# Implementing arbuscular mycorrhiza fungi in an environmental risk assessment – case study with benomyl considering effects on spore germination and symbiosis in natural soil

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## Background

Arbuscular mycorrhizal fungi (AMF) are ubiquitous in terrestrial ecosystems and form symbioses with most plants, thereby can enhance nutrient uptake, pest tolerance, and yield. Despite their ecological importance, AMF have been recognized as non-target soil organisms relevant for environment risk assessment (ERA) of plant protection products (PPP) within the EFSA soil opinion (2017). The current OECD nitrogen transformation test (OECD Test Guideline (TG) 216), used to assess PPP effects on soil microorganisms, does not address impacts on AMF. To close this gap, the project MICROSOIL (1) proposes to include a Spore Germination Test (ISO 10832) as a Tier 1 screening method. However, this method does not capture the symbiosis between AMF and host plants. Therefore, a symbiosis test method was developed within the project ERAMYC (2) for assessing the effects of PPP on the symbiotic phase of AMFs.

Here, we observed the effect of a PPP in artificial substrate and natural soil on AMF using both methods, the Spore Germination Test and the AMF Symbiosis Test.

## Materials and Methods

**Test organism:** *Funneliformis mosseae* was used as AMF. The inoculum originated from the International Bank for Glomeromycota (https://www.i-beg.eu). Leek, *Allium ampeloprasum* L. (Flora Lusitana Lda., France) was used as host plant for spore propagation and the AMF Symbiosis Tests. Prior to use, leek seeds were surface sterilized by immersion in 1% sodium hypochlorite solution.

**Test substance:** The fungicide benomyl was used as it is mentioned as a reference substance in ISO 10832 and showed a clear dose-response relationship on spore germination in two independent preliminary experiments (data not shown).

**Test substrate:** Artificial substrate (AS) with a 2.5% peat content prepared following the recommendations of the OECD TG 232 and natural reference soil (RefeSol 02A). RefeSol 02A is a stagnic Luvisol containing 2.3% sand, 82% silt and 15.7% clay. The pH of the reference soil was at 7.1 (measured in $CaCl_2$) at test initiation.

**Reference:** The OECD TG 216 Test was conducted to compare AMF sensitivity to the standard soil microbial assay used in ERA.

**Test concentrations:** All tests were performed with concentrations ranging from 0.256 to 10.0 mg/kg dry weight (dw) soil.

**Spore Germination Test (ISO 10832):**

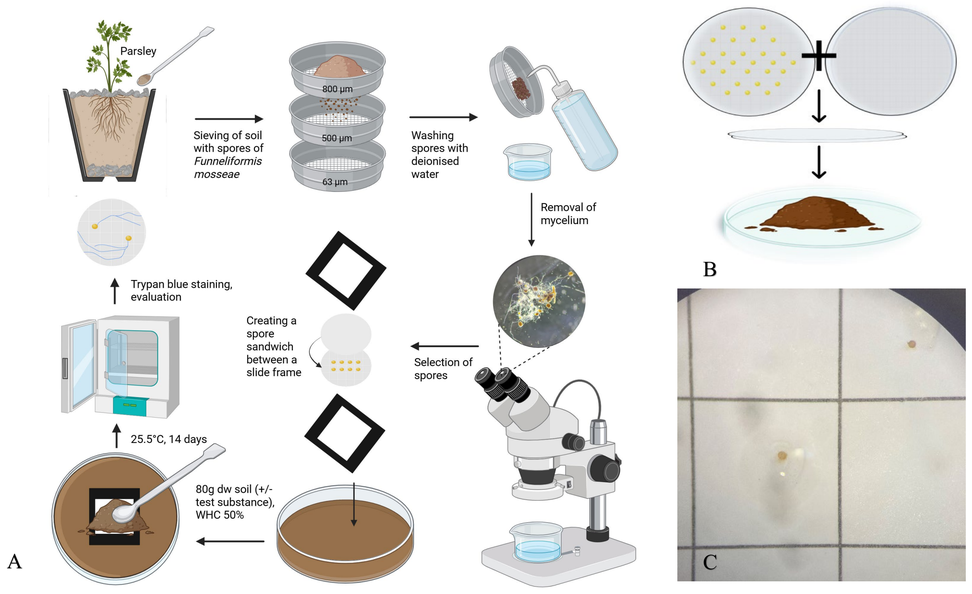


**Figure 1**: (A) General test procedure of the Spore Germination Test using *F. mosseae* from Reiermann et al. (2025). (B) Model preparation of a single spore sandwich). (C) Actual distribution of spores during assay on a nitrocellulose filter, observed under the Stemi 305 EDU binocular microscope at 40x magnification. (A) and (B) were created with BioRender.

## Materials and Methods

### AMF Symbiosis Test:

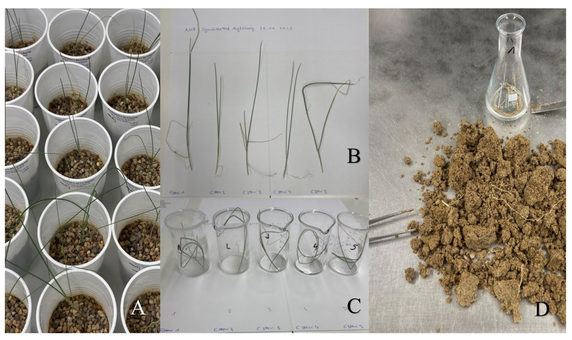


**Figure 2**: AMF Symbiosis Test (AS, 2.5% peat content): Harvesting of leek seedlings, roots and their sample processing. (A) Containers prior to harvesting; (B) harvesting of leek seedlings; (C) determination of seedling fresh mass prior to drying; (D) collection of root fragments for subsequent staining.

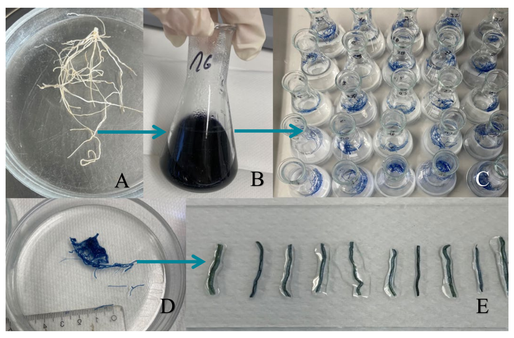


**Figure 3:** Preparation workflow for root colonization assessment. (A) Freshly harvested and cleaned roots of leek . (B) Staining procedure of roots. (C) Stained roots for storage. (D) Sampling of roots from one replicate, followed by excision of ~1 cm segments from different sections along the root system. (E) Final preparation for colonization assessment by *F. mosseae.*

The test was performed following the ERAMYC protocol (Figure 2, 3). After a 56-day exposure, endpoints include intra-radical colonization, extra-radical mycelium production, and plant biomass.

## Results

- OECD TG 216: There was no statistically significant different from the control and no deviation above 25% from the control after 28 and 56 days.
- In the Spore Germination Tests for the fungicide benomyl an $EC_{50}$ of 4.5 mg/kg dw soil in artificial substrate and of 3.2 mg/kg dw soil in natural soil was determined after 14 days exposure.
- AMF Symbosis Test (ERAMYC protocol): The most sensitive parameters were total colonization and root colonization both with comparable effect concentrations. The $EC_{10}$ was at 0.5 mg/kg and $EC_{50}$ at 0.8 mg/kg in artificial substrate (2.5% peat). No reliable results due to poor dose response in natural soil (Figure 4).

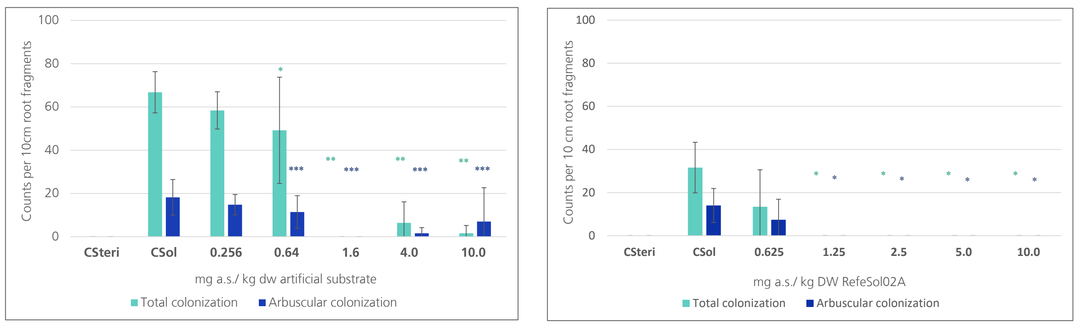


**Figure 4:** Counts of *F. mosseae* for total AMF colonization and for arbuscular colonization in artificial substrate (left) and natural soil (right). Total AMF colonization (*p < 0.05; **p < 0.01) in bright blue and arbuscular colonization (***p < 0.001) in dark blue.

- Extra-radicular mycelium length: Less sensitive compared to total colonization and no clear dose response relationship. Therefore, no effect concentration could be determined. Difficult to assess endpoint, while very time consuming.

## Conclusion

- For the fungicide benomyl no effect was observed in the N-Transformation Test (OECD TG 216) but in both tests using AMF showing the importance of considering AMF in environmental risk assessments next to e.g., OECD TG 216.
- The AMF Symbiosis Test is time consuming and somehow difficult to assess, but not more sensitive than the Spore Germination Test (ISO 10832) using benomyl. Testing pesticides with other mode of actions would be necessary to fully access the reliability of the AMF Symbiosis Test.

### References

1 Schlich et al. (2025) Correctly assessing the performance and threats of microorganisms in agricultural soils – identifying meaningful endpoints under field-relevant pesticide, biocide and pharmaceutical exposure. German Environment Agency. Texte 93/2025. https://www.uba.de/n118669de

2 Natal da Luz (2026) Test battery for the effect determination of chemicals in soils: Suitability of test systems with mycorrhizal fungi for the risk assessment. German Environment Agency. Texte 10/2026. https://www.uba.de/n306194de

3 Reiermann et al. (2025) The spore germination test (ISO 10832) with *Funneliformis mosseae* for use in an environmental risk assessment for chemicals-necessary adaptations and exemplary testing. Integr Environ Assess Manag. DOI: 10.1093/inteam/vjaf146

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