C: Biotests and field efficacy trials of fungal BCAs against scarabaeid larvae

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Specific scope

This standard provides protocols for laboratory and field evaluation of the efficacy of fungal biocontrol agents against scarabaeid larvae in the soil.

Specific approval and amendment

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Introduction

Solid efficacy data are a prerequisite for to the registration of plant protection products in the European Union. The requirements for efficacy data of plant protection products are covered under Council Directive 91/414 of 15 July 1991 concerning the placing of plant protection products on the market (Council of European Union 1991), demanding a proof of the benefits arising from the tested preparation.

For fungal BCAs the efficacy evaluation should generally begin with biotests screening for the most virulent strain of an insect pathogen or the most efficacious formulation of a product. Subsequently, an efficacy evaluation needs to be carried out under field conditions to confirm the product efficacy and define the required application dose.

Currently, no specific guidelines for the conduction of biotests with fungal BCAs active against scarabaeid larvae (white grubs) exist. An EPPO Standard for the efficacy evaluation of white grubs has been drafted (Rieckmann *et al.* 1999), but the applicability of the suggested methods for fungal BCAs is doubtful (Strasser *et al.* 2005).

This RAFBCA Standard provides methods for the laboratory screening and field efficacy assessment of fungal BCAs against scarabaeid larvae such as *Melolontha* spp., *Phyllopertha* spp. and *Amphimallon* species.

Laboratory biotests

Overview of procedure

Scarabaeid larvae are collected in the field and are quarantined individually after transfer to the laboratory. The virulence/efficacy of different fungal strains or produced formulations is compared by exposing the larvae to soil inoculated with a defined BCA concentration. Larvae are kept individually in plastic containers over the observation period and larval deaths are recorded.

Mortality data is then subjected to statistical analyses for comparison of median times required to kill test insects.

Collection and quarantining of larvae

The number of larvae required for the bioassay depends on the number of tested strains/formulations. For each tested strain/formulation and the control treatment approximately 50 larvae are required.

Determine the number of larvae needed for the experiment and collect required larvae from an infested field. Refer to EPPO Standards PP1 (2004b) for the identification of species to make sure all collected larvae belong to the species intended to control.

Place larvae individually in sterilised cylindrical polypropylene containers (dimensions: ∅ ca. 3 cm, height ca. 5 cm). Cover with moist peat by filling up containers to approximately two thirds of their height [use RAFBCA Standard Appendix B: Monitoring of fungal biocontrol agents in the soil (Laengle *et al.* 2005a) to verify that the peat is free of the fungal pathogen].

Feed all larvae by placing a piece of carrot (approx. 0.25 cm^3) in each container, then cover all containers with a moist cloth or paper towel and incubate at 10 °C. Soil and covering need to be kept in moist (not wet) condition, carrot pieces are replaced at least weekly.

After a quarantine period of 6 weeks, counted from the date of collection in the field, the larvae can be used for the bioassay.

Production of inoculum

Fungal inoculum used for the bioassay can either be available as a commercial product or needs to be produced on a laboratory scale.

If no formulated spore product or specific protocols for spore production are available then the conidia needed for the bioassay are produced by growing the BCA strains on Potato-Dextrose Agar (PDA), Sabouraud-2-%-Glucose Agar (S2G), or another suitable medium. If larger spore quantities are needed, the production may also be done by growing the fungi on substrates, such as sterilised parboiled rice.

After sporulation of fungi wash conidia off agar plates by flooding with 10 ml of sterile 0.1 % (w/v) Tween® 80 solution. If other substrates such as rice are used, adjust amount of wash-off solution accordingly. After wash-off, transfer spore suspension to sterile centrifuge containers. Wash spores by two centrifugation steps, each time disposing the supernatant and re-suspending the pellet in 0.1 % (w/v) Tween® 80 solution.

Determination of spore density and viability

For both commercial spore products and conidia produced on a laboratory scale the spore density and viability has to be determined. This is very important as only defined inoculum concentrations can assure comparability of bioassay data.

For commercial spore formulations as well as laboratory-produced conidia perform quality control as described by RAFBCA Standard Appendix A: Quality control of fungal biological control agents (Laengle *et al.* 2005b). Because viability determination takes up to 4 days it is necessary to plan experiments accordingly to ensure viability data are available at the start of the bioassay (if conidia are obtained by the above described wash-off procedure it may be necessary to remove a conidia sample for viability testing a few days before washing off).

Inoculation of substrate

After the density and viability of the obtained conidia has been determined, prepare a spore suspension in 0.1 % (w/v) Tween® 80 solution containing 5×10^6 viable conidia per mL for each fungal strain/BCA formulation tested. Pure 0.1 % (w/v) Tween® 80 solution is used as control.

Blend the spore suspension with dry peat soil that previously has been tested for the absence of the evaluated entomopathogenic fungi using RAFBCA Standard Appendix B: Monitoring of fungal BCAs in the soil (Laengle *et al.* 2005a). To obtain a final spore density of 10^6 spores per gram soil dry weight, 20 mL of spore suspension have to be added per 100 g of soil.

It is crucial that a homogenous spore distribution is ensured. Sterile polypropylene bags have are useful for blending the soil/peat

with the conidia. Make sure that control substrates are always prepared prior to inoculation with spore suspension to avoid cross-contamination.

Bioassay setup and layout

Prepare 30 sterile polypropylene containers (dimensions: ∅ ca. 3 cm, height ca. 5 cm) per tested strain/formulation and control. Place scarabaeid larvae (quarantined for at least six weeks; see above) in containers. Arrange containers with larvae in separate plastic trays for each treatment (no randomization is recommended due to the risk of cross-contamination).

Cover larvae with moist peat that had been inoculated with 10^6 conidia per gram soil dry weight, filling up containers to approximately two thirds of their height. Controls are always prepared first to avoid contamination. Then the inoculated substrates are added to the containers previously placed in trays as described above.

Feed all larvae by placing a piece of carrot (ca. 0.25 cm^3) in the containers, then cover all containers with a moist cloth or paper towel and incubate at 10 °C.

Bioassay maintenance and data collection

Check the bioassay containers every 3 days for dead larvae, replace carrot pieces at least weekly. Keep covering and soil moist (not wet) throughout the trial period by spraying with tap water.

Record time of death for all larvae and remove containers with dead larvae from the experiment. The cause of death is determined by visually differentiating between larvae killed by the tested entomopathogen and death resulting from other causes. Typically, cadavers of larvae killed by fungi are hardened by dehydration and characterised by the emanating fungal mycelium. In doubtful cases verification may be obtained by placing cadavers on selective media (see RAFBCA Standard Appendix B: Monitoring of fungal biocontrol agents in the soil; Laengle *et al.* 2005a) or by microscopic examination if trained personnel is available.

Data Analysis

Generally, mortality data is analyzed by Kaplan-Meier survival analysis to obtain median survival times (LT_{50}). Statistical differences among treatments or between treatments and controls are tested by log rank comparison (Pyke & Thompson 1986, Motulsky 2004).

Software recommended for the data analysis is Graphpad Prism 4.0 interface. PriProbit (written in Mathematica 4, Wolfram

Research, Inc., Champaign, IL; see Throne *et al.* 1995) has also proven to be useful.

Regardless of the used software, it is important to differentiate larval deaths caused by the tested entomopathogen from natural control mortality, i.e. larval deaths resulting from other, undetermined causes. In statistical terminology "other deaths" are referred to as "censored events" (Motulsky 2004).

Note that confidence limits for median survival times (LT_{50}) are calculated based on the assumption that mortality curves have the shape of an exponential decay curve by most software. If this condition is violated then confidence limits may not be used. Other results (log rank comparison, median survival times) are based on non-parametric methods and are not affected by the shape of the mortality curve (Motulsky 2004).

Results should be given as LT_{50} obtained at a given spore concentration and should always be reported together with p-values calculated by log rank comparisons. For more guidance on analysis of bioassay data refer to Goettel & Butt (2000).

Field efficacy trials

Background

Proposals for an EPPO guideline for the assessment of insecticide efficacy against scarabaeid larvae (Rieckmann *et al.* 1999) are based on relatively small plot sizes and assume that the use of replicates compensates for variations (EPPO Standard PP1/152(2) Design and analysis of efficacy evaluation trials; European and Mediterranean Plant Protection Organization 2004b). This approach has not proven to be useful, at least not for the efficacy assessment of fungal BCAs (Strasser *et al.* 2005). The following chapter provides a procedure that takes into account the high patchiness of larval occurrence as well as the relatively high mobility of scarabaeid larvae in the soil by using larger plot sizes and high sampling intensity.

Overview of procedure

Once an efficacious strain and formulation has been selected by the above specified laboratory bioassay, their efficacy has to be assessed under field conditions. The procedure described in this guideline aims at the generation of dose dependent data on the efficacy of scarabaeid control with fungal BCAs under field conditions. Guidance is given with regard to the selection of trial fields, trial design as well as on data collection and analysis.

The selected trial field is assessed for pre-application larval density, and is then split into four subdivisions. Subsequently, three subdivisions (plots) are treated with different doses of the BCA formulation, the fourth subdivision serves as a control. The effect of the treatments on larval mortality as well as feeding damage by larvae is assessed and data are statistically analysed. The outcome of the study should result in a recommendation for the amount of BCAs needed to sufficiently control scarabaeid larvae.

Selection of trial field

The selected trial field should have a history of grub damages. Cultural conditions such as soil type, fertilization, slope or tillage should be uniform across the trial field (Rieckmann *et al.* 1999). The trial field should have a size of 5000 square meters (0.5 hectare) or more.

Pre-application assessment of scarab density

Assess the pre-application density of scarab larvae by digging 50 cm × 50 cm sample holes to a depth of at least 50 cm and place the removed soil on plastic tarps. Manually screen the removed soil for scarabaeid larvae and record the number of target larvae as well as the density of other scarabaeid larvae that may be present in the soil [compare European and Mediterranean Plant Protection Organization (2004b) for guidance on the differentiation of common genera]. At least 10 such soil samples uniformly distributed over the trial field have to be taken. The sampling should be done no longer than a week prior to the application of the products. It is important to choose a sampling date that ensures that larvae are present in the upper soil horizons. In the case of *Melolontha* spp., for instance, the soil temperature should be above approximately 8 °C.

The larval density is given per square meter as an average of the 10 samples with standard deviation. If mycosed larvae are found they should be reported separately.

The larval density should be uniform across the trial field, no gradient in the number of larvae in each sample should be detectable in any direction across the field. Furthermore, the average larval density in the trial field should be higher than 30 larvae per square meter. If the conditions of uniformity and the minimum larval density are not met the trial should not be used for efficacy trials.

Treatments

Choose three different doses of BCA to be applied to the plots. The chosen doses should be based on the doses recommended for

comparable commercial fungal BCAs. Doses should include the recommended dose, as well as a lower (e. g. half of recommended dose) and a higher (e.g. double to fivefold of recommended) dose. Doses should be reported as kilograms (kg ha^{-1}) or litres (L ha^{-1}) per hectare.

The control treatment should be exactly the same as the BCA treatments (recommended dose), except for the absence of the active ingredient of the BCA. If available, a comparable reference product may also be included for comparison of efficacy.

Trial layout

Subdivide the trial field into equal plots with a size of at least 1000 square meters per plot. The buffer zone between plots should be at least 3 m wide.

Randomly assign treatments with different doses of BCA as well as the control treatment to the respective subdivisions. Conduct applications with appropriate equipment and record application details thoroughly. Then plant/sow cultures for which the efficacy of protection is to be assessed in accordance with local agricultural practices. Record all agronomic details as well as weather conditions at the time of application. Also refer to EPPO Standard PP1/181(3) Conduct and reporting of efficacy evaluation trials including good experimental practice (European and Mediterranean Plant Protection Organization 2004a) for further recommendations.

Efficacy assessment

The efficacy of the product is by taking at least 10 samples per trial plot. Sampling spots have to be uniformly distributed over trial plots. Larvae are recorded in three different categories that comprise (i) healthy larvae, (ii) larvae killed by the BCA (mycosed larvae), and, if present, (iii) larvae killed by other causes. Sampling should be done prior to the harvest of the crop.

Additionally, assess efficacy of crop protection by recording crop damage characterised as feeding damage by scarabaeid larvae. Make sure damages caused by other pests such as mice or wireworms are not mistaken for damages caused by scarabaeid larvae. For the analysis of efficacy evaluation trials compare EPPO Standard PP1/152(2) Design and analysis of efficacy evaluation trials (European and Mediterranean Plant Protection Organization 2004c).

Quantification of BCA in the soil

Take at least 30 soil cores per treatment and control and analyse as described in RAFBCA Standard Appendix B: *Monitoring of fungal biocontrol agents in the soil* (Laengle *et al.* 2005a).

Analysis of fungal BCA density in the soil

Calculate the amount of BCA propagules per gram soil dry weight as described in RAFBCA Standard Appendix B: *Monitoring of fungal biocontrol agents in the soil* (Laengle *et al.* 2005a).

Analysis of efficacy data

Calculate the BCA induced larval mortality as percentage of the total number of larvae found in each sampling hole and average over each treatment. Calculate standard deviation.

The correlation between *B. brongniartii* density in the soil and the mortality of *M. melolontha* can be described by the calculation of a dose-mortality curve using, for instance, the statistical software Graphpad Prism 4. Set minimum (bottom) and maximum (top) values of the curve to 0 and 100 percent mortality, respectively. Then enter mortality data (y-axis) for each applied dose (x-axis) to obtain LD_{50}-values (dose that kills 50 % of targets) with 95 % confidence limits. Likewise, enter mortality data as a dependent variable of the BCA propagule concentration (from the soil layer in which larvae are most likely to be active) to estimate the propagule concentration required to kill 50 % of larvae (LC_{50}). For details on the analysis of dose-mortality data refer to Motulsky & Christopoulos (2004).

Interpretation of results

Dose response curves can be used to predict the efficacy of a certain application dose or BCA propagule density and to set dose recommendations for a product.

For fungal BCAs a field prevalence of above 20 % in the first year of application is considered sufficient to provide an effective long-term control of scarabaeid larvae (Strasser 1999). An initial dose recommendation in the range of the LD_{50} should therefore result in a good medium-term to long-term control of the target and a down-regulation of the target population. In subsequent years, or when the objective is the protection of a risk area from the invasion of pests, the recommended application dose might also be lower.

References

Council of the European Union 1991. Council Directive of 15 July 1991 concerning the placing of plant protection products on the market. *Official Journal of the European Communities L* (230): 1–32.

European and Mediterranean Plant Protection Organization (2004a) Conduct and reporting of efficacy evaluation trials including good experimental practice. PP 1/181(3). *EPPO Standards. Efficacy Evaluation of Plant Protection Products. Volume* 1: General Standards. EPPO, Paris.

European and Mediterranean Plant Protection Organization (2004b) Efficacy evaluation of insecticides: white grubs (Scarabaeid larvae). EPPO Standards PP1 - Efficacy Evaluation of Plant Protection Products. *Bulletin OEPP / EPPO Bulletin*: in press.

European and Mediterranean Plant Protection Organization (2004c) Design and analysis of efficacy evaluation trials. PP 1/152(2). *EPPO Standards. Efficacy Evaluation of Plant Protection Products. Volume* 1: General Standards. EPPO, Paris

Goettel M. S., Butt T. M. (2000) Bioassays of entomogenous fungi. In: Navon A., Asher K. R. S. (Eds.). *Bioassays of Entomopathogenic Microbes and Nematodes*. CABI Publishing, Wallingford: 141–195.

Laengle T., Raffalt J., Pernfuss B., Strasser H. (2005a) Appendices: Risk Assessment of Fungal Biocontrol Agents Standards. B: Monitoring of fungal BCAs in the soil. *Sydowia* **57** (1): 78–83.

Laengle T., Raffalt J., Pernfuss B., Strasser H. (2005b) Appendices: Risk Assessment of Fungal Biocontrol Agents Standards. A: Quality control of fungal biological control agents. *Sydowia* **57** (1): 67–77.

Motulsky H. (2004) Graphpad Prism Version 4. Statistics Guide. Statistical analyses for laboratory and clinical researchers. http://www.graphpad.com/manuals/Prism4/StatisticsGuide.pdf (last accessed: 7 Jan 2005).

Motulsky H., Christopoulos A. (2004) Graphpad Prism Version 4. Fitting Models to Biological Data using Linear and Non-Linear Regression. A practical guide to curve-fitting. http://graphpad.com/manuals/Prism4/RegressionBook.pdf (last accessed: 13 Aug 2004).

Pyke D. A., Thompson J. N. (1986) Statistical Analysis of Survival and Removal Rate Experiments. *Ecology* **67** (1): 240–245.

Rieckmann W., Block T., Heimbach U., Lein K. A., Lauenstein G., Mathes P., Schmid C., Steck U. (1999) Deutscher Vorschlag für eine EPPO-Richtlinie zur Prüfung von Insektiziden gegen Engerlinge in ackerbaulichen Kulturen. I22. BBA, Braunschweig, Germany. http://www.bba.de/eppo/i_22.pdf (last accessed: 9 Jan 2005).

Strasser H. (1999) Beurteilung der Wirksamkeit des biologischen Pflanzenschutzpräparates Melocont-Pilzgerste zur Maikäfer-bekämpfung. *Der Förderungsdienst* 5: 158–164.

Strasser H., Zelger R., Pernfuss B., Laengle T., Seger C. (2005) EPPO based efficacy study to control *Phyllopertha horticola* in golf courses. *IOBC wprs Bulletin* 28 (3): 189–192.

Throne J. E., Weaver D., Chew V., Baker J. E. (1995) Probit analysis of correlated data: multiple observations over time at one concentration. *Journal of Economic Entomology* **88**: 1510–1512.

Annex I: Media and solutions

Sabouraud-2%-Glucose Agar

30 g Sabouraud-2%-Glucose-Bouillon

(MERCK 108339.0500)

15 g Agar

1 L deionised water

Sterilise for 20 min at 121 °C, 1.2 bar. The medium has a clear yellowish appearance after sterilisation. If the medium turns dark brown it has been overheated and has to be disposed.

Potato-Dextrose Agar

39 g Potato-Dextrose Agar
(MERCK 1.10130.0500)
1 L deionised water
Sterilise for 20 min at 121 °C, 1.2 bar.

Tween® 80 Solution 0.1 % (w/v)

Prepare a flask with 1 L of deionised water. Weigh 1 g of Tween® 80 (Merck 822187) into a beaker, dissolve in a portion of the prepared water, then blend in the flask before sterilising for 20 min at 121°C / 1.2 bar. The solution has a milky appearance after autoclaving and will become clear when cooling down.