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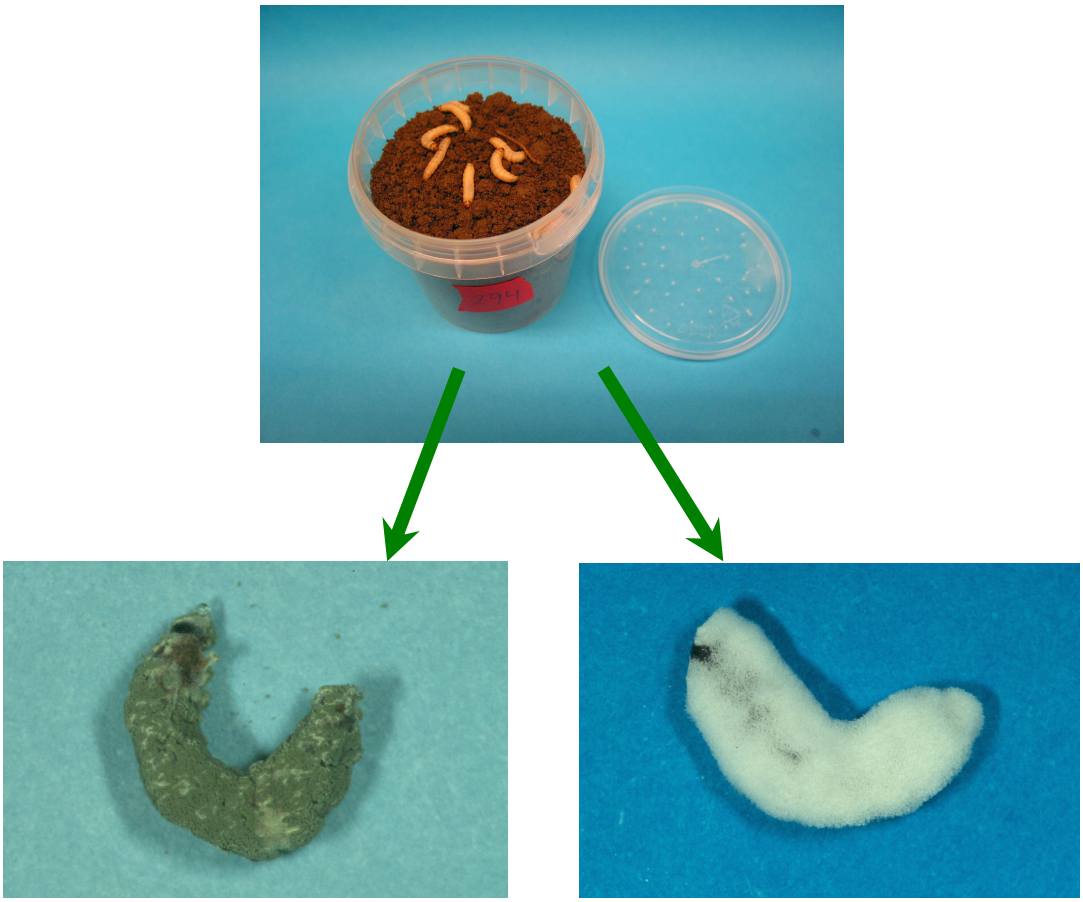
Laboratory manual, January 2007

Deliverable 5.1, VegQure, DARCOF III: Research in Organic Food and Farming (FØJO III)



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Summary

Descriptions of methods and recommendation of laboratory procedures for the isolation of soil borne entomopathogenic fungi (specifically *Beauveria* spp. and *M. anisopliae*) are presented. For screening of occurrences of indigenous populations of entomopathogenic fungi the insect bait method is recommended. Further recommendations are: 1) Collect sufficient number of soil samples to cover the area of investigation; 2) if the bait method is used, apply sufficient individuals of bait insects to each sample to increase the likelihood of isolating the fungi present. Descriptions of isolation methods, statistical analyses of the data and preparation of media and bait insects are given.

Introduction

Entomopathogenic fungi are natural enemies of insects and arachnids and the fungi contribute to the regulation of their host populations. In agriculture, the fungi have been observed to cause mortality in pest populations and several fungal species have been investigated for their potential as biological control agents. The traditional approach in biological control with entomopathogenic fungi has been to apply the fungal material (usually conidia) to the cropping system, using an inundative or inoculative biological control strategy (Eilenberg *et al.*, 2001). Thus these approaches do not exploit the indigenous reservoir of fungi that is already present in the cropping system.

Another biological control strategy is conservation biological control. Eilenberg *et al.* (2001) defined this strategy as: "*Modifications of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effects of pests*". Therefore knowledge of the community of natural enemies in the agroecosystem as well as the effect of the agronomical practices on these organisms is essential to use a conservation biological control strategy. This manual will focus on methods to obtain knowledge of the community of entomopathogenic fungi in soils. The manual will be limited to selected taxa of fungi belonging to the order Hypocreales in the division Ascomycota.

Entomopathogenic fungi occur naturally as infections in insect or arachnid hosts. Thus sampling of host individuals can reveal information about host range and prevalence of fungal species as pathogens in natural host population. However, several entomopathogenic fungi only occur as infections in living hosts for a relatively short period of time during their life cycle. The remainder of the life cycle these species presumably lurk as dormant conidia in the soil in the vicinity of the dead host cadaver. Limited saprobic growth is some times possible from resources contained in the host cadaver. Most fungi from the order Hypocreales are only known in their anamorphic (asexual) life cycle in Europe, thus only mitosporic conidia are formed. The dead host cadavers will mostly fall to the ground and thus a reservoir of fungal material is present in the soil environment. Further dispersal from cadavers as focal points presumably occur due to weather (rain and wind), soil manipulation and also insect activity (Meyling *et al.*, 2006).

Conidia produced on the surface of dead host cadavers are relatively long lived. These structures represent the freeliving infective stages, as defined by Anderson & May (1981), of the pathogen and need to come in contact with a susceptible host in order to grow and

proliferate successfully. In the laboratory, however, the conidia from hypocrealean entomopathogenic fungi can also germinate, grow and conidiate *in vitro* on artificial rich media. These two methods of germination are manipulated for isolation of entomopathogenic fungi from the soil environment.

Methods for isolation of entomopathogenic fungi from soil samples

Selective media

A wide range of fungi occur in the soil environment and they have various ecological functions. Most of these fungi, along with a range of bacteria, can grow on artificial media *in vitro*. These abilities have long been exploited to isolate microorganisms from soil samples and specific media have been developed to select for certain groups of microorganisms. Some media for the selective isolation of entomopathogenic fungi have also been developed. Bacteria can be inhibited by the application of broad-spectrum antibiotics such as chloramphenicol, tetracycline or streptomycin (Goettel & Inglis, 1997). The main remaining obstacle in using this isolation method is that the hypocrealean entomopathogenic fungi grow relatively slowly in comparison to the ubiquitous opportunistic saprotrophic fungi found in the soil environment. Thus the contents of the media need to include substances that prevent these fungi from overgrowing the species of interest. Generally, the species *Metarhizium anisopliae*, *Beauveria bassiana* and *B. brongniartii* have been investigated the most.

Media for isolation of Metarhizium spp.

Goettel & Inglis (1997) provide a list of suitable selective media for *Beauveria* and *Metarhizium* (Goettel & Inglis, 1997, p. 248). The suggested medium for isolation of *Metarhizium* spp. is often called Veens semiselective medium (Hu & St Leger, 2002) to refer to its first description by Veen & Ferron (1966). The medium contains the antibiotics chloramphenicol as well as the fungicides dodine and cyclohexamide (Goettel & Inglis, 1997). In different laboratories modifications have usually been made to optimise isolation results based on experience. For example, Hu & St. Leger (2002) used Veens medium to isolate *M. anispoliae*, but omitted cyclohexamide to study the occurrences of other fungi than *M. anisopliae*. In our laboratory, a modified medium has been developed to re-isolate applied

conidia of *M. anisopliae* in order to estimate persistence in the soil as well as vertical movement (Vestergaard & Eilenberg, 2000). The procedure to make this medium is described in Appendix A.

Media for isolation of Beauveria spp.

During the last decades research groups have been developing biocontrol programmes for the control of soil dwelling larvae of scarabaeid beetles, principally the cockchafer, *Melolontha melolontha*. This group of beetles is frequently infected in the field by the pathogen *B. brongniartii* and this particular fungal species has been developed as a biocontrol agent in Switzerland and Austria. In order to monitor the fate of applied fungal material in the soil, a selective medium was developed. Originally described by Strasser *et al.* (1996) this medium has been used in several studies (Enkerli *et al.*, 2004; Keller *et al.*, 2003; Kessler *et al.*, 2003; Kessler *et al.*, 2004). At KVL, this medium is also used for the detection of survival of applied *B. brongniartii* material, but has also been successfully used to isolate *B. bassiana* from phylloplanes of different plant species (Meyling & Eilenberg, 2006a). This medium is described in Appendix A.

Insect bait method

The use of selective media exploits the saprotrophic abilities of hypocrealean entomopathogenic fungi. However, to exploit the ability of the fungi to infect host, the insect bait method can be used. This method was originally developed to isolate entomopathogenic nematodes from soil samples, but fungi were sometimes additionally isolated (Zimmermann, 1986). Thus Zimmermann (1986) suggested that this method could also be a standard isolation method for entomopathogenic fungi. For the method to be feasible insects, which are easily reared and are susceptible to the fungi, must be used. The traditional bait insect is the highly susceptible larvae of the wax moth, *Galleria mellonella,* (Lepidoptera: Pyralidae) but also mealworm larvae, *Tenebrio molitor* (Coleoptera: Tenebrionidae), are suitable. Baiting soil samples with larvae of *G. mellonella* is a widely applied tool to screen for indigenous species of entomopathogenic fungi (Vanninen *et al.*, 1989; Vänninen, 1996; Chandler *et al.*, 1997; Bidochka *et al.*, 1998; Klingen *et al.*, 2002; Keller *et al.*, 2003; Meyling & Eilenberg, 2006b). Method for rearing *G. mellonella* is presented in Appendix C.

Few studies have evaluated the use of several bait insects from different taxa. Klingen *et al.* (2002) found that dipteran larvae isolated fungi differently than *G. mellonella*. More specifically, larvae of *Delia floralis* (family Anthomyiidae) isolated *Tolypocladium cylindrosporum* more frequently than did *G. mellonella* (Klingen *et al.*, 2002). Thus the use of insect baits can also be considered to be a selective isolation method. However, the "*Galleria* bait method" appears to be more sensitive than traditional plating on media (Keller *et al.*, 2003) and is therefore useful for isolation and identification of the spectrum of entomopathogenic fungi indigenously present in soils.

Recommendations for the use of the insect bait method

Since Zimmermann (1986) recommended the insect bait method for the selective isolation of entomopathogenic fungi, numerous studies have been carried out using insect baits, especially *G. mellonella*. In 1998, a further set of recommendations was published by the International Organisation for Biological Control (IOBC) (Zimmerman, 1998). Here, the recommendations are:

- Air-dry soil samples and re-moisturise the samples afterwards to appropriate levels to avoid infections by entomopathogenic nematodes
- Use 5-10 larvae per sample
- Replicate baiting of each sample 5 times
- Incubate the samples at 20-22^{o}C in the dark and invert the individual containers every day during the first week
- Inspect the samples for the first time after 5 days and repeat this every 3-4 days for 3 weeks after initial baiting
- Surface sterilise the dead bait larvae with 1% Na-Hypochlorite prior to incubation in moist chamber

Based on my own experience, the following comments are given to the usage of the insect bait method to screen soil samples for entomopathogenic fungi. These comments are supplementary to the recommendations given by Zimmermann above.

- There is no need to first dry the sample and the re-moisture it. However, it takes some experience to get a feeling of what the best moist contents of the soil should be. As a rough guideline, the soil should not be too damp and not leave too much condensation on the inside of the container. However, some condensation on the inside of containers is desirable. The soil should not be so moist that clumps are formed. Remember to punch air holes in the lids of containers
- It is important to turn the containers regularly in the beginning of the baiting period (first week) to make bait insects penetrate as much soil as possible while they are still vigorous
- If *G. mellonella* larvae are used, select medium sized larvae and prepare them by heat treatment in warm water to prevent extensive webbing in the soil. The method was used successfully by Meyling & Eilenberg (2006b) and is described in Appendix 2
- Use 10 bait larvae as some always disappear or die of causes other than mycosis
- There is no need to inspect the samples until after 1 week because no larvae die of mycosis during the first 5 days at 20-22^{o}C
- Replicating baiting of each sample is fine if the number of samples is low. Otherwise, do the replication in the field and take more samples. Then there is no problem of relatedness of the results during statistical analysis
- Surface sterilisation is fine to prevent external saprophytic fungi from growing on the dead cadaver. However, if the larvae are indeed killed by entomopathogenic fungi that have penetrated the body of the insect they will immediately emerge from the cadaver keeping other opportunistic fungi at bay. Furthermore, individual surface sterilisation of large numbers of larvae will be a huge amount of work that does not provide much information if many soil samples are to be screened. Thus surface sterilisation should be considered critically and evaluated with regards to the number of samples in the study

Advantages and disadvantages of using different isolation methods

Each isolation method will have some advantages and disadvantages. These have to be kept in mind while selecting the most suitable method to be used for a specific study.

Soil suspensions on selective media

Advantages	• Quantitative data • Parametric data; use of standard statistical analyses (e.g. ANOVA)
Disadvantages	• Overgrowth of opportunistic soil fungi on media • Small soil sample (1 g); risk of not sampling the fungus because entomopathogenic fungi are usually clumped in the soil • Dilution effects: zero-values when in fact the fungus is present because only a diluted sample is taken from 1 g sample of the original sample

Insect bait method

Advantages	• Use of *G. mellonella* is a very sensitive detection method • Entomopathogenic fungi are selectively isolated
Disadvantages	• Some insect species may select for specific fungal pathogens • Moist soil may enhance the infection of nematodes and not fungi • Difficult to quantify inoculum levels

Soil sampling, types of data and their analysis

Traditional analyses

Any investigation of the occurrence of entomopathogenic fungi in soil needs to consider appropriate methods for statistical analysis of the data. The most widespread and always applicable way to analyse occurrence data is by the use of frequencies (qualitative data) and chi-square tests. No considerations about distribution of data are necessary for this type of test. Since frequency based data only inform about +/- occurrence it is essential that a

sufficient number of samples are included in the analysis and that an appropriate effort for isolation of fungi from each soil sample is applied. Chi-square test needs to have at least numbers larger than 5 in each cell (or 20% of cells) to be reliable thus the number of positive samples needs to exceed 5. For instance, if the "true" occurrence of a fungus is approximately 10%, then at least 50 samples need to be included for isolation. If the bait technique is used then enough individuals of bait insects need to be applied to each sample to yield reliable data. For example, Chandler *et al.* (1997) only used one *G. mellonella* larva for each sample and found low frequencies compared to other studies. This result is probably due to the death of larvae of other causes than fungal infections thus presumably underestimating the occurrence of fungi.

When the soil dilution plating method is used the procedure is more tedious than the bait method (preparation of soil subsamples, determination of water content, dilution and plating of suspensions as well as the initial production of media). Thus lower numbers of samples are usually included than is possible if the insect bait method is used. The dilution plating method yields quantitative data that can readily be analysed by parametric methods (e.g. ANOVA) normally after transformation of the data to stabilise variances.

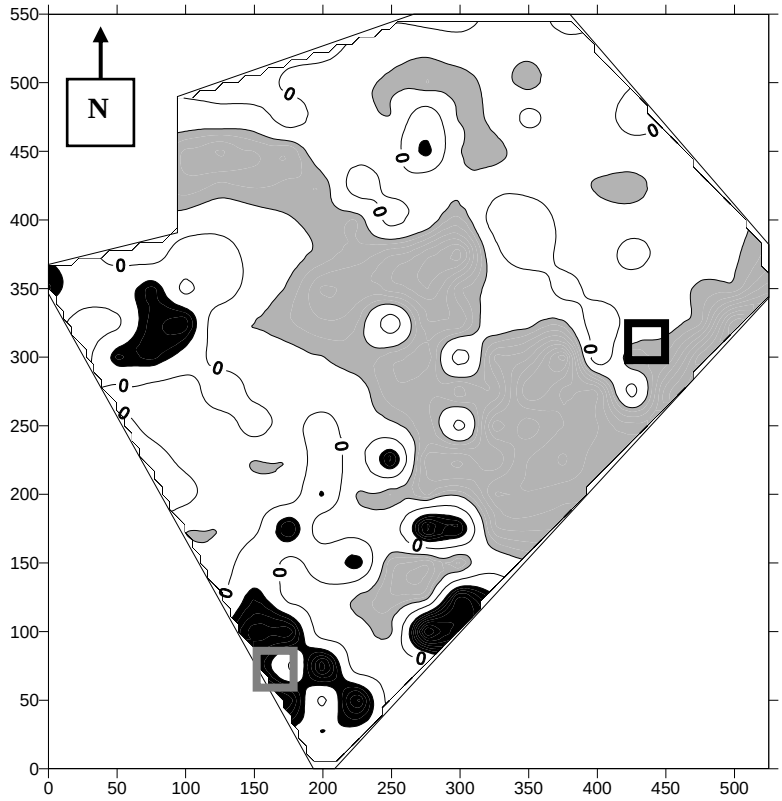
Distribution patterns

Most published studies have reported on collection of soil samples with no particular references to the spatial distribution of the individual samples. Normally, the samples have been described with respect to the type of habitat from which they were collected, e.g. agricultural field soil, forest soil, etc. Meyling & Eilenberg (2006b) collected soil samples from specific points in a sampling grid based on GIS (Geographical Information Systems). The individual sampling points could be identified by GPS (Global Positioning System). Thus the occurrence of entomopathogenic fungi in each sample could be related to a specific coordinate and a map of the occurrences could be created. The occurrence data were analysed as quantitative data since the number of dead larvae (0 - 10) was included in the analyses. Spatial statistics, where both data values as well as locations of the data in two-dimensional space are included, were applied to the data. The method used is called SADIE and has been developed for analysis of count data such as the occurrence of insects in traps etc, but the method was also found to be useful for data on occurrence of entomopathogenic fungi. More information of the statistical method can be found on the website of Professor Joe Perry:

http://www.rothamsted.bbsrc.ac.uk/pie/sadie/SADIE_home_page_1.htm and in Perry *et al.* (1999).

The main advantage using the method is that it allows for the identification of clusters of patches and gaps of the organisms and makes explicit tests of whether the data follow a random distribution or are clumped. In the study by Meyling & Eilenberg (2006b) the fungus *Beauveria bassiana* was found to be distributed in clumps in one year and clusters of patches and gaps of the fungus were found in specific parts of the investigated field (Figure 1). Furthermore, sampling of soil in selected patch and gap areas and subsequent isolation of entomopathogenic fungi confirmed the identification of the areas as areas of high and low occurrence of *B. bassiana* (Meyling & Eilenberg, 2006b; Figure 1). These explicit results could not have been obtained if knowledge of the location of each sampling point had not been available. Such data provide the opportunity to correlate occurrences to other spatial factors in the cropping system and subsequently develop hypotheses about factors that could effect the distribution of entomopathogenic fungi. For example, combination of data of selected insect populations (hosts) and fungal inoculum could provide results of the correlations in distribution between the populations of these organisms.

Figure 1



Horizontal distribution of the entomopathogenic fungus *B. bassiana* in the field at Bakkegården in September 2002 calculated by the statistical software SADIE. The scale of the figure is in metres. Clustering indices equal to 0 are represented by contour lines accompanied by 0. Grey shaded areas represent 'gaps' i.e. areas where clustering indices are below –1.5 (areas with lesser occurrence of *B. bassiana* than what should be expected from a random distribution). Black areas are 'patches', i.e. where clustering indices are above 1.5 (areas with higher occurrence of *B. bassiana* than what should be expected from a random distribution). The two small squares enclose the area of sampling with reduced distances conducted in September 2003 and these data confirmed the existence of patches and gaps. Data are presented in Meyling & Eilenberg (2006b).

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SM

Selective medium

- Suspend 32.5 gram SDA (Sabouraud Dextrose Agar) in 500 ml distilled water in a blue cap bottle.
- Add 1 ml Dodine (a fungicide to inhibit fungal growth)
- Mix the medium and mark the blue cap bottle with autoclave tape.
- Autoclave the medium for 20 min at 120 °C 20 bar.
- (Remember that the lid of the blue cap bottle has to be loose during autoclaving)
- Cool the medium after autoclaving to approx. 60°C and add:
- 500 µl Chloramphenicol (antibiotic, inhibits bacteria)
- 500 µl Streptomycin sulphate (antibiotic, inhibits bacteria)
- Invert the bottle gently and pour the plates.

Solutions:

Chloramphenicol: 1g in 10 ml 96% ethanol.
Streptomycin sulphate: 0,5 g in 10 ml sterilized distilled water.
Dodine: 5 g in 45 ml distilled water.

BSM

Selective medium for *Beauveria* spp.

5 g Peptone	Dissolve in 500 ml dem. water
10 g Glucose	pH adjusted to 6.3 with 1 M HCl
6 g Agar (no.1, Oxoid)	Autoclave for 20 min. at 120 ^{o}C

When the medium has cooled to 50-60 ^{o} C add:

0.5 ml a' 0.6 g/ml Streptomycin
0.5 ml a' 0.05 g/ml Tetracycline
0.5 ml a' 0.1 g/ml Dodine
2.5 ml a' 0.05 g/5 ml Cyclohexamide

Invert gently the bottle without making air bobbles and pour the plates

Appendix B

Treatment of *Galleria mellonella* larvae to prevent webbing in soil

When *G. mellonella* are reared at 20^{o}C, four week old larvae are most suited for baiting to avoid that they pupate in the soil. To prepare the heat treatment:

1. Place a beaker with 500 ml of water in a water bath at 56^{o}C.

2. Take the number of larvae (+10%) from the rearing containers and place them in a box. Place a sheet of paper in the box and the larvae will crawl under this for hiding. Thus they do not crawl out.

3. Remove the paper and shake the box so that the larvae can not cling to webbing in the box. Pour all the larvae into the beaker with hot water. Let them remain in the water for 10 seconds, maximum 15 s. Pour the water through a sieve and cool the larvae in cold running water for 30 s. Place the larvae on dry tissue paper and place them in the dark for 3-5 hours.

4. When the larvae have recovered from the treatment (they may appear dead at first) place them in the containers with soil. Do not invert the samples until the following day as the larvae may be squashed and die.

This description is based on recommendation from Ingeborg Klingen, Norway, and *Woodring, J. L. & Kaya, H. K. 1988. Steinernematid and Heterorhabditid Nematodes: A handbook of biology and techniques, Fayetteville, Arkansas: Arkansas Agricultural Experiment Station. Pp. 1-30*

Appendix C. Rearing of the wax moth, Galleria mellonella

Rearing can be performed in plastic boxes incubated in the dark in a climate controlled room at 20°C. Adult moths should be provided with a solution of water and honey. Under the lid of the box containing the adults, strips of folded paper can be provided for oviposition. The females will attempt to place their eggs in crevices as the folded paper represents. The paper can then easily be removed with the eggs attached.

Paper strips with eggs can be placed in a new box with a ball of food for early instars (see below). When the eggs hatch, the neonate larvae will themselves move to the food and start feeding. When the larvae have reached approximately 1 cm in length they can be provided with food for late instars (see below). Larvae of approximately 2.5-3 cm in length (4 weeks after hatching) are suitable for baiting soil samples.

Food for early instar larvae

180 g honey	260 g whole grain wheat flour
180 g glycerine	80 g dry brewers yeast
50 g bee wax	50 g wheat bran

Honey, glycerine and bee wax are melted in a cooking pot (don't boil). Remove from heat. Add brewers yeast and then whole grain wheat flour. Then add wheat bran. Mix thoroughly. Form the mixture into balls. These can be kept in the fridge until use.

Food for late instar larvae

280 g honey
240 g glycerine
40 g dry brewers yeast
400 g blended dry dog food (e.g. Pedigree Junior)
100 g rolled oats
100 g wheat bran

Mix honey, glycerine and brewers yeast and add blended dog food (must be blended to powder). Add rolled oats and wheat bran. If the mixture is too greasy add more oats and wheat bran. Keep the food refrigerated and add the food to boxes with late instar larvae.