B: Monitoring of fungal biocontrol agents in the soil

T. Laengle[1], B. Pernfuss[1], J. Raffalt[2] & H. Strasser[1]

[1] Institut für Mikrobiologie, Leopold-Franzens Universität Innsbruck, Technikerstr. 25, *A-6020 Innsbruck, Austria*
[2] *F. Joh. Kwizda GmbH, Division Agro, Dr. Karl Lueger-Ring 6, A-1011 Wien, Austria*

Specific scope

This standard provides a protocol for the monitoring of fungal BCAs in the soil using cultivation techniques and selective media.

Specific approval and amendment

First approved in January 2005.

Introduction

The persistence of fungal BCA progagules in the soil is a prerequisite for successful control when the fungus is applied to the soil before the crop is sown (Vänninen *et al.* 2000).

Likewise, for a comprehensive environmental risk assessment of fungal biocontrol agents it is important to have knowledge on their establishment potential and their persistence in the environment (van Lenteren *et al.* 2003).

This RAFBCA Standard provides a method for the quantification of fungal biocontrol agents in the soil and thereby enables the generation of important data for the risk assessment and the efficacy evaluation of a product.

Overview of procedure

A precondition for a reliable quantification of fungal BCAs in the soil is the collection of representative soil samples. These soil samples are suspended in an extraction solution. A dilution series of each extract is prepared and each dilutions is plated on selective media. After incubation of the dilution plates the number of fungal colonies formed by the BCA is counted and the amount of BCA per gram soil dry weight is calculated.

Soil sampling

Entomopathogenic fungi have been described to appear in clusters and form conidial pellets in the soil (Callot *et al.* 1996). Thus, it is necessary to obtain representative soil samples by pooling a number of soil cores from across the whole trial site. The classical sampling method used for the physical-chemical characterisation of soils (Oehlinger 1986) is appropriate also for the analysis of fungal and bacterial densities (Strasser 1995).

Use a soil corer (minimum core diameter 1 cm) to sample the soil down to a depth of 30 cm in agricultural fields (10 cm in pastures and meadows). If soil conditions do not allow sampling to the recommended depths, the actually reached depth is recorded.

Split soil cores into segments representing the 0–10 cm and 10–30 cm soil layers to obtain information on the depth distribution of the BCA (the size of the segments may be varied depending on the type of field sampled).

At least 40 soil cores per hectare, but not less than 15 soil cores per sampled field or treated area, should be taken. Cores pooled in a sealable inert plastic bag for each depth layer.

Sample preparation and storage

Dry soil samples at room temperature for 1–2 days, pass through a 2 mm mesh sieve, and mix thoroughly. If sieved samples are not processed immediately they should be stored for no longer than 2 months at 4 °C.

Determination of soil dry weight

After air drying and sieving take three sub-samples of 5 g from each homogenously mixed soil sample and place each sub-sample in a dry, pre-weighed glass petri dish (P). Record the exact amount of soil added to each petri dish (S_{airdry}) and dry soil in petri dish at 108 °C for 24 h.

After drying allow to cool down in a desiccator and weigh petri dishes with dry soil ($P+S_{ovendry}$). Use the following formula for the calculation of the ratio (r) between air-dried and oven-dried soil.

$$r = \frac{S_{airdy}}{(P + S_{ovendry}) - P}$$

This ratio is later used as correction factor for the calculation of fungal BCA per gram soil dry weight.

Dilution plating

Extraction

Take three subsamples of 10 g from each homogenously mixed soil sample and place each subsample in a sterilised 100 mL Erlenmeyer flask (wide neck). Record the exact amount of soil added to each Erlenmeyer flask.

Add 40 mL of sterile Tween® 80 Solution 0.1 % (w/v) to each flask and shake at 150 rpm for 30 min at 25 °C on a horizontal shaker. Then place flasks into an ultrasonic bath for 30 seconds.

Dilution series

Immediately prior to use shake Erlenmeyer flask for 60 s and then allow to stand for another 30 seconds. Prepare a 1 : 5 of the soil suspension by pipetting 1 mL from that liquid surface into 4 mL of sterile Tween® 80 solution in a glass test tube.

Mix thoroughly on a vortex mixer, and for a second dilution step, transfer 1 mL of this suspension to another test tube containing 4 mL Tween® 80 solution.

The original soil suspension together with the first two dilutions allow a detection of approximately 5×10^2–1.5×10^5 colony forming units (cfu) of BCA per gram soil. If a higher density of BCA is expected, repeat the dilution process to obtain further dilutions.

Plating and incubation

Inoculate 4 BCA-selective or semi-selective agar plates (see Annex II) with 50 µL of each of the prepared soil suspensions (original soil suspension in Erlenmeyer flasks as well as the dilutions thereof) and spread evenly with a Drigalski spatula.

Incubate plates at a temperature suitable for the tested BCA (for instance 25 °C and 60 % RH for *Metarhizium* spp. and *Beauveria* spp.) until colonies become clearly visible (check growth at least every two days).

Counting of colonies

At the end of the incubation period, record the number of colonies on each plate. It is important that the person responsible for the counting of colonies has been properly trained for this task, because the identification of BCA colonies based on their morphology requires some experience, especially, if the medium used is not sufficiently selective.

Analysis of results

Only use the dilution plates with the number of colonies ranging between 20–100 as more colonies per plate may result in reciprocal inhibition.

For each counted plate calculate the number of colony forming units per gram soil dry weight ($cfu_{dry\ soil}$) for each counted plate using the formula

$$cfu_{dry\ soil} = \frac{Soil_{\ flask}}{(40 + Soil_{\ flask})} \times cfu_{plate} \times d \times r \times \frac{1}{R}$$

where $Soil_{flask}$ is the soil in grams, placed in the Erlenmeyer flask in gram, d is the dilution rate of the soil suspension (i.e. 1 for undiluted, 5 for the first dilution of 1:5, etc.), r is the weight ratio between air-dry and ovendry soil as described above, and R is the recovery rate determined as described in Annex I.

The median value of all replicates of one soil sample is then calculated and reported together with the 25% and 75% quartiles. The calculation of mean values is only permissible if data is normally distributed in all samples.

References

Callot G., Vercambre B., Neuveglise C., Riba, G. (1996) Hyphasmata and Conidial Pellets: An Original Morphological Aspect of Soil Colonization by *Beauveria brongniartii*. *Journal of Invertebrate Pathology* **68**: 173–176.

European Commission (2003) Proposal for a Council Directive amending Annex VI to Directive 91/414/EEC as regards plant protection products containing micro-organisms. Document COM(2003) 814 final, Brussels, 22. 12.2003. http://europa.eu.int/eur-lex/en/com/pdf/2003/com2003_0814en01.pdf (last accessed 18 Oct 2004)

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

Oehlinger R., Eibelhuber A., Fischerlehner J. (1986) Bodenprobennahme fuer Enzymaktivitaetbestim-mungen. *Mitteilungen der Österreichischen Bodenkundlichen Gesellschaft* **33**: 255-283.

van Lenteren J. C., Babendreier D., Bigler F., Burgio G., Hokkanen H. M. T., Kuske S., Loomans A. J. M., Menzler-Hokkanen I., van Rijn P. C. J., Thomas M. B., Tommasini M. G., Zeng Q.-Q. (2003) Environmental risk assessment of exotic natural enemies used in inundative biological control. *BioControl* **48**: 3–38.

Strasser H., Forer A., Schinner F. (1996) Development of media for the selective isolation and maintenance of virulence of *Beauveria brongniartii*. In: Jackson T., Glare T. (Eds.) *Microbial Control of Soil Dwelling Pests*. AgResearch, Lincoln, New Zealand: 125–130.

Strasser H. (1995) Bekämpfung von Engerlingen des Maikäfers und Gartenlaubkäfers mit *Beauveria brongniartii*. Endbericht. Bund-Bundesländer-Kooperation L925/94. *Project report. Innsbruck*: 1–30.

Vänninen I., Tyni-Juslin J., Hokkanen H. (2000) Persistence of augmented *Metarhizium anisopliae* and *Beauveria bassiana* in Finnish agricultural soils. *BioControl* **45**: 201–222.

ANNEX I: Determination of recovery rate

To get accurate information on the BCA density in the soil it is necessary to determine what precentage of the BCA in the soil can be detected with the method described in this RAFBCA Standard. The recovery rate of a specific BCA from soil is determined as follows.

Prepare a spore suspension by washing off spores from a surface culture of the BCA with Tween® 80 solution.

Determine spore viability in the suspension as described in RAFBCA Standard Appendix A. Quality control of fungal biocontrol agents (Laengle *et al.* 2005).

Prepare 10 mL of a spore suspension containing 10^6 viable spores per mL. Add 10 mL of this suspension to 100 g of oven-dried, BCA-free soil, thereby inoculating the soil with 10^5 spores per gram dry weight.

Blend material as homogenously as possible. This can, for instance, be done by manually blending in a plastic bag followed by placing the inoculated soil in a 250 mL flask on an overhead shaker for 1 hour.

Analyse the inoculated soil for its BCA content as described in this RAFBCA Standard.

Compare results ($cfu_{\text{inoculated soil}}$) with the expected number of 105 cfu per gram soil dry weight using a One-Sample t-Test. If no significant difference is detected the recovery rate (R) is set at 1, if a significant difference is detected then calculate the recovery rate as follows:

$$R = \frac{cfu_{inoculated\ soil}}{10^5}$$

This rate is then used as shown in the formula for the calculation of the BCA density per gram soil dry weight ($cfu_{\text{dry soil}}$). Additionally, the recovery rate has to be reported along with the results of the BCA monitoring in the soil samples.

ANNEX II: Media

The recipe for the preparation of selective media for *Beauveria* spp. (Strasser *et al.*, 1996) is listed in the following paragraph and may be used as basis for the development of selective media for other entomopathogenic fungi. For instance, the addition of 20 g L^{-1} glucose will make the medium more suitable for the growth of *Metarhizium* spp.

Beauveria-selective 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. After autoclaving, the medium is supplemented with Cycloheximide (70 mg L^{-1}), Streptomycin (100 mg L^{-1}), Tetracycline (50 mg L^{-1}) and Dodine (100 mg L^{-1}). The antimicrobials are added as an adequate sterile filtered stock solution, except for Dodine, which is dissolved in 1.5 mL ethanol 96 % (v/v) and then added to the medium. Make sure that the temperature of the agar is in the range of 50 °C – 60 °C when the stock solution is added.

0.1 % (w/v) Tween® 80 solution

Dissolve 1 g of Tween® 80 in deionised water and sterilise for 20 min at 121 °C, 1.2 bar. The solution has a milky appearance after autoclaving and will become clear when cooling down.