different storage conditions

Conclusion

The visible effect of *B. brongniartii* in the treated fields starts in the second generation of the maybeetles after the application of the fungus.

There is a correlation between the density of white grubs at the beginning of the experiments and the concentration of *B. brongniartii*-spores in soil.

The method of tilling has an influence on the number of *B. brongniartii*-spores in the soil.

In the Kaiserstuhl area, the application of *B. brongniartii* should be done in early spring when the precipitation is still high and the soil moist.

In order to guarantee a high quality of *Beauveria* Schweizer, the product has to be stored permanently in the dark, at low relative humidity and at a low temperature.

Acknowledgements

I would like to thank Mr. Zimmermann and Mr. Schnetter for the good cooperation and discussions, Mr. Fröschle, Mr. Glas and Mr. Meinert for the promotion during my work and the discussion and ideas whenever it was necessary. Finally, I would like to thank the Ministry of Agriculture of Baden-Württemberg for funding the project.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.85 – 88

Applying *Beauveria brongniartii* into the planting holes – results of a recent trial

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Abstract: Considering that the application of *Beauveria* products was less successful so far, the mode of application had to be thought over. When a farmer told the author about a successful experiment with a *Beauveria* application into the planting holes of young apple trees a new trial was designed. Thus a similar experiment was done with potted vines. The *Beauveria* products were applied at planting, the White Grubs were added into the soil three weeks later and the assessment was done another 80 days later. Regarding the small number of recovered living White Grubs in the treated plot, the experiment was successful. The results ought to be verified by further trials in glasshouses or in the field. Up to now the usual applications of *Beauveria brongniartii*-products were financed by the local government. This financing was done in the frame of experiments that were necessary, because no pesticide was registered for this purpose. If this mode of application will render *Beauveria* products more effective and less expensive, farmers in Baden-Württemberg possibly should be able to pay for the *Beauveria* products themselves. Precondition for this is a registration of such products in Germany.

Key words: *Melolontha*, white grubs, potted vines, *Beauveria brongniartii,* mode of application

Introduction

Farmers are appreciating the possibility of controlling white grubs with *Beauveria* products in spite of the poor results of the usual mode of application of *Beauveria brongniartii* (SACCH.) PETCH against white grubs under the prevailing climatic conditions in the infested areas of Baden-Württemberg. A fruit grower tried to improve the efficacy by applying the *B.brongniartii* infected barley kernels directly into the planting holes of apple trees. Trees treated in this way were not damaged (Epp, oral comunication). In a nursery in Switzerland Thuja plantlets have been completely protected in this way (Matzke, oral comunication).

Beyond these promising reports there are at least two more reasons to change the usual mode of application. Up to now the government of the state Baden-Württemberg has paid all the *Beauveria* infested barley kernels applied in the state, but this financing of the application will be stopped when Azadirachtin is registered in Germany for this purpose. Regarding the slow mode of action and the costs of the application the farmers are refusing to pay for it themselves. That means that probably from next year onwards no such products will be applied in Baden-Württemberg. It is possible that applying the product into the planting hole may render a more effective and less expensive protection of individual plants.

Material and methods

The experiment was carried out at the State Institute of Viticulture and Enology in Freiburg, Germany. Fifty grafted and rooted vine plants of the variety Bronner (ten each treatment) have been planted on May 28th 2001 separately in 5 l – pots (20 cm diameter) filled with compost, peat and sand (80:10:10). The mode of application of the products is described in table 1. The pots were placed in front of a glasshouse and kept sufficiently wet.

Table 1: Experimental design

Product	Formulation	Amount/vine	Mode of application
1) untreated			
2) Fytovita	wp	1 %	dipping
3) Melocont	infested barley kernels	10 g	scatterring
4) BEAUVERIA SCHWEIZER		10 g	
5) BEAUVERIA SCHWEIZER + NeemAzal-T/S*		10 g	

* a carrot slice was dipped into a suspension of 2 ml of NeemAzal-T/S in 200 ml of water

The grubs were collected the next day in the Kaiserstuhl area. 195 grubs of the second instar were separately put into boxes with native soil, kept in the laboratory and fed with slices of carrots. Dead or ill grubs were eliminated on May 30th, June 5th and 13th. None of the grubs had died of *B. brongniartii* infection. On June 18th the apparently sound grubs were added into the soil of the pots. Per treatment altogether 25 grubs were used, three of them into the pots "a" to "e" and two into the pots "f" to "j".

The assessment was done on August 30th by evaluating the vine plants for grub damage. The number of grubs were counted whether they were infested by *B. brongniartii* or living or not recovered at all. The grubs recovered living were kept in boxes with the potted soil and fed with slices of carrots for three weeks in order to see whether they were infected. The dead ones and the pupae were removed. Those that were still living got new carrot slices at each assessment date.

Results

In the untreated control three vine plants were undamaged, three plants were dead. No dead plants were found in the plots treated with the *Beauveria*-products formulated on barley kernels (Table 2). The effects on the grubs were more distinct. Whilst in the untreated plot half of the grubs were recovered living and apparently sound, in the plots treated with Melocont, BEAUVERIA SCHWEIZER or BEAUVERIA SCHWEIZER + NeemAzal-T/S only 11 out of 75 grubs were recovered, out of which 9 were infected, only 1 pupa and 1 apparently sound grub (Table 3). The results with Fytovita ranged between the untreated control and the treatments 3, 4 and 5, regarding both the plants and the grubs.

None of the grubs recovered living during the following three weeks in the laboratory died of *B. brongniartii* infection. One grub developed to the pupal instar (Table 4).

Table 2: Effects on the vine plants

Product		Number of vine plants undamaged	damaged	dead
untreated	a – e	1	4	0
	f – j	2	0	3
Fytovita	a – e	0	3	2
	f – j	0	5	0
Melocont	a – e	2	3	0
	f – j	3	2	0
BEAUVERIA SCHWEIZER	a – e	3	2	0
	f – j	3	2	0
BEAUVERIA SCHWEIZER	a – e	4	1	0
+ NeemAzal-T/S	f – j	2	3	0

Table 3: Effects on the grubs

Product		Number of grubs living	infected	not recovered
untreated	a – e	7	0	8
	f – j	6	0	4
Fytovita	a – e	5	0	10
	f – j	1	1 pupa	8
Melocont	a – e	0	4	11
	f – j	0	2	8
BEAUVERIA SCHWEIZER	a – e	0	1	14
	f – j	0	0	10
BEAUVERIA SCHWEIZER	a – e	2*	1	12
+ NeemAzal-T/S	f – j	0	1	9

* one apparently sound grub and one pupa

Discussion

The effects of the various products with *Beauveria* infested barley kernels equally succeeded in avoiding dead vines and more distinctly in causing mortality of the White Grubs. Contrary to the results of Darwish & al., (2001) adding a carrot slice dipped in a suspension of NeemAzal-T/S gave no better result. They used an amount of 75 or 150 ppm NeemAzal-T/S and dried the carrot slices for two ours. In the trial reported in this paper the slices have not been dried, but the amount of NeemAzal-T/S (10 g/l active ingredient) was 2%. It has to be discussed whether NeemAzal-T/S can enhance the efficacy. According to the results of the experiment referred to in this paper the application of NeemAzal-T/S as an additional stress factor may be unnecessary. Whether this is also true in the field must be examinated.

Even if – applying the Schneider-Orelli's formula - the efficacy figures are rather low, this tentative experiment was successful. The relatively poor results with the novel product Fytovita are due to the mode of application. Dipping grafted and poorly rooted vine plants

into a suspension is insufficient. Scattering the wettable powder into the planting holes surely will also do successfully.

These results and the reports of the farmers cited seem rather promising. If there is the unanimous intention to favour the application of *B. brongniartii* in Baden-Württemberg, scientific trials – in the glasshouse or in the field – should be performed. In particular all the problems of minimizing the amount of products applied to the single plants must be solved. A lot of experiments regarding the various crops like fruit trees, grafted vines, grafted and rooted vines, nursery plants, bush fruit etc. have to be done.

Table 4: Assessment of the grubs recovered living

Date	Product	Number pf grubs			
		total	consumption of food	no consumption of food	
			living	living	dead
02.09.	untreated	13	10	2[1]	1
	Fytovita	6	3	3	0
	BEAUVERIA SCHWEIZER + NeemAzal-T/S	1	0	0	1
06.09.	untreated	11	11	0	0
	Fytovita	6	4	1	1
09.09.	untreated	11	10	1	0
	Fytovita	5	2	2	1[2]
13.09.	untreated	11	9	2	0
	Fytovita	4	2	2	0
16.09.	untreated	11	10	1	0
	Fytovita	4	1	2	1
20.09	untreated	11	10	1	0
	Fytovita	3	2	1	0

[1]) one pupa

[2]) death caused by *Beauveria brongniartii*

Acknowledgements

I am obliged to Dr. Zimmermann who gave me advice in planning and evaluating the experiment; he also gave me the products Fytovita and Melocont, to the Institute of Viticulture and Enology in Freiburg for the vine plants, the pots, and the earth, and to the co-workers Schaber and Trescher of that institute for their help. Mr. Schaber and Misses Maass of our own Institute gratefully assisted with collecting and surveying the grubs.

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Biology, ecology and production of *Beauveria brongniartii*

Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.91 – 96

Microsatellite markers in *Beauveria brongniartii*: a sensitive and efficient method for strain characterisation and identification

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Abstract: We have established a fast and reliable genotyping method for strain identification of the fungus *B. brongniartii.* This organism is used as biocontrol agent (BCA) to control the European cockchafer (*Melolontha melolontha*) a pest in orchards, grasslands, and forests. The typing method is based on analyses of 6 microsatellite loci shown to be highly polymorphic between *B. brongniartii* strains (Enkerli *et al.* 2001).

Here we report on the application of these microsatellite analyses for determination of the persistence of *B. brongniartii* BCA strains in the field. In 1998/1999, *B. brongniartii* isolates were collected from various field test sites in Switzerland which were treated either in 1985, 1988, 1991 or 1992 with *B. brongniartii* BCA strains. Data revealed that applied BCA strains were still present at all test sites. At some sites the *B. brongniartii* population consisted of the BCA strains exclusively whereas at other sites indigenous strains were present in addition to BCA strains. This study demonstrates the persistence of *B. brongniartii* BCAs in the field and the value and applicability of microsatellite markers to monitor BCAs.

Key words: *Melolontha melolontha*, biological control, BCA persistence, monitoring

Introduction

The entomopathogenic fungus *Beauveria brongniartii* (Sacc.) Petch (Deuteromycotina, Hyphomycetes) is used as biocontrol agent (BCA) to control the larvae of the European cockchafer (*Melolontha melolontha)* in grasslands and orchards. A commercial product based on barley kernels colonised by *B. brongniartii* is available in Switzerland since 1991. It is applied by using an adapted seed drilling machine. Another application method based on spraying blastospores on swarming beetles which subsequently carry the BCA to the breeding sites was used earlier in Switzerland. Pilot field trials were performed 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 1992, Keller *et al.* 1979). Although the efficacy of these two *B. brongniartii*-based biological control methods has been demonstrated in numerous field trials (Keller 1992, Keller *et al.* 1997, Zelger 1996), it has not been possible to prove that the applied BCA strain was responsible for the observed control of the pest.

Microsatellite markers are a valuable tool for studies in plant, animal and fungal biology such as genome mapping, population genetics, or identification of individuals (Goldstein & Schlötterer 1999). Microsatellites are tandemly repeated DNA sequence motifs of 1 to 6 bases length. Microsatellite loci occur throughout the genome. Different alleles of a specific microsatellite locus may be distinguished based on different numbers of repeat units resulting in different lengths of the microsatellite alleles. The length of microsatellites tend to be highly polymorphic between individuals, a characteristic which can be used for the differentiation and identification of individuals (Figure 1). For analysis microsatellite loci are individually amplified by PCR using pairs of oligonucleotide primers specific to the unique DNA

sequences flanking the microsatellite (Figure 1). Subsequently the sizes of the amplification products are determined for the distinction of individual alleles. The genotype of an organism is then described by the alleles detected at the microsatellite loci analysed thus providing a genetic fingerprint for the organism.

We recently have isolated microsatellite markers in *B. brongniartii* to provide a fast and reliable method for strain characterisation and identification (Enkerli *et al.* 2001). The microsatellite loci analysed in the present study display a high level of length polymorphism. This makes them well suited for studies on genetic diversity of natural *B. brongniartii* populations or investigations on the fate of applied *B. brongniartii* BCA strains in the field (Enkerli *et al.* 2001). Here we report on the use of these molecular markers to identify *B. brongniartii* BCA strains recovered between 6 and 14 years after their application in the field.

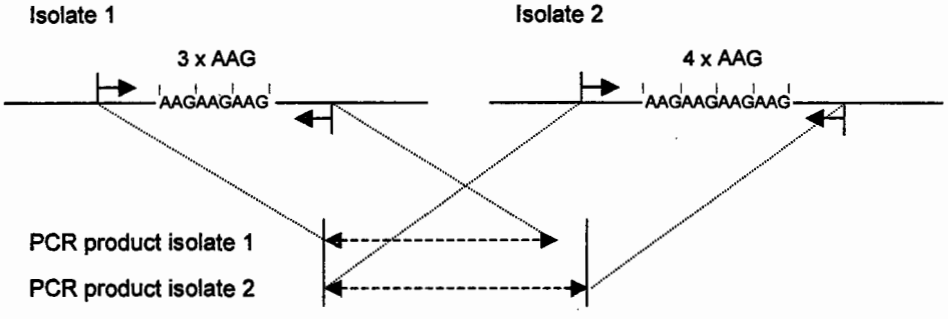

Figure 1. Length polymorphism of a microsatellite locus containing repetitions of an AAG sequence motif. The locus is PCR amplified from genomic DNA of isolate 1 and 2 using a primer pair (⇆) hybridizing to the flanking sequence of the microsatellite. Different numbers of repeat units in the two isolates result in different sizes of the PCR products (←--→).

Materials and methods

In spring 1998 and spring 1999 soil samples were taken from nine different locations in Switzerland (Keller *et al.* 2000) treated with blastospores in 1985/1988 (Keller *et al.* 1997) or with fungus kernels in 1991/1992 (Keller *et al*, in preparation). *B. brongniartii* was isolated from the soil samples (Keller *et al.* 2000) and maintained on solid complete medium (Riba & Ravelojoana 1984) at 22° C in the dark. The four different *B. brongniartii* BCA strains and the nine application sites are listed in Table 1. The genotypes of the BCA strains defined by the six microsatellite markers are shown in Table 2 except for isolate 256 which is not available anymore. DNA isolation as well as amplification and analysis of the microsatellite markers were performed as described by Enkerli *et al.* (2001).

Table 1. Location of test sites, type and year of application, and year of isolation of *B. brongniartii* isolates investigated.

Location	Type of application	Year of application	Year of isolation	BCA strain applied
Zuben	blastospores	1985	1999	129, 119, 132
Dünnershaus		1985	1998	129, 119, 132
Happerswil		1985	1998	129, 119, 132
Schönholzerswilen		1988	1998	129, 119, 256
Bleiken 1		1988	1998	129, 119, 256
Guggenbühl	fungus kernels	1991	1998	129
Neukirch		1991	1998	129
Bleiken 2		1992	1998	129
Biessenhofen		1992	1999	129

Table2. Genotypes of BCA strains applied

BCA strains[a)]	Microsatellite loci & their allel sizes [bp]						Genotype
	Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6	
129	223	103	196	168	160	172	A
119	214	103	190	159	160	172	B
132	202	106	196	165	166	172	C

[a)] except for strain 256 which was not available

Table 3. Primer sequences for the six microsatellite loci investigated

Marker	Forward primer (5'-3')[a]	Reverse primer (5'-3')[a]
Bb1F4	GATCCTTCGTCACCTGCTC	CGGTGTTGGAGAGCTATTGT
Bb2A3	CACTTCAGTTATCATGCTCGAA	ATCTGGCACGTCAAGTGTCT
Bb2F8	GCACACTTTCGCTGTGTCAT	ATGATGTCTGCCACGTCTGA
Bb4H9	CTGCTCAGTGCTCATTGCTC	ACAGCTCGGCATTGATGATT
Bb5F4	CTCGATCACTTTTCCATCAA	TGGTTGGGTCATGTTAGTCA
Bb8D6	TTTGTCCGAAGTTGTCTCAGT	TGCATTGTGAAAGGTAATGC

a) Enkerli *et al.* (2001)

Results

B. brongniartii isolates have been obtained in 1998/1999 from 9 different test sites at different locations in Switzerland (Table 1). Five of the test sites were treated with blastospores in 1985/1988 (Keller *et al.* 1997) and four test sites were treated with fungus kernels in 1991/1992 (Keller *et al.* in preparation). To test whether these isolates represent the applied *B. brongniartii* BCA strains or other *B. brongniartii* isolates their genotypes were investigated by analysing 6 microsatellite loci (Table 3). The genotypes were compared to the genotypes of the *B. brongniartii* BCA strains applied at the different sites (Tables 1 & 2).

Different abundances of the applied BCA genotypes were detected among the isolates originating from test sites treated with blastospores (Fig. 2). The blastospore application in 1985 consisted of a 1:1:1 mixture of the three BCA strains 129, 119, and 132 (genotypes A,

B and C) and the application in 1988 consisted of a 1:1:1 mixture of the three BCA strains 129, 119, and 256 (genotypes A and B, third strain was not available). At all test sites investigated one or more of the genotypes of the applied BCA strains could be identified (Fig. 2). At two locations (Zuben, Happerswil) all the genotypes of the applied BCA strains were present, whereas at the other sites only the genotype of a single BCA strain was isolated. Genotypes other than the ones of the BCA strains were identified at three locations (Zuben, Dünnershaus and Bleiken). One of the genotypes isolated from the location Bleiken might represent the genotype of the third strain applied which is not available anymore.

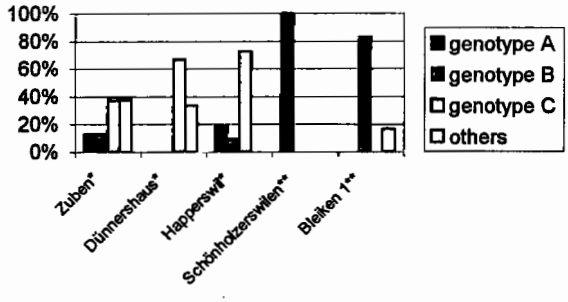

Figure 2. Relative genotype frequencies of *B. brongniartii* isolates originating from locations treated with blastospores in *1985 (genotypes A, B, C) or **1988 (genotypes A and B)

The relative distribution of different genotypes detected among the isolates originating from test sites treated with fungus kernels are shown in Figure 3. The fungus kernels applied in 1991/1992 were inoculated with only one BCA strain (genotype A). At two locations (Guggenbühl, Neukirch) all the genotypes of the isolates represented the genotype of the BCA strain applied whereas at the two other locations (Bleiken, Biessenhofen) also other genotypes were identified beside the genotype of the BCA strain.

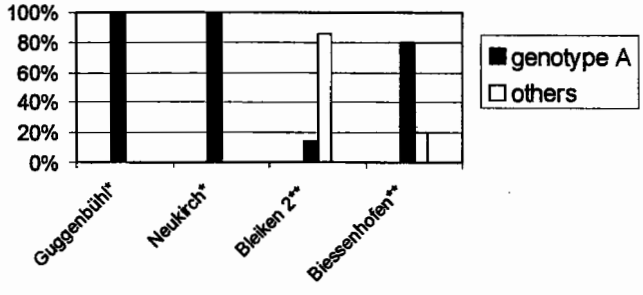

Figure 3. Relative genotype frequency of *B. brongniartii* isolates originating from locations treated with fungus kernels (genotype A) in *1991 or **1992

Discussion

In this study we demonstrated the feasibility of microsatellite analysis for monitoring the fate of *B. brongniartii* BCA strains in the field. Analysis of six microsatellite loci among the isolates obtained from test sites treated with blastospores or fungus kernels revealed that applied BCA strains can persist in soil for at least 14 or 7 years respectively. Eighty-one percent of the isolates re-isolated from the treated sites had a genotype identical to the genotype of one of the applied BCA strains.

A total of 15 isolates with a genotype other than any of the BCA strains applied were identified. These isolates originated from five test sites whereas in the other four test sites only isolates with a genotype identical to the BCA strains were identified. The 15 isolates either represent indigenous isolates or they represent isolates that have arisen from genetic interaction between BCA strains and indigenous isolates. However since microsatellite loci tend to display high mutation rates it is also possible that mutation events might have caused the differences in the genotypes (Henderson & Petes 1992, Levinson & Gutman 1987). Unfortunately, we have no isolates available that were collected before the applications. Such isolates would allow to determine which of the genotypes or alleles were present in the population before the application. Thus based on our current information it is not possible to determine the history of the isolates with a genotype other than any of the BCA strains.

Once developed microsatellite markers are a fast and efficient tool to genetically analyse large numbers of samples. They can provide detailed information due to their multiallelic nature and high level of polymorphism. This study demonstrates that microsatellite markers are well suited to identify and monitor BCA strains in the field.

Acknowledgments

This work is part of the EU-project BIPESCO (FAIR6-CT98-4105) and supported by the Swiss Federal Office for Education and Science (BBW Nr. 98.0068).

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.97 – 101

Growth and persistence in the Aosta Valley of highly virulent and genetically recognizable strains of the entomopathogenic fungus *Beauveria brongniartii.*[*]

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Abstract: To verify the growth and persistence of highly virulent and genetically recognizable strains of *Beauveria brongniartii* for the microbiological control of *Melolontha melolontha* six experimental fields were chosen in the Aosta valley. The fields were infested by *M. melolontha* and there was a natural absence of the *B. brongniartii* strains used in this research as checked by RAPD-PCR analysis.

In these fields, a conidial mixture of 5 *B. brongniartii* strains (C/2, F, K/2, N/3, W/2), previously grown on rye kernels, was spread, with a conidial load of 1.0 x $10^9/m^2$. For one year, every month with the exception of the winter period, the soil of each experimental field was sampled, in order to ascertain the growth and persistence of the strains inoculated. *B. brongniartii* strains were isolated from each soil sample and submitted for genetic characterization by RAPD-PCR analysis. From the the results obtained it appears that strain F showed a good aptitude for growth and persistence in all the fields where it was inoculated; strains C/2 and W/2 were found only in some of the fields where they were inoculated; strain K/2 showed little adaptation capacity while strain N/3 was never isolated from the experimental fields.

From the results obtained from these investigations we can conclude that *B. brongniartii* strain F is likely to be the most suitable of the strains studied for the successful microbiological control of *M. melolontha* in the Aosta valley.

Key words: *Beauveria brongniartii*, highly virulent strains, genetically recognizable strains, growth in soil, persistence in soil.

Introduction

In the last few years our Department has undertaken various studies on the genetic characterization of different strains of the entomopathogenic fungus *Beauveria brongniartii* obtained from infected larvae of *Melolontha melolontha* in the Aosta Valley (Cravanzola *et al.*, 1997; Ozino *et al.*, 1998; Piatti *et al.*, 1998).

The RAPD-PCR method used in this research allowed us to obtain genetic markers useful for identification of these strains in experimental trials for the microbiological control of *M. melolontha*.

This study was set up to select among the highly virulent and genetically recognizable *B. brongniartii* strains those strains which are suitable for successful microbiological control in the particular pedoclimatic conditions of the Aosta Valley. The main aim of this research was to verify the growth and persistence ability of the highly virulent and genetically recognizable strains in the soil of the Aosta Valley.

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[*] Research funded by a grant of the Regione Autonoma Valle d'Aosta

Materials and methods

In Jovençan (Aosta) 6 orchards with a surface of $625m^2$-$1732m^2$ each were chosen for the investigations. The chosen fields were assayed by $1/6m^2$ drillings (on average 10 per field) and found to be infested by *M. melolontha* L. with an average density of 11 larvae/m^2.

A conidial mixture of 5 strains (C/2, F, K/2, N/3, W/2) of the entomopathogenic fungus *B. brongniartii* (Sacc.) Petch was spread in the 6 experimental fields considered. The 5 strains were chosen for their virulence level among 58 isolates obtained from infected larvae of *M. melolontha* in the Aosta Valley (Piatti *et al.* 1998). Bioassays confirmed the high virulence level of the 5 strains: 90% for strains F, K/2, N/3, W/2 and 85% for strain C/2, values that show the percentage of *M. melolontha* larvae killed by *B. brongniartii* within 45 days of incubation.

In May 2000 a conidial mixture of the 5 strains, previously grown on rye kernels, was spread in the experimental fields, with a conidial load of $1.0 \times 10^9/m^2$ ($1,0 \times 10^5$/g d.w.), with 2.0×10^8 conidia/m^2 from each strain. The inoculation of the mixture in the soil was preceded by an estimate of any *B. brongniartii* naturally present in the 6 experimental fields, by the classical methods of isolation of microbes from soil samples.

After inoculation, growth and persistence of the 5 strains in the field was studied. The presence of the 5 strains was estimated in the months following inoculation (July 2000, August 2000, September 2000, October 2000) and in April 2001 after winter when the soil was covered by snow or was frozen.

Fungal isolations from soil samples (9 for each field along diagonals) collected in the experimental fields was performed on selective Sabouraud dextrose Agar medium as described by Veen & Ferron (1966). The *B. brongniartii* colonies obtained were submitted to DNA extraction as described in the protocol of Rogers and Bendich (1988), modified by Henrion *et al.* (1994). Extracted DNA was submitted to genetic characterization with RAPD-PCR analysis in order to determine suitable molecular markers to identify and track, after inoculation in the ground, the 5 strains used.

The reaction conditions were 16mM $(NH_4)_2SO_4$, 67mM Tris-HCl pH 8,8 (25°C), 0,01% Tween 20, 3mM $MgCl_2$, 200µM each of dATP, dCTP, dGTP, dTTP, 0,6µM 10-base primer, 1,5U of Biotherm Red DNA Polymerase (Fisher Molecular Biology, Trevose, USA), 10-40ng of genomic DNA in a final reaction volume of 25µl.

RAPD amplification was performed with 4 primers belonging to the OPM set (Operon Technologies), OPM2 (5'-ACAACGCCTC), OPM3 (5'-GGGGGATGAG), OPM6 (5'-CTGGGCAACT), OPM14 (5'-AGGGTCGTTC), and able to characterize C/2 and N/3 strains, F strain, W/2 strain, and K/2 strain respectively.

Thermal cycling was performed as previously described (Cravanzola *et al.* 1997) in a Perkin-Elmer Cetus thermal cycler (model GeneAmp PCR System 2400).

Amplification products were separated by electrophoresis in 1,2% agarose gels using TAE buffer and visualized with ethidium bromide. The size of the fragments was estimated using a 1Kb DNA ladder from Advanced Biotechnologies.

Results

The fungus was not isolated from the experimental fields which had been checked for the natural presence of any *B. brongniartii* (evaluation made before inoculation in the soil with the 5 considered strains). The results obtained in the experimental fields regarding the growth and persistence of the 5 strains of *B. brongniartii* considered, are explained in Table 1.

From an evaluation of the conidial load values obtained in different isolations performed in one year, strains F, C/2 and W/2 seem to have good growth and persistence capacity. In particular strain F, a year after inoculation, reached the same load as at inoculation.

Table 1. Conidial load (conidia/g soil) of *Beauveria brongniartii* (Bb) strains present in the 6 fields taken as a whole in the period July 2000-April 2001.

	Inoculation load	**Conidial load of the strains in the period July 2000-April 2001**				
	May 2000	Jul 2000	Aug 2000	Sep 2000	Oct 2000	Apr 2001
BbF	2.0×10^4	1.7×10^4	1.2×10^3	6.3×10^3	3.2×10^3	2.5×10^4
BbC/2	2.0×10^4	1.2×10^4	3.0×10^3	1.7×10^3	3.2×10^3	8.3×10^3
BbW/2	2.0×10^4	8.6×10^3	3.0×10^2	6.3×10^3	1.3×10^3	8.3×10^3
BbK/2	2.0×10^4	0.0	0.0	5.9×10^2	6.3×10^2	2.7×10^3
BbN/3	2.0×10^4	0.0	0.0	0.0	0.0	0.0

Strain K/2 showed considerable difficulty in soil adaptation while strain N/3 was not able to develop in the experimental fields. A total of 123 isolates were obtained; their genetic characterization shows that 43.1% belonged to strain F, 30.9% to strain C/2, 23.6% to strain W/2, 2.4% to strain K/2 and 0% to strain N/3. Figure 1 shows RAPD profiles of the 5 strains obtained by using 4 primers belonging to the OPM set.

Table 2 gives the frequency of each *B. brongniartii* strain appraised, on an overall basis in the 6 fields considered, in the period July 2000-April 2001. Strain F showed, compared with strains C/2 and W/2, more constant progress with fairly high frequency values; in particular, a year after inoculation, strain F reached a frequency of 56.2%.

Table 2. Frequency of the *Beauveria brongniartii* strains in the period July 2000-April 2001 expressed as a percentage of the values obtained in the 6 fields taken as a whole.

	Jul 2000	**Aug 2000**	**Sep 2000**	**Oct 2000**	**Apr 2001**
BbF	45.3%	26.6%	42.3%	38.5%	56.2%
BbC/2	32.1%	66.7%	11.5%	38.5%	18.8%
BbW/2	22.6%	6.7%	42.3%	15.3%	18.8%
BbK/2	0.0%	0.0%	3.9%	7.7%	6.2%
BbN/3	0.0%	0.0%	0.0%	0.0%	0.0%

Table 3 shows the frequency of each strain estimated in each field over the period July 2000-April 2001 as a whole. Again strain F, which was always present, prevailed over the others with rather high frequencies, with the exception of field 2.

The values given in the table, calculated using the Duncan test, show that strain F is different from all the others because of its better capacity of adapting in the considered fields; the values of strains C/2 and W/2 are the same as are those of strains K/2 and N/3.

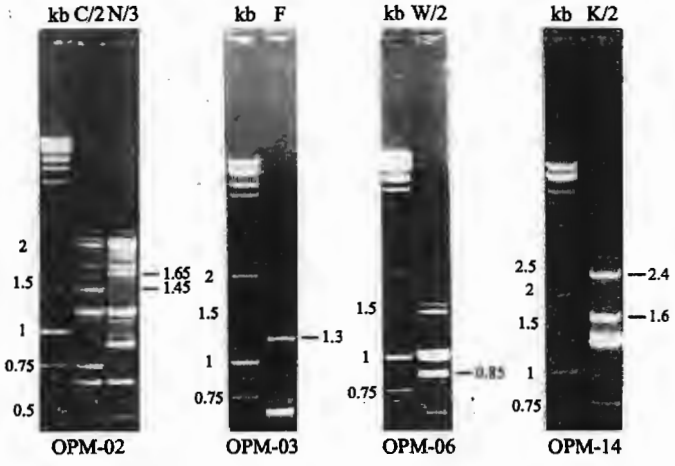

Figure 1. RAPD profiles of the 5 selected strains of *Beauveria brongniartii* obtained with the primers OPM-02, OPM-03, OPM-06 and OPM-14. The RAPD markers specific for the 5 strains are indicated. Band 1.65kb: specific for the strain N/3. Band 1.45kb: specific for the strain C/2. Band 1.3kb: specific for the strain F. Band 0.85kb: specific for the strain W/2. Simultaneous presence of bands 2.4kb and 1.6kb: specific for the strain K/2.

Table 3. Frequency of the strains of *Beauveria brongniartii* in the fields expressed as a percentage of the values obtained in the period July 2000-April 2001.

Strains	Experimental fields						Mean
	1	2	3	4	5	6	
BbF	80%	10.7%	55.6%	57.9%	43.3%	53.1%	50.1%[a]
BbC/2	0.0%	60.7%	33.3%	26.3%	13.3%	28.1%	27.0%[b]
BbW/2	0.0%	28.6%	11.1%	15.8%	43.3%	12.5%	18.5%[b]
BbK/2	20%	0.0%	0.0%	0.0%	0.0%	6.3%	4.4%[c]
BbN/3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%[c]

[a-b-c] Means within rows without a common superscript are significantly different (P<0.05)

Discussion

This research points out that different highly virulent strains of *B. brongniartii* isolated from infected *M. melolontha* larvae in the Aosta Valley, can show, after soil inoculation, different behaviour patterns of growth and persistence capacity, including negative results.

This must be considered when selecting strains for use in microbiological control, especially when it is performed in particular pedoclimatic conditions such as those of the Aosta Valley (Bondaz *et al.*, 1991; Cravanzola *et al.*, 1995; Ozino, 1995).

The results obtained point out that strain F can guarantee successful microbiological control in the Aosta Valley due to its good growth and persistence and its capacity to adapt to the environment. The behaviour of strains C2 and W2 was more modest and less constant than that of strain F. However, they should not be undervalued due to their fairly good environmental adaptation. This suggests that in programs for the microbiological control of *M. melolontha* in the Aosta Valley it is possible to use the 3 strains contemporaneously.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.103 – 107

Virulence and attenuation of entomopathogenic *Beauveria brongniartii* strains

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Abstract: Variability in fungal morphology and physiology after repeated subculturing on artificial media is a well-known phenomenon and, in the case of pathogenic fungi, may result in altered, usually reduced virulence. In our study highly virulent single spore isolates of *B. brongniartii* were cultivated up to eighteen times on Sabouraud glucose agar and on Chitin peptone agar, respectively. Physiological tests were carried out in order to see if there is a change in the carbon metabolism. By comparing the carbon utilisation patterns it was intended to find new key virulence determinants, which could help to accelerate the definition of fungal virulence and the monitoring of quality control during production process. The host-insect pathogen relationship was defined in bioassays with *M. melolontha* and on the basis of these data adequate conditions for the handling and storage of the entomopathogen *B. brongniartii* were found.

Key words: *Beauveria brongniartii*, *Melolontha melolontha*, virulence, attenuation, carbon utilisation

Introduction

In the scale of the funded EU-RTD Project Fair–PL98-4105 "Biocontrol of Important Soil Dwelling Pest" new Biocontrol Agents (BCA's) will be developed. One of the main issues in the project is to study basic mechanisms that are responsible for the virulence of *Beauveria brongniartii* against the target host *Melolontha melolontha*. The isolation of a fungal strain from an insect cadaver, its taxonomical identification and the necessary tests to define its virulence are essential and time consuming. Until the commercial production of a BCA can start many passages of the entomopathogens without any contact to the target host are necessary. The host-insect pathogen relationship must be defined in bioassays and the best conditions for handling and storage of entomopathogens are important to avoid attenuation. The production process itself asks for the monitoring of key virulence determinants in special and the quality control of products is essential for the consistent efficacy of the BCA.

Material and methods

Beauveria brongniartii strains

Highly virulent isolates of *B. brongniartii* IMBST 95031 (No. 1), IMBST 95041 (No. 2), FAL 166 (No. 3), FAL 546 (No. 4) were used in this study. The *B. brongniartii* strains IMBST 95031 and IMBST 95041 were isolated from pasture soil infested with *Melolontha melolontha* (Scarabaeidae) in Kramsach (Tyrol), Austria. FAL 166 and FAL 546 are commercial single spore isolates obtained from FAL-Reckenholz, Zürich, Switzerland.

Cultivation

B. brongniartii isolates were cultivated on Sabouraud-2-Glucose agar (S2G) and Chitin-peptone agar (CiP) at 25°C until spores could be harvested (20 to 50 days). S2G broth was purchased (Merck 1.08339) and 18 g l^{-1} agar was added. Chitin-peptone agar was composed of 10 g l^{-1} chitin (Sigma C-7170), 5 g l^{-1} peptone (Merck 7214) and 18 g l^{-1} agar (pH 5.6).

The strains were successively subcultured up to fourteen times on CiP and on S2G agar in intervals of 21 days in minimum. Periodically transfers were carried out by inoculating agar plates with 200 µl of spore suspension in 0.1% Tween 80 using a Trigalsky spatula. All passages were incubated at a temperature of 25°C and 80% r. h.

Carbon utilisation patterns

Airborne conidia were used to inoculate BIOLOG SFP- and SFN microtiter plates. Following Dobranic & Zak (1999) the tetrazolium dye MTT – Dimethylthiazolyl-diphenyl-tetrazolium bromide was used as a redox indicator. With the named two plate systems the metabolisation of 130 carbon sources was investigated. All experiments were done twice.

Data analysis was performed by calculating the average of relative carbon utilisation, whereby the absorption [OD_{590} nm] of the reference well without a carbon source was subtracted from the values measured for each well containing a carbon source. The second highest absorption for each strain and experiment, respectively was set to 100%. These data were grouped into four categories, which are defined as follows: < 10%, < 40%, < 70% and > 70% carbon utilisation.

Data of absolute carbon utilisation (OD_{590} minus OD of the reference well) were statistically analysed by student's t-test.

Melolontha melolontha *larvae*

White grubs of second or third instar larvae (L2 or L3) were collected in the field and placed individually into rearing boxes (height: 8 cm; diameter: 6 cm). The boxes were filled with peat substrate, a mixture of weakly and strongly decomposed bog peat with a pH of 5.5 - 6.0. For feeding the larvae one slice of carrots per week and individuum was used. The substrate was changed on requirement.

The larvae were kept at a temperature of 20 - 22°C and a relative humidity of 70% in the dark for six weeks (quarantine) before using them for bioassays.

Bioassays

Conidia of *B. brongniartii* were washed off the plates under sterile conditions using 10 ml of 0.1% of Tween 80 and an inoculation loop. The spore suspension was collected with a pipette and homogenised by shaking for 15 min. The number of conidia was counted using a hemocytometer and the suspensions diluted with Tween 80 (0.1%) to 1 x 10^7 ml^{-1}.

For the bioassays 10 - 30 ml suspension in a beaker of about the size of the rearing containers were used. The white grubs (15 larvae per treatment) were dipped individually for 5 seconds into the suspension. The drop of excess liquid was stripped off on a paper towel and the larvae were returned into new boxes. Larvae dipped into 0.1% Tween 80 served as a control. *M. melolontha* larvae were incubated at 20-22°C in the dark and checked weekly for mortality rates.

Results and discussion

Bioassays

The survival rates of *M. melolontha* larvae 6 weeks after the inoculation with *B. brongniartii* strains grown on S2G-medium were in the range between 20 and 30%. Strain No. 2 (IMBST 95041) killed 80% of the larvae within a period of 6 weeks and was the most virulent strain, whereas strain No. 3, the least virulent isolate in this bioassay, killed 32% of the larvae in the same period (Figure 1). *M. melolontha* 3rd instar larvae showed no symptoms of infection by fungi or other micro-organisms during quarantine.

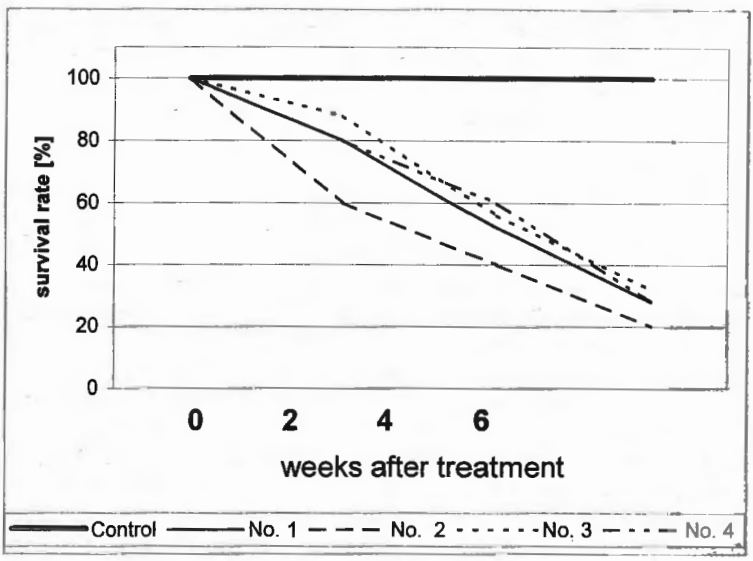

Figure 1. Virulence of *B. brongniartii* strains IMBST 95031 (No. 1), IMBST 95041 (No.2), FAL 166 (No. 3) and FAL 546 (No. 4) grown on S2G medium (second sub-culture) against *M. melolontha* 3rd instar larvae.

Conidia of the same *B. brongniartii* strains but successively subcultured eight times on S2G-medium were tested for their virulence against *M. melolontha* 3rd instar larvae according to the method described above. Survival rates of *M. melolontha* after 14 weeks were in the range between 84 (strain No. 3) and 96% (corrected for mortality in the control), thus corresponding to a significant reduction of fungal virulence compared to conidia from the second passage (Table 1). The time course of the appearance of infection was also strongly retarded.

Attenuation of virulence was retarded when successive subculturing was done on CiP-agar. Despite six transfers to CiP-agar, a loss of virulence of *B. brongniartii* strains could be avoided (Data not shown). With any further passage on S2G-agar, a significant decrease of virulence could be observed. After 18 passages a delay in LT_{50} was recorded for the cultures

Table 1. Survival rate of *M. melolontha* 3rd instar larvae after dipping in 1 x 10^7 ml^{-1} conidia of *B. brongniartii* strains IMBST 95031 (No. 1), IMBST 95041 (No.2), FAL 166 (No. 3) and FAL 546 (No. 4) freshly grown on S2G medium (second subculture) and after eight and fourteen successive subcultures.

	Larvae survival rate [%] six weeks after dipping		
	2nd subculture	8th subculture	14th subculture
IMBST 95031	30	96	90
IMBST 95041	20	30	70
FAL 166	32	94	70
FAL 546	28	88	33

grown on S2G and CiP, whereby the latter nutrient medium stabilized virulence best. *B. brongniartii* showed only a marginal loss of aggressiveness if compared to a control strain, which was directly isolated from a mycosed larva. Nevertheless, a clear attenuation of virulence could be observed for both strains subcultered on S2G-agar.

Carbon utilisation patterns

Monosaccharides, like ribose and xylose were metabolised best by all *B. brongniartii* isolates, irrespective of whether they were freshly cultivated or sub-cultured several times. Tween 40 and tween 80, which are metabolised to a high degree, are wax like substances. They might have an impact on the natural infection process of entomopathogenic fungi as the infection of insects is transcuticular and adhesion of conidia to the waxy, hydrophobic cuticle is essential for subsequent invasion and establishment of pathogenesis.

Carbon sources, which might represent virulence determinants, i. e. N-acetyl-glucosamine, a fission product of chitin, were metabolised better when fungi were sub-cultured while the metabolisation of carbon sources like xylitol and adonitol decreased with successive sub-culturing, which might indicate an attenuation of virulence (Table 2).

These findings have now to be verified and the data of *M. melolontha* bioassays done with *B. brongniartii* isolates grown on S2G- and on CiP-medium will be compared with the carbon utilisation patterns using adequate statistical methods. The aim of this study was to prevent attenuation of virulence and to find key virulence determinants, which are easy to analyse and are suitable to supplement or to replace bioassays in future.

The principal barrier to fungal infection is the insect cuticle, which is primarily composed of lipids, proteins, and chitin (Richards, 1978). The corresponding enzymes, lipases, proteases and chitinases have been assumed to play a major role in insect cuticle penetration by entomopathogenous fungi and thus have received a great deal of attention. Braga et al. (1999) studied the oxygen consumption of *Metarhizium anisopliae* during growth on different carbon sources. The authors found that the growth of the fungus on chitin was characterized by the long duration of the acceleration phase if compared to the growth on other carbon sources. However, it was observed that conidia increased their respiratory rate in medium containing chitin, this being the major characteristic of the break of dormancy, which was more rapid than in the presence of glucose. Total oxygen consumption in the medium containing chitin was higher than observed in glucose for several hours. The authors argumented that chitin when autoclaved in liquid medium, undergoes some hydrolysis with release of the N-acetylglucosamine monomer, which may have stimulated germination of *M. anisopliae* conidia. The presence of N-acetylglucosamine in the medium accelerated germination and reduced the duration of the lag and acceleration phase.

Table 2. Relative carbon metabolisation of *B. brongniartii* isolates successively sub-cultured on S2G-medium. Increasing and decreasing metabolisation rates are indicated only if the metabolisation was generally high (more than 70%) and the alteration was highly significant (less than 10% and more than 70% relative carbon metabolisation), respectively.

Successive subculturing of *B. brongniartii* and metabolisation rate of carbon sources			
	Generally > 70%	increasing	decreasing
Tween 40	+		
Tween 80	+		
D-Xylose	+		
D-Ribose	+		
L-Arabinose		+	
Palatinose		+	
L-Erythritol		+	
L-Lactic acid		+	
N-Acetyl-glucosamine		+	
D-Glucose		+	
Methyl-D-glucoside		+	
Adonitol			+
D-Mannose			+
D-Trehalose			+
Gentiobiose			+
Xylitol			+

On the basis of the presented results it can be deduced that in the insect host-pathogen relationship of *M. melolontha* and *B. brongniartii* the induction of chitinase plays a major role, at least if the goal is to slow down attenuation of virulence.

Acknowledgements

The presented work was funded by the European RTD programme FAIR6-CT98-4105 BIPESCO.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.109 – 113

Interactions of *Beauveria brongniartii* with soils under different application regimes

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Abstract: Field trials revealed that growth of *Beauveria brongniartii* in the soil depends on temperature, clay content and catalase activity. We conducted incubation experiments to verify the findings from the field under constant conditions. The production of colony forming units was distinctly higher at 22°C than at 12°C after one month of incubation. These differences are getting smaller after 3 months indicating that growth rate is reduced at sub-optimal temperature. The growth of *B. brongniartii* in three different native soils (sand, loam, clay) differs, but do not correlate with clay content. As these differences disappear in semi-sterilised soil, biotic factors are suggested to have major influence rather than soil texture.

Key words: fungal growth, soil temperature, microbiological activity, soil texture

Introduction

Beauveria brongniartii is an entomopathogenic fungus which has been used for several years in Switzerland to control the larvae of the European cockchafer (*Melolontha melolontha*). Since 1991, a product based on barley kernels colonised with the fungus is commercially available. Application of the biocontrol agent is achieved by tilling barley kernels overgrown with fungal mycelium into the soil. Subsequently the fungus grows and sporulates on the kernels and builds up the inoculum. For an efficient control of the host, a large amount of conidia has to be present in the soil under a wide range of environmental conditions. Field trials in Switzerland revealed that the growth and sporulation of *B. brongniartii* on the barley kernels in the field depend from factors such as temperature, clay content and catalase activity of the soil (Kessler & Keller in press). In this paper we present results from experiments verifying these factors under constant conditions in the laboratory.

Methods

We selected soils from three meadows in the northeast region of Switzerland (TG1, TG3, TG4). The soil samples were taken from a soil depth of 5 to 18 cm. The soils differed mainly in soil texture, content of organic material and catalase activity (table 1). The soil was filled into cylindrical plastic containers (4.5 cm ∅, 6 cm heights) and three fungus kernels (Beauveriapilz® from E. Schweizer Samen AG, Thun, Switzerland) were placed into each container ensuring that it was completely covered by the soil. For a second treatment, the soil samples were autoclaved twice for 20 minutes at 120°C before inoculation in order to produce "semi-sterile" soil. For both treatments the containers were placed into a larger plastic box together with a container filled with water to guarantee high air humidity. The boxes were incubated in darkness at 12 or 22°C for 1 and 3 months respectively. Each treatment was repeated 5 times. After incubation the soil of each container was transferred to an 300 ml

Erlenmeyer flask and 100 ml of tap water with 0.1% Tween 80 were added (Fornallaz 1992). The flasks were shaken on a horizontal shaker at 110 rpm for 3 hours. 1 ml of each suspension was diluted 1:1000 for the native soil and 1:10000 for the semi-sterile soil. 0.1 ml of each dilution was plated on a Petri dish with 9 cm diameter with selective medium (Sabouraud-2-Glucose-agar, Strasser *et al.* 1996) in five replicates and incubated at 22°C. After 10 days the number of colony forming units (CFU) were counted. The dataset was transformed by $\log_{10}$ function prior using ANOVA. Post Hoc test was performed using the Newman-Keuls-test.

Table 1: Soil characterisation and mean of soil parameters

Soil	Characterisation	Clay %	Sand %	Organic %	pH	Salt µ Si	Catalase O_2/min
TG1	*Sandy loam*	19.7	40.4	3.8	7.0	239.4	2.80
TG3	*Loamy sand*	12.7	56.6	3.4	6.7	242.8	3.03
TG4	*Loamy clay*	42.5	20.5	8.1	6.3	259.9	6.08

Results

Native soil: At temperatures of 22°C the growth of *B. brongniartii* was better than at 12°C [$F(1,115)=39{,}23$, $p<0.0005$]. After 1 month incubation at 22°C we counted in native soil 8.7×10^5 CFU g^{-1} soil on average, corresponding to 1.4×10^7 CFU/kernel. At 12°C we measured 2.3×10^5 CFU g^{-1} soil, and 3.8×10^6 CFU/kernel respectively. Two months later the number of CFU increased at both temperatures up to 6.2×10^5 CFU g^{-1} soil at 12°C and up to 1.4×10^6 CFU g^{-1} soil at 22°C. The difference of growth between the two incubation temperatures was significantly higher after one months than after three month of incubation [$F(1,47)=4.97$; $p<0.03$] (Table 2 and Figure 1).

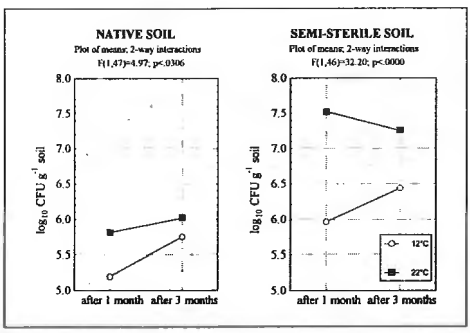
Table 2: Production of colony forming units (CFU) on barley kernels inoculated *with B. brongniartii* in native and semi-sterile soils (mean of all three soil types) at two different incubation temperatures. Significant differences are indicated (Newman-Keuls Post Hoc test, $p<0.05$).

Incubation time	Soil	Temperature	CFU/kernel	CFU g^{-1} soil	Log_{10} CFU g^{-1} soil	p<0.05
1 month	Native	12	3.8×10^6	2.3×10^5	5.19	A
	Native	22	1.4×10^7	8.7×10^5	5.81	B
	Semi-sterile	12	1.8×10^7	1.1×10^6	5.97	B
	Semi-sterile	22	5.7×10^8	3.4×10^7	7.55	E
3 months	Native	12	1.0×10^7	6.2×10^5	5.75	B
	Native	22	2.3×10^7	1.4×10^6	6.02	B
	Semi-sterile	12	5.6×10^7	3.3×10^6	6.43	C
	Semi-sterile	22	3.6×10^8	2.2×10^7	7.25	D

Semi-sterile soil: In semi-sterile soils the growth of *B. brongniartii* was at both incubation temperatures significantly higher than in native soil [$F(1,92)=463{,}07$, $p<0.0005$]. After one month we counted 3.4×10^7 CFU g^{-1} soil at 22°C, and 1.1×10^6 CFU g^{-1} soil at 12°C. After three

months the number of CFU of *B. brongniartii* was lower at 22°C than after one month. Like in native soils, the difference of growth at different incubation temperatures was significantly higher after one months than after three month [$F(1,46)=32.20$, $p<0.00005$] (Table 2 and Figure 1)

We measured a significant difference of fungal growth in the three different native soils [$F(2,47)=5.28$, $p<0.01$]. After one month we measured the highest density in TG4 (loamy clay) ($7.8x10^5$ CFU g^{-1} soil), whereas the maximum was reached after 3 month in TG1 (loamy sand) ($1.4x10^6$ g^{-1} soil). The growth of *B. brongniartii* on the kernels could not be directly connected with clay content in the soil (TG3<TG1<TG4). However, we measured a significant increase of the number of CFU from one to three months in the soils with less organic material (TG1 and TG3), but not in the soil with a higher content of organic material (TG4). The differences of fungal growth in soils of different texture and content of organic material disappeared when the experiments were conducted in semi-sterilised soil [$F(2,46)=0.37$, $p>0.69$] (Figure 2).



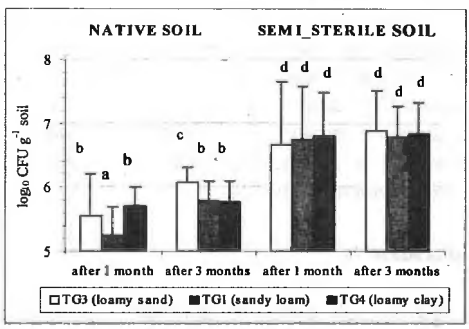


Figure 1 (left): Growth of *B. brongniartii* at two different incubation temperatures in native and semi-sterile soils. (Significant two-way interactions by ANOVA, $p<0.05$). Figure 2 (right): Growth of *B. brongniartii* in three different soils (TG1: sandy loam; TG3 loamy sand; TG4: loamy clay) under native and semi-sterile conditions. Significant differences are indicated (Newman-Keuls Post Hoc test, $p<0.05$).

Discussion

Field experiments in Switzerland revealed that the production of the inoculum of *B. brongniartii* after the application with fungus kernels is related to temperature, clay content and microbiological activity of the soil (Kessler & Keller in press). The presented results confirm this model at least for the factors temperature and microbiological activity. The optimal growth temperature of *B. brongniartii* range around 22-25°C (Aregger-Zavadil 1992). At 12°C we measured a lower density of *B. brongniartii* than at 22°C after the same incubation period. The fact that the difference is getting smaller the longer the kernels are incubated, suggests that sub-optimal temperatures will slow the growth rate but not the maximum of inoculum potentially attained as it was demonstrated for hyphal growth of *B. bassiana* (Studdert & Kaya 1990). However, sporulation may be more affected by sub-optimal temperatures than hyphal growth, so that we expect not only growth rate but also inoculum potential is reduced at temperatures below 5°C (Aregger-Zavadil 1992). Therefore

the temperature is a crucial factor for a successful application of the fungus kernels. Recent experiments have demonstrated that application in spring and summer are more successful than application in late autumn (Kessler & Keller in press). After an application in autumn, the growth of the fungus on the kernel will be slow and drag on for months into a period when the temperatures are even lower 5°C and makes sporulation unlikely.

We measured a distinctly higher production of CFU in semi-sterile soil than in native soil. Conidia of *B. brongniartii* and *B. bassiana* are known to be able to germinate and grow in sterile soil (Aregger-Zavadil 1992; Lingg & Donaldson 1981). Sterilisation of the soil increases the availability of soluble nutrients which can be used by the fungus for further growth and increase the production of conidia. A study on conidia production of *B. brongniartii* in sterilised soil and in sterile peat revealed significantly less conidia production in the nutrient-poor peat (Aregger-Zavadil 1992). The hypothesis that the higher nutrient level in semi-sterile soil enhances CFU production may be supported by the observation that we detected less CFU after three months than after one month at 22°C. At optimal growth temperature the nutrient reserve may be quickly depleted and germinated conidia have lost their viability after three months. Nevertheless we measured no significant differences between the three soil types in sterile soil as it could be observed in native soils. Therefore the differences might be caused by biological factors and are not a consequence of soil texture. Furthermore the number of CFU was still increasing after one month of incubation in soils with less organic material (TG1 & TG3), whereas no further growth could be detected in soils with higher content of organic material and catalase activity (TG4). Therefore it is concluded that biological activity in the soil influence the development of *B. brongniartii* in the soil.

Conclusions

Fungus kernels should be applied in spring and summer, when soil temperature favour growth and sporulation of *Beauveria brongniartii*. Application in autumn have no success due to sub-optimal soil temperatures. The influence of different soils on the growth of *B. brongniartii* may be caused rather by biotic soil factors than by soil texture.

Acknowledgements

The work is part of the EU-project BIPESCO (FAIR6-CT98-4105) and supported by grants from the Swiss Federal Office for Education and Science. We are grateful to Dr. H.R Oberholzer for measuring the catalase activities.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp.115 - 119

Different formulations of the entomopathogen *Beauveria brongniartii*: first results on persistence, virulence and application.

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Abstract: Standard application of the entomopathogenic fungus *Beauveria brongniartii* for the control of white grubs makes use of biomass produced on barley kernels for soil treatments. Problems not resolved yet of this practice include limited application techniques and the heterogeneity of the fungal barley kernels. In the course of the EU-project "BIPESCO" we tested purified blastospores ("hyphal bodies") and aerial conidia as well as five different formulations based on biomass, res., as alternative biocontrol agents. For the persistence-tests with the two spore types 2 x 10^5 cfu/g soil were added to pots containing 1 kg of either "non-sterile" (native) or "semi-sterile" (treated thermally) soil. Two soils from different apple plantation areas with slightly different physical-chemical characters were used in parallel, but did not show any difference on fungal persistence. Significant amounts of conidia were re-isolated 12 and 60 days post-application, res., from both "non-sterile" and "semi-sterile" soils. Blastospores were re-isolated only from "semi-sterile" soils. From "non-sterile" soils no re-isolation was possible, indicating strong inhibition by native micro-organisms. Infection of white grubs by blastospores was successful only in "semi-sterile" soils too. The five formulations were produced by PTU-Vienna and consist basically of 2% fungal mycelium (with small amounts of hyphal bodies) and a constant proportion of nutrients (yeast extract and glucose). Different organic and inorganic substances were added to increase shelf life. Dependent on these auxiliary materials the water solubility of the pellets was quite heterogeneous. A liquid application of the five formulations was possible using a standard spray nozzle. Using the pellets in their solid state the virulence towards cockchafers was on an average in the range of 35-65 % , with one formulation showing only 15% infection. On the other hand the virulence of the liquid formulations was rather low (ca. 10 %). On solid S2G-medium pellets produced 45 - 468 x 10^9 conidia/g after 8 weeks. The persistence of the pellets in "semi-sterile" soils was very low. From "non-sterile" soils no fungal propagules were re-isolated.

Key words: *Beauveria brongniartii*, formulation, persistence, application

Introduction

The success of a microbial Biological Control Agent (BCA) depends strongly on the kind of formulation, which is used to increase its stability. Shelf life before and after application, resistance against UV-radiation and other physical and chemical stresses, persistence in the soil, water solubility, rate of sporulation etc. are among the most important features of the microbial BCA`s, which can be altered by technological methods.

At present the fungus *Beauveria brongniartii* (Sacc.) Petch. -used for biocontrol of cockchafer larvae in different European countries- is commercialised as biomass on sterilised barley kernels, which act as inoculum for soil treatment (trade names: Beauveria-Schweizer®,

Engerlingspilz®, MELOCONT®-Pilzgerste). Infection of the larvae occurs through the spores produced on the surface of the kernels.

The aim of this study was to compare the persistence in the soil of the products already on the market with "naked" aerial conidia, hyphal bodies (or "blastospores") produced in liquid culture, and five newly developed formulations based on *B. brongniartii* biomass.

Material and methods

Fungal strain

Beauveria brongniartii (Sacc.) Petch. strain IMBST 95041 was used to produce the different formulations. The strain was isolated by H. Strasser from pasture soil infested with *Melolontha melolontha L.* in Kramsach/Tyrol, Austria.

Production of fungal propagules

Fungal biomass on barley kernels was obtained from Agrifutur slr (Alfianello, Italy). Conidia were produced on Sabouraud-2-Glucose-Agar (S2G, Strasser et al. 1996) and harvested after three to five weeks. Submerged spores (called "hyphal bodies" or "blastospores") were produced in the liquid media FSM by shaking on a longitudinal shaker for a week. S2G (Sabouraud-2-Glucose-Agar): 10 g peptone from meat pancreatically digested, 20 g glucose, 18 g agar, 0.05 g cyclohexamide, 0.1 g dodine, 0.1 g streptomycin, 0.05 g tetracycline; pH 6.3. FSM (Fluid Sabouraud Medium; Difco): 30 g/L (5 g bacto casiton, 5 g bacto peptamin, 20 g bacto dextrose). The spore numbers produced from the pellet-formulations on S2G-medium were counted from isolated colonies (10 repeats).

Assays on fungal soil persistence

Fungal persistence was tested in plastic pots containing 1 kg of either non-sterile or semi-sterile soil, res. 5 pots were used in parallel for each experiment. For the blastospore experiment $1x10^8$ bl were used for each pot in order to get a final concentration of $1x10^5$ bl/g soil, diluted in 100 ml distilled water and poured carefully on the surface of the pots. In the barley kernel and pellet experiments, one grain was put into the pot in a depth of 10 cm. The pots were incubated at room temperatures for three months. Re-isolation was measured using the selective S2G-medium (for details see Zelger & Schweigkofler 2001).

Bio-assays on fungal virulence

Virulence of the five formulated products towards adult cockchafers (*Melolontha* spp.) was tested in a solid and a liquid state, res. For solid state, one pellet was added to a plastic box filled with ca. 2 cm of peat containing one chafer. For the liquid application, 1 g of the product was dispersed in 1 L of distilled water and sprayed on the chafers using a hand-hold spray can with a nozzle type "eros". After application the chafers were isolated and put into the plastic boxes. Blastospores were tested against 3rd. instar larvae.

Results and discussion

The persistence of *B. brongniartii* Petch (Sacc.) in two soil types was tested using pot trials. The entomopathogenic fungus was formulated as follows: biomass grown on barley kernels, pure conidia, pure hyphal bodies (or "blastospores") and five different types of pellets, consisting of 2 % biomass, nutrients (yeast extracts and glucose) and different stabilising co-formulants. In semi-sterile soils conidia and hyphal bodies were still detectable in significant numbers 12 and 60 days after the addition of 2×10^5 cfu/g soil (Fig.1). Biomass on barley kernels at the start of the experiment contained only low levels of colony forming units (cfu's), due to heavy sporulation in the soil this value increased already at day 12 and remained stable till day 60 post-application.

Similar results were obtained with conidia and biomass on barley kernels in non-sterile (native) soils. In contrast, hyphal bodies were not recovered from this soil type on day 12 and 60 post-application, respectively. This lower fitness may be explained by the thinner and less stable cell wall of the hyphal bodies, which are produced only in liquid media. In non-sterile soils antagonistic micro-organisms might rule out the hyphal bodies much easier than the conidia or physical-chemical stress might lead to decomposition (for details see Zelger & Schweigkofler, 2001).

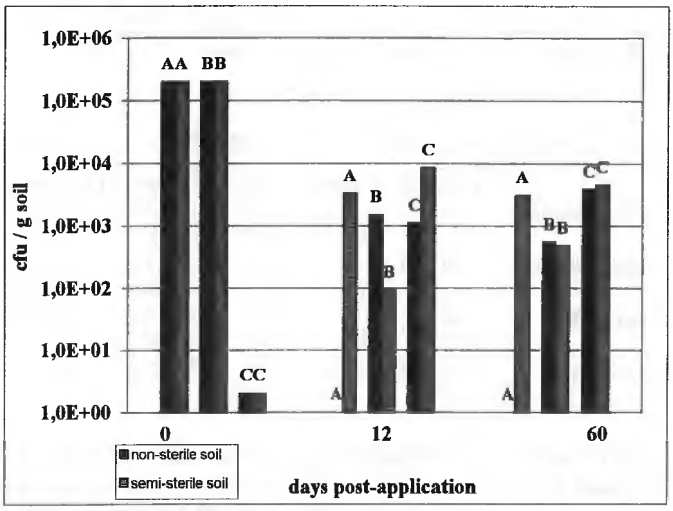

Figure1. Persistence of different fungal propagules (biomass on barley kernels, conidia and hyphal bodies) in non-sterile and semi-sterile soils using pot-trials.

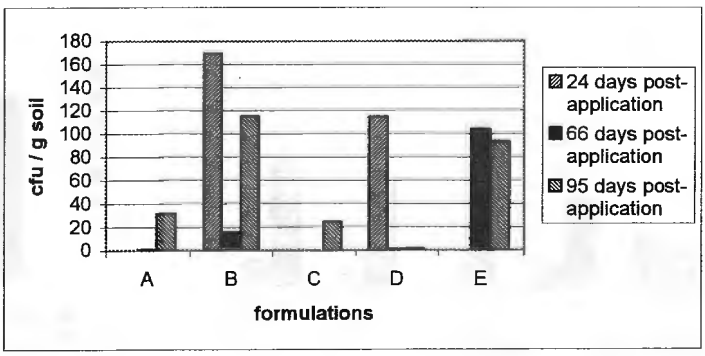

Figure 2. Persistence of five pellet-formulations based on *B. brongniartii*-biomass in semi-sterile soils using pot-trials.

Re-isolation rates from the five pellet-formulations in "semi-sterile" soils are shown in Fig. 2. Numbers of cfu's/g soil were very low for all the different types of pellets, reaching

values one to two orders of magnitude lower than the barley kernels. From "non-sterile" soils no fungal propagules were isolated at all. Similar to the situation with blastospores, fungistatic and/or antagonistic effects of the in-situ microflora seems to prevent sporulation of *B. brongniartii*.

Like biomass on barley kernels also biomass formulated as pellets has to sporulate to infect insects. When incubated on S2G-medium at 25 °C for five weeks, the pellets produced up to 1.5 x 10^9 conidia (Table 1), corresponding to 45 – 470 x 10^9 conidia/g pellet. Sporulation in the soil is assumed to occur at much lower levels, as indicate also the data shown in Fig. 2. On barley kernels, incubated on a wet paper at 25°C ca. 2x10^7 conidia were produced, corresponding to ca. 4 x 10^8 conidia/g barley kernel.

Table 1. Sporulation of five *B. brongniartii*-formulations (pellets) on S2G-medium.

	Form A	**Form B**	**Form C**	**Form D**	**Form E**
Conidia/single pellet min	0.36 x 10^9	0.10 x 10^9	0.56 x 10^9	0.36 x 10^9	0.23 x 10^9
Conidia/single pellet max	2.90 x10^9	3.41 x 10^9	1.53 x 10^9	0.82 x 10^9	0.92 x 10^9
Conidia/single pellet mean	1.46 x 10^9	1.34 x 10^9	1.12 x 10^9	0.57 x 10^9	0.51 x 10^9
Conidia/g pellet mean	432 x 10^9	147 x10^9	468 x 10^9	45 x 10^9	86 x 10^9

The virulence of the five pellet-formulations against adult cockchafers (*Melolontha hippocastani*) was tested in a solid state and as a dispersion in water (1 g/L). As shown in Fig. 3a, in four out of five cases the solid state application yielded a higher mortality rate, ranging from 33 % (Form B) to 66 % (Form C). The respective values for water dispersed pellets ranged between about 5 to 10 % mortality. Form A applied in solid state showed only low virulence (15 %), with the dispersed form slightly higher mortality rates were measured (20 %).

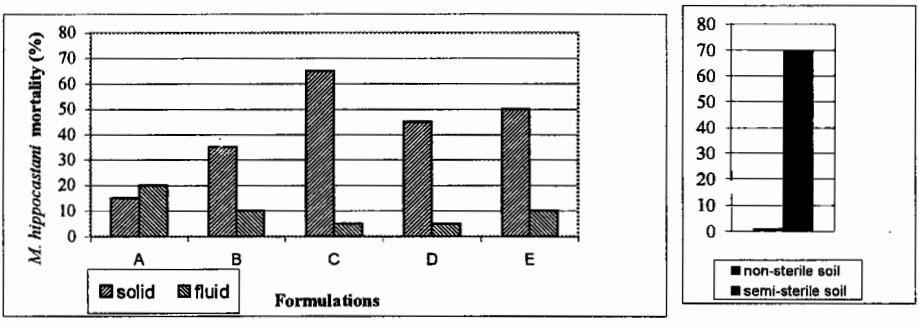

Figure3a (left).Virulence of *B. brongniartii* formulated in pellets against adult cockchafers (applied in a solid and a dispersed form).
Figure 3b (right). Virulence of hyphal bodies against 3rd. instar larvae.

The low virulence of the dispersed forms might be explained at least in part by the heterogeneous solubility of the pellets in water, depending on the composition of the auxiliary material. Application of the dispersed material (Form A-E) on Petri dishes containing S2G-medium using a commercial hand-hold spray with a standard nozzle however resulted in high colony forming (data not shown).

Addition of hyphal bodies to soils containing 3rd instar *Melolontha* larvae resulted in high mortality in semi-sterile soil only. In non-sterile soils no increase of mortality rate was observed (Fig. 3b). This results correlate well with the persistence data described above. Virulence of purified conidia of strain IMBST 95041 against *Melolontha* larvae reaches similar or slightly higher values as shown by several groups (H. Strasser, J. Eilenberg, pers. comm., own unpublished data).

The results presented in this study indicate a concrete ability of *B. brongniartii* conidia to be established in the soil in relevant numbers to act as a BCA. The low persistence of blastospores represent a serious obstacle for its use for biocontrol despite its simple and cheap production. Formulations of fungal propagules must still be optimised with main emphasis on application techniques and soil persistence.

Acknowledgements

The presented work was funded by the European RTD programme FAIR6-CT98-4105 BIPESCO.

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Integrated Control of Soil Pests "Melolontha"
IOBC wprs Bulletin Vol. 25 (7) 2002
pp. 121 – 125

Solid substrate fermentation versus liquid fermentation

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Abstract: The mass production of entomopathogenic fungi is possible by (1) solid substrate fermentation, often also called surface culture and (2) liquid fermentation or submerged culture. Both techniques are used by companies. The choice of the production technology has a huge impact on the whole commercialization process. The method selected depends on various parameters and necessary post-fermentation processes. However, economic considerations are of major importance. In this presentation, the characteristics of both techniques are described, including the production equipment, the importance of media selection and the advantages or disadvantages/problems of both fermentation methods.

Key words: Entomopathogenic fungi, *Beauveria brongniartii*, commercialization, mass production, solid substrate fermentation, liquid fermentation

Introduction

A successful commercialization and use of entomopathogenic fungi (EPF) depends on economic mass production, efficient formulations and suitable application techniques. However, any commercialization process starts with the development of an effective and economic production technology, which is directly influencing the final price of a commercial product. Generally, the method used for mass production of an entomopathogenic fungus depends on biological, technical and economic parameters, such as (1) the growing behaviour of the fungus, i.e. its ability to produce aerial conidia or submerged spores, (2) the available equipment for scaling up, (3) the intended use and the end product desired and (4) economical calculations. Furthermore, also post-fermentation processes have to be considered, such as (1) separation of spores, e.g. by centrifugation, filtration or sieving, (2) formulation and drying, (3) packaging, (4) application techniques and (5) storability. In the following, a short overview is presented on some specific characteristics of solid substrate and liquid fermentation, including the necessary equipment, the media used and the advantages or disadvantages.

Solid versus liquid fermentation

Solid substrate fermentation (SSF) of fungi has a long history. It is used for example for production of food, enzymes and fungal metabolites, organic acids or fungal biocontrol agents. At present, SSF is also used for the production of mycoinsecticides, e.g. for *Beauveria bassiana*, *B. brongniartii*, *Metarhizium anisopliae* and *Verticillium lecanii*.

Generally, SSF consists of a two phase production system. In the first phase, the fungal inoculum is produced in liquid culture, which is then used for inoculation of the solid substrate. Two different types of fermentation procedures are distinguished:

(1) Production of mycelium and conidia on sterile, nutritive substrates, such as barley, rice or wheat bran. The conidia are either harvested from the substrate by separation and then formulated or the fungus overgrown substrate is used as final product.
(2) Production of mycelium and conidia on inert, non-nutritive materials, such as vermiculite, which is drenched with a liquid medium. The advantage is, that the medium can be adjusted more precisely to the requirements of the fungus.

The liquid fermentation (LF) or submerged production of fungi is also a well-known and established technology, which is used by industrial companies for manufacture of bakers and brewers yeast, production of fungal metabolites, bioconversion of organic molecules and the production of antibiotics. Also bioinsecticides based on *Bacillus thuringiensis* are produced by LF. However, the production of a mycoinsecticide in liquid culture may differ from many established processes, because the living biomass is produced rather than any fermentation by-product. Most of the EPF are able to grow in liquid culture, which is comparable to the multiplication of the fungus in the hemolymph of the host insect. Generally, EPF are growing as blastospores, also called hyphal bodies, hyphal biomass or pellets. Also the production of submerged conidia has been studied and evoked great interest, as these propagules differ from blastospores in their morphology and stability (Hegedus et al. 1992). LF was used for production of the first mycoinsecticide registered in Germany, BIO 1020® by BAYER, based on *M. anisopliae*. Currently, two commercial mycoinsecticides are produced by liquid fermentation: PreFeral® (*Paecilomyces fumosoroseus*) from THERMO TRILOGY and Vertalec® (*Verticillium lecanii*) from KOPPERT B.V.

Production equipment

For SSF, a great variety of production technologies, including various vessel types and other equipment, is available. Some of these methods have also been used for the production of EPF. The process of development is still lasting. The equipment includes bags, trays and stationary or rotary solid substrate fermenters. An extensive list of vessel types is presented by Aidoo et al. (1982). Recently, new types of vessels have been developed and described, e.g. the so-called Zymotis fermenter by Roussos et al. (1993), a new SSF technology developed by the company PROPHYTA or new techniques used by the spawn-producing industry. It was claimed by the US company MYCOTECH, that they have the most effective SSF technology regarding EPF for the conidia production of *B. bassiana*. The yields are about 1×10^{10} spores per gram of substrate, i.e. about 1×10^{13} per kg (Wraight et al., 2001). The advantage of the new solid substrate fermenters is, that a scaling-up and an efficient control of temperature, moisture and aeration is possible. However, also the capital investments are higher compared to the bag technique. Nevertheless, this development will certainly have an impact on the production and commercialization of mycoinsecticides. A special liquid surface culture technique for production of aerial conidia is used by the Czech company FYTOVITA. This technique is a combination of surface production using a liquid medium, i.e. aerial conidia of *B. bassiana* and *B. brongniartii* are produced on the surface of a sterile nutrient solution in inflated plastic bags (e.g. Boverol®).

In contrast to the diversity in the technical equipment for SSF, a cascade of fermenter vessels, and separators are necessary for LF. The technology and the experience are available. The equipment contains flasks, bottles or technical fermenters of different size (1 l up to 30.000 l and more). There are only minor changes in the development of new vessel types. The reason may be, that LF is a well established technology, including the scaling up.

Media

Costs, availability and simplicity are key factors for the development of a production medium in SSF as well as in LF. Any medium that is used for SSF or LF without considering the problems of scaling up and commercialization is of limited value. For production of EPF, a wide variety of solid substrates has been tested and is actually used. For example, low-cost production media are: Rice, rice bran, various cereals and waste products from the food industry. For maximum sporulation, a good surface area to volume ratio and an optimal substrate/water ratio is important. Further details and a list of media have been compiled by Jenkins et al. (1998). As inert, non-nutritive substrates generally clay granules are used which are drenched with a nutrient solution. Currently, this technique is used by the company Natural Plant Protection (NPP) for the production of *B. bassiana* (Ostrinil®) and *B. brongniartii* (Betel®)(Guillon, 1997).

Inexpensive and readily available nutritive substrates are also a prerequisite for the media development in LF. The first liquid media for the production of blastospores of *B. bassiana* and *M. aniopliae* were developed by Samsinakova (1964) and Adámek (1963). Lateron, Catroux et al. (1970) published a liquid medium for submerse production of *Beauveria brongniartii*. The media used today mostly contain glucose, sucrose, starch, malt extract or molasses as carbon sources and ammonium salts, corn steep liquor, peptone or soybean meal as nitrogen source. Also, various waste products from the food industry or animal manure have successfully been used for blastospore production (Stephan & Zimmermann, 1997).

Advantages and disadvantages

In nature, aerial conidia are the only infective propagule, and are adapted to their environment. Therefore, most of the biocontrol products available are produced by SSF or on surface culture. As already mentioned, a great variety of methods and technologies is available, starting from autoclavable plastic bags to newly developed, microprocessor-controlled solid substrate fermenters. Also, many simple and cheap media have been tested and are used presently. This offers a great variety of possibilities and the selection of the most suitable and economic technology, depending on the fungus, the desired product and general cost investments.

A prerequisite for the use of SSF is, that the selected fungus isolate is able to produce high amounts of conidia per gram or cm^2 substrate. A disadvantage compared to LF is the relatively long duration of the fermentation, which lasts about 2 weeks. In the simple solid substrate fermentation technique using plastic bags, a direct control of the production parameters is not possible. Here, also problems with contaminations may occur. However, if the fungus overgrown kernels are directly used, e.g. for soil application of *B. brongniartii* against white grubs, further post-fermentation processes can be reduced or are even not necessary as the sporulation of the fungus on the kernels takes place in the soil after application. For the new generation of SSF, higher capital investments are necessary, including the separation technique for harvesting of conidia. When the conidia are produced in trays also special harvesting techniques are necessary.

The advantages of the LF are that a scaling up in conventional tank fermenters is possible up to 30.000 liters and more. The medium used can be adapted to the relevant species or isolate, and all parameters necessary for growth can be controlled, such as temperature, pH and oxygen supply. There is only a low risk for contamination. Another advantage is the relatively short duration of fermentation runs of usually three days, and the separation of the fungal biomass from the nutrient medium also allows a separation from any possible toxic metabolites.

On the other side, the disadvantages are that high capital investments and high start-up costs are necessary. Yet, this problem may be overcome by outsourcing of the production process. As for SSF, isolate selection is very important. Isolates may vary greatly in their ability to produce blastospores or submerged conidia, and their production depends on several parameters, such as the selected isolate itself, the composition of medium or the stirring technique. However, high virulent isolates, which produce only limited amounts of conidia but high amounts of blastospores or biomass, are useful for LF. A disadvantage may be, that the number of popagules produced per ml seems to be restricted to about 1 - 3 x 10^9, i.e. 1 - 3 x 10^{12} per liter, because of the increasing amount of biomass resulting in stirring problems. These might perhaps be overcome by so-called airlift or internal loop-fermenter technologies. In many papers, the limited stability of blastospores compared to aerial conidia is mentioned. However, it has been demonstrated, e.g. by Stephan & Zimmermann (1998), that successful drying of submerged spores is possible using certain protectants and spray-drying adjustments. For certain granular formulations, not the number of blastospores or submerged conidia but the biomass produced may be important. Formulation of moist biomass could be more advantageous than using dry conidia, which have to be remoistened again after harvesting.

Conclusions

Entomopathogenic fungi, such as *Beauveria bassiana*, *B. brongniartii* or *Metarhizium anisopliae*, can be produced either by solid substrate or liquid fermentation At present, SSF techniques are used more frequently than LF for mass production of EPF. The production of conidia or blastospores per g substrate or ml, the available technology and, especially, economic considerations play a major role. For any economic calculation, the number of spores produced per g, ml or cm^2 in relation to the number of effective spores applied per ha is important. The technology used for mass production in connection with economical considerations has a great impact on the successful commercialization of a biocontrol agent. Only a low-cost mycoinsecticide with high quality, efficacy and good storability will be successful on the market.

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The IOBC/WPRS Bulletin is published by the International Organization for Biological an Integrated Control of Noxious Animals and Plants, West Palearctic Regional Section (IOBC/WPRS

Le Bulletin OILB/SROP est publié par l'organisation Internationale de Lutte Biologique et Intégré contre les Animaux et les Plantes Nuisibles, section Régionale Ouest Paléarctique (OILB/SROP)

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ISBN 92-9067-144-9

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