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Full Length Article

Establishment of a Quality Test for Arbuscular Mycorrhizal Inoculum

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Abstract

Commercial arbuscular mycorrhizal inocula are widely used in agriculture, landscaping, horticulture, and forestry. However, the quality of the majority of these inocula has not been assessed. Quality control is vital, both for the manufacturers and users, to understand the effects of the inocula and whether they correspond to those described by the manufacturers. It is important to carry out tests under conditions similar to those used in practice. In this study, a quality test of one commercial arbuscular mycorrhizal inoculum was designed to investigate (1) the arbuscular mycorrhizal fungi present, and (2) the efficacy of the inoculum. The small-scale experiment used three plants: tomato, onion and marigold. Only one morphotype of AMF spore has been observed, many empty brownish "balls" were mixed with arbuscular mycorrhizal fungal spores. The arbuscular mycorrhizal fungal species found was not the species described by the manufacturer, and its concentration was much lower than that stated. The inoculum failed to form mycorrhizae in two of three soils types. No significant growth has been obtained after the application of the inoculum. The performance of the inoculum contradicted that described by the manufacturer; no effect on growth of the three plants was found. The reasons for this are discussed, and suggestions for the manufacturer are given.

Keywords: Arbuscular mycorrhiza; Commercial inoculums; Quality control; Fungal component; Inoculum efficacy

Introduction

A mycorrhiza is a symbiotic association composed of plant roots and fungal hyphae; about 90% of the world's land plants belong to families that are commonly mycorrhizal (Read *et al.*, 2000). Mycorrhizae can be regarded as 'the chief organs involved in nutrient uptake of most land plants' (Harley and Smith, 1983).

Arbuscular mycorrhizal (AM) fungi can greatly increase a plant's uptake of soil nutrients, especially phosphorus (Baird *et al.*, 2010). As an important rhizospheric microorganism, AMF plays an important role in improving the rhizosphere soil environment, changing the forms of heavy metals in the rhizosphere and affecting the uptake and accumulation of heavy metals by plants (Amir *et al.*, 2013). AMF can improve the ability of plants to resist fungi, bacteria, viruses, and nematode diseases (Whipp, 2004). The presence of AMF enhanced the ability of trees to resist environmental stress, such as salt stress (Turkmen *et al.*, 2008; Peng *et al.*, 2011; Evelin *et al.*, 2012). However, VAM can be lost due to soil disturbances such as mining, construction or erosion, strong acid or chemical treatments, pesticides, excessive heat, drought or flooding, or the denial of oxygen or water by asphalt (Adholeya *et al.*, 2005). Therefore, the use of AMF inocula can reduce the use of artificial fertilization and pesticide, which is of great significance in the ecological environment and the protection of biological resources.

AMF are incapable of saprotrophic survival and can only be grown with a host plant (Douds *et al.*, 2000). Numerous methods are used to prepare and apply mycorrhizal fungal inoculum. The technical sophistication of these approaches varies greatly. Production of mycorrhizal inoculum for commercial purposes has evolved considerably in recent years ranging from fungal propagation in on-site nursery plots (Estrada *et al.*, 2013), pot culture techniques (Mosse, 1956; Gilmore, 1968; Morton *et al.*, 1993; Feldmann and Grotkass, 2002), hydroponic (Janda *et al.*,1999), aeroponic (Sylvia and Hubbel, 1986; Hung and Sylvia, 1988; Jarstfer and Sylvia, 1995), to axenic in vitro production in root organ culture and in vitro propagation systems of the root–organ cultures (Bécard and Piché, 1992; Declerck *et al.*, 1996; Adholeya *et al.*, 2005). As a good plant growth promoter and soil conditioner, AMF inocula has developed a variety of mature products which were applied in agriculture up to 2001 (Gianinazzi and Vasatka, 2004).

The inocula products are marketed as plant and soil enhancement materials, useful for a wide range of applications, in sustainable agriculture, erosion control and restoration, landscaping, horticulture, and forestry, among others. However, the inocula may have positive, neutral, or negative effects on plant growth (Schwartz *et al.*, 2006).

Therefore, there needs to be some quality control, by a neutral body, with analyses of the AMF contents and performances of the inocula. Gianinazzi and Vosatka (2004) also stressed the importance of instituting industry-wide quality control measures to ensure the production of viable mycorrhizae that meet the expected requirements of end-users. However, such tests have been rarely done.

The aims of this study were to test the AMF components of a biological inoculum called *ENTRIVIT*® to identify whether the AMF in *ENTRIVIT*® could form arbuscular mycorrhizae with selected plants and to assess whether the growth of the inoculated plants was enhanced. The data are important, as they have allowed us to (1) give manufacturers advice for the improvement of the quality of their product, and (2) to suggest the appropriate use of this product.

Materials and Methods

Experimental Procedure

The test product, *ENTRIVIT*® which we gained the permission to make a test was a biological inoculum from a Biotechnology Company of Germany. The product description indicates that it consists of seven AMF species and that it is a water-soluble powder that can be used in greenhouses and in the field.

AMF Components of *ENTRIVIT*®

Morphological investigation: *ENTRIVIT*® was dissolved in water to form a solution. The solution was sieved in a series from 150 µm to 40 µm. Spores were collected from each sieve and their morphologies were analyzed under a Normarski interference contrast microscope (Standard 14, ZEISS West Germany) at 1000 × magnification.

Molecular Identification

DNA from single AMF spores was extracted using the ChargeSwitch gDNA Plant kit (Invitrogen, Germany), following the manufacturer's instructions. PCR followed Kruger *et al.* (2012). Purified PCR products were sequenced by the LMU Sequencing Service (Großhaderner Straße 2, Planegg-Martinsried, Munich, Germany). The Blastn was carried out in GenBank (http://www.ncbi.nlm.nih.gov/).

Spore Concentrations

0.5 g *ENTRIVIT*® was dissolved in water to form a solution. The solution was sieved in a series from 150 µm to 40 µm. The spores were collected from the sieves into a small Petri dish. Single spores were taken out using a pipette and the amount of spores was counted. The amount of spores was multiplied by 2 to provide the concentration of spores in 1 g of *ENTRIVIT*®. This procedure was repeated 10 times.

Growth Experiment

Experimental design: The growth experiment was conducted under controlled greenhouse conditions. This experiment had three factors: (1) plants (3 levels: tomato (*Solanum lycopersicum*), onion (*Allium cepa*), and marigold (*Tagetes erecta*); (2) mycorrhizal inoculum (2 levels: *ENTRIVIT*® and control (no *ENTRIVIT*®); and (3) soils (3 levels: cultivation, plant, and farmland soils). Three replicates of each treatment were performed, with 30 plants in each replicate. Plants were grown for 12 weeks in 5 × 5 × 6 cm boxes (Romberg, Germany). Tap water was used for watering and no additional nutrient solution was given during the experiment.

Onion (Barletta, Germany), tomato (Rio Grande, Germany) and marigold (Cormen, French) were selected because the plants can be easily colonized by AMF, according to literature (Omirou *et al.*, 2016). In addition, according to the manufacturer's suggestions, the amounts of *ENTRIVIT*® that should be applied to the three plants are different (Table 1). Cultivation, plant and farmland soils are commonly used for greenhouse, garden and larger cultivation areas, respectively. The soluble nutrient contents in the cultivation and plant soils were given in (Table 2).

Application of *ENTRIVIT*®

Seven days after sowing, *ENTRIVIT*® was applied to the young seedlings, according to the manufacturer's recommendations (Table 3). For each treatment, the *ENTRIVIT*® was firstly dissolved in 200 mL water. Then, 2 mL solution was applied to the soil of each plant using a pipette (the tips of the pipettes were cut so that the spores did not become blocked in them). The solution was put into the soils at 4–5 different places around the root systems at a depth ca. 0.5 cm.

Harvest

Six whole plants in each treatment were harvested 2 weeks, 4 weeks and 12 weeks after the application of *ENTRIVIT*®. Shoots were dried at 60ºC for 72 h. Then, the shoot dry weight of each plant was measured.

The roots were washed, dried with filter paper and conserved in 70% ethanol for the colonization examination. Young roots from different parts of the root system were cut for staining. To check whether the control (no inoculation) tomato, onion and marigold plants had been colonized by the inoculum (through wind or watering procedures), the roots of six randomly chosen plants grown in all three soil types at each harvest were also checked. Root segments were cooked in 10% KOH for 4 min and then cooked in ink-vinegar (5%) for 4 min. Then they were washed in water containing a few drops of vinegar for 20 min, according to Vierheilig *et al.* (1998).

ENTRIVIT® Concentration Experiment

Seven weeks after sowing, the roots of all the plants developed extensively, forming a root ball in the cultivation and plant soils. Three plants of each plant species were taken from the soil and were inoculated with 10 ×, 20 × and 50 × the recommended amount of *ENTRIVIT*® (Table 4). The *ENTRIVIT*® powder was directly applied on the surface of the root balls to test in which concentration our plants could be inoculated successfully, and to compare with the statement as the manufacturer suggested.

Statistical Analyses

Significant differences ($P < 0.05$) among treatments were identified according to the least significant difference method (LSD method), carried out in SPSS13.0.

Results

Spore Morphotypes

One spore morphotype was observed (Fig. 1); the spores were globose, yellowish, and 96–110 µm in diameter. The spores' walls were 4–9 µm thick, and yellowish to brownish. They had 1–2 (4) laminated walls, and the inner walls were darker than the outer walls. The walls of the spores extended into the hyphal attachments. The hyphal attachments were 6–9 µm, with a wall thickness of 1.5–2.5 µm.

There were some brownish and dark brown "balls" of variable sizes in *ENTRIVIT*® (Fig. 2). They were found both as individual balls and clustered, were transparent, and were all empty. It is most likely that they were not AMF.

Spore Concentrations

It was found that 1 g of *ENTRIVIT*® contained ca. 50–80 spores. This is much lower than the minimal 200 spores/g, described by the manufacturer.

Molecular Identification

The only morphotype found in *ENTRIVIT*® was sequenced and its accession number in GenBank (NCBI) is KC970586. This sequence had 100% query coverage and 99% maximal identity with identified sequences of *Rhizophagus irregularis* (accession No. JN417510–JN417518), and with the unidentified sequences of different *Glomus* spp. isolates (accession No. s: FM865612, FM865614, FM865615, FM865551, and FM865558) in NCBI, according to the nucleotide BLAST search.

Mycorrhizal Colonization

Two weeks after the application of *ENTRIVIT*®, arbuscular structures and hyphal coils were observed in the roots of marigold in the farmland soil.

Table 1: The amounts of *ENTRIVIT*® that should have been applied to the onion, tomato and marigold plants, according to the manufacturer's recommendations

Plants	Amount (g)	
	200 seedlings	338 seedlings
Tomato	9	13
Onion	11	15
Marigold	7	13

Table 2: Soluble nutrient concentrations in the cultivation and plant soils

Nutrients	Concentrations (mg/L)	
	Cultivation soil	Plant soil
N	140	280
P (P_2O_5)	80	195
K (K_2O)	190	430

Table 3: The actual amounts of *ENTRIVIT*® applied to the tomato, onion and marigold plants in the growth experiment and the corresponding spore concentrations, according to the manufacturer's description

Plants	Amount applied (g)		Spores per plant
	90 seedlings	270 seedlings	
Tomato	4.5	13.5	7–9
Onion	5.	16.5	9–11
Marigold	3.5	10.5	7–8

Table 4: The amounts of *ENTRIVIT*® applied to each plant in the concentration experiment

Plants	Amount applied (g)		
	10 ×	20 ×	50 ×
Tomato	0.45	0.9	2.25
Onion	0.55	1.1	2.75
Marigold	0.35	0.7	1.65

No arbuscules, hyphal coils or vesicles were found in the roots of the tomato or onion plants, in any of the soils at this time. However, four weeks after the application of *ENTRIVIT*®, the roots of tomato, onion and marigold plants in the farmland soil contained arbuscules and hyphal coils. No arbuscules, hyphal coils or vesicles were observed in the roots of any of the plants in the cultivation or plant soils, after even 12 weeks from the application of *ENTRIVIT*®. No AMF colonized the control plants.

In the *ENTRIVIT*® concentration experiment, 12 days after application, arbuscules, hyphal coils and vesicles were observed in the roots of the tomato plants that were inoculated with 50 × the recommended dosage of *ENTRIVIT*®, but only in the cultivation soil (Fig. 3a–d). No arbuscules, hyphal coils or vesicles were observed in the roots of the onion or marigold plants, in either the cultivation or plant soils. The same results were obtained 5 weeks after inoculation.

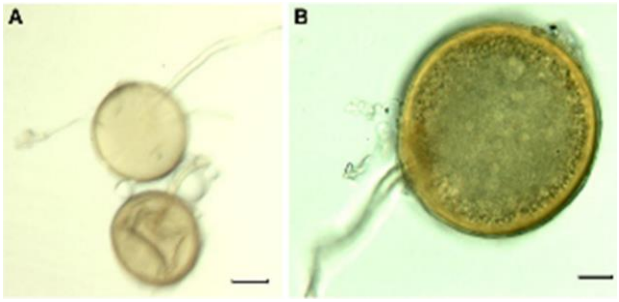

Fig. 1: Yellowish arbuscular mycorrhizal fungal spores with laminated walls and hyphal attachments. The scale bar in (a) is 25 µm, and in (b) it is 10 µm

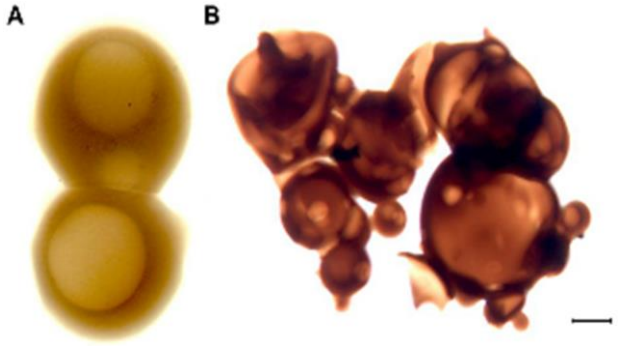

Fig. 2: Brownish empty "balls" (a) and dark brown empty "balls" (b) in *ENTRIVIT*®. The scale bar is 10 µm

Growth Performance

The shoot dry weights between the inoculated and control marigold plants, in the farmland, plant and cultivation soils, at the three harvest time points (2, 4, and 12 weeks) were not significantly different (Fig. 4a–c). Similarly, there were no significant differences in the shoot dry weights between the inoculated and control tomato (Fig. 5), or onion (Fig. 6), plants in all three soil types, at the three harvest time points.

Discussion

At least two reasons could cause the lack of mycorrhizal formation of the tomato, onion and marigold in the cultivation and plant soils in present study. Firstly, the low spore concentrations of *ENTRIVIT*®, actual concentration of 50–80 spores/g vs supposed concentration of 200 spores/g minimum by the manufacturer meant that the plants were inoculated with 2–5 spores each. Secondly, soluble P in the cultivation and plant soils was likely too high, which could cause the inefficient colonization by AMF, because colonization of roots by mycorrhizal fungi has been shown to be negatively correlated with P fertilization (Treseder, 2004). Soluble P concentrations in the cultivation (169 µm) and plant (411 µm) soils were 17–822 times higher than the range of P concentrations (0.5–10 µm) found in most common AMF habitats (Smith and Read, 2008).

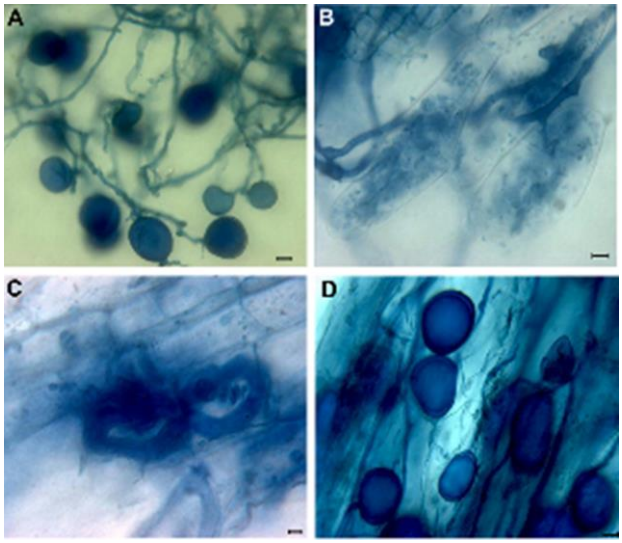

Fig. 3: Arbuscular mycorrhizal fungal (AMF) structures in the roots of tomato plants inoculated with 50 × the recommended dosage of *ENTRIVIT*® in the cultivation soil 12 days after the inoculation: spores (a); arbuscules (b); hyphal coils (c); and vesicles (d). The scale bar in (a) and (d) is 32 µm, in (b) it is 12 µm, and in (c) it is 4 µm

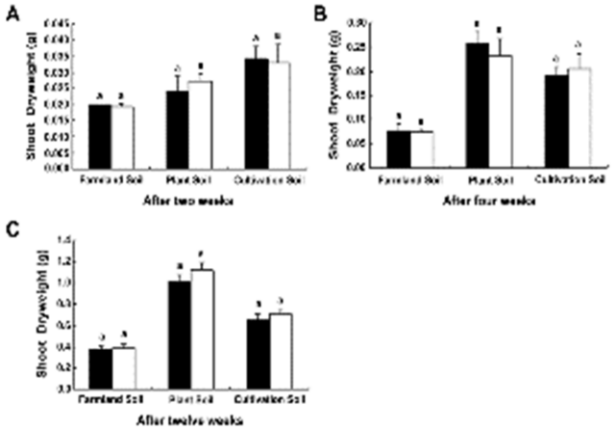

Fig. 4: Shoot dry weights of the marigold plants in the three soil types after harvest at 2 (a), 4 (b), and 12 (c) weeks. Key: black, with the *ENTRIVIT*® inoculum; and white, control. There were no significant differences between the shoot dry weights of the inoculated and control plants in any of the soils types, or at any harvest time (represented by the 'a' above the columns)

The available P in the cultivation and plant soils (80 ppm and 195 ppm, respectively) also exceeded the 40 ppm normally used for normal practice (Ravnskov and Larsen, 2005).

The arbuscular structures observed in the roots of three plants in the farmland soil could have been caused either by AMF that already existed in the farmland soil, or by the AMF in *ENTRIVIT*®. The source of the AMF in roots of

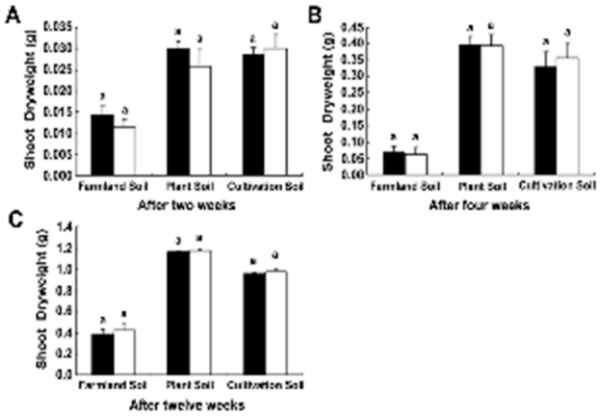

Fig. 5: Shoot dry weights of the tomato plants in the three soil types after harvest at 2 (a), 4 (b), and 12 (c) weeks. Key: black, with the *ENTRIVIT*® inoculum; and white, control. There were no significant differences between the shoot dry weights of the inoculated and control plants in any of the soils types, or at any harvest time (represented by the 'a' above the columns)

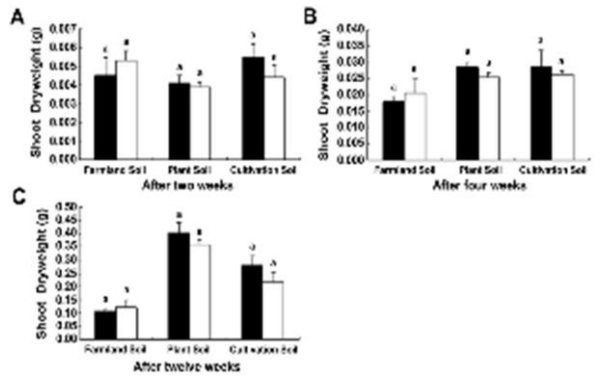

Fig. 6: Shoot dry weights of the onion plants in the three soil types after harvest at 2 (a), 4 (b), and 12 (c) weeks. Key: black, with the *ENTRIVIT*® inoculum; and white, control. There were no significant differences between the shoot dry weights of the inoculated and control plants in any of the soils types, or at any harvest time (represented by the 'a' above the columns)

three plant species needs to be analyzed through further molecular investigations. However, the lack of growth differences between the inoculated and control plants indicate that, if the AMF were from *ENTRIVIT*®, they had no effect on plant growth during the study period. Vitality of AMF spores in the product could directly contribute to this result. Because the vitality of spores, which could be related either with the choice of carrier substrate of the inoculants as the substrate should provide a stable environment for the microbial fractions to disperse and dissolute within soil (Malusá *et al.*, 2012), or with the product shelf-life, the stabilization of the products with time seems to be highly variable because of a very short shelf-life, or the absence of 'use-by' dates (Owen *et al.*, 2015). In addition, the way that the *ENTRIVIT*® inoculum was applied could also have impact on the results. The distinct lack of consistency in-application rates and methods for the different products is of concern (Owen *et al.*, 2015).

In the *ENTRIVIT*® concentration experiment, the AMF of *ENTRIVIT*® colonized only the roots of the tomato plants successfully within 12 days of the application in the cultivation soil, but this was not observed in the plant soil. This result demonstrates that the AMF in *ENTRIVIT*® respond differently depending on the plant types and the soil types. Hart and Forsythe (2012) reported that mycorrhizal growth dependency of host species can have a bearing on the success of AM colonization. The importance of plants within bio-inoculant design and testing is highlighted by the contradictory results obtained when using differing plant species. Soil type has been shown to be a major factor determining microbial community structure, as well as plant growth and rhizosphere nutrient dynamics (Oehl *et al.*, 2010; Marschner *et al.*, 2011). Bashan (1998) showed how soil type affected the persistence of *Azospirillium brasilense* in root-free media using 23 different soil types. Our results indicate that AMF from *ENTRIVIT*® could function under certain spore concentrations and soluble nutrition conditions, which is consistent with the conclusion by Adholeya *et al.* (2005) that the efficacy of any fungal or bacterial strain utilized within inoculants is also subject to numerous soil, crop and environmental factors, from crop species compatibility, size and effectiveness of indigenous microbial populations, soil fertility and management.

The fact that only one morphotype of AMF was observed in *ENTRIVIT*® strongly contrasts the manufacturer's description of seven different AMF species in the product; the morphotype was successfully identified through molecular methods, similar to *R. irregularis* and *Glomus* spp.. The *Glomus* spp. are individual subcultures, with the identifier DAOM197198; this has been regarded as a *G. intraradices*-like fungus and was recently described as *G. irregular* (Stockinger *et al.*, 2009) and identified as *R. irregularis* (Kruger *et al.*, 2012).

The success of commercial bio-inoculants should be reflected in an economic gain, either through improved yields or reduced inorganic fertilizer applications, or both. In present study, no significant differences between the inoculated and control plants in terms of the shoot dry weights in the cultivation and plant soils have been observed. There is no consensus on the efficacy of bio-inoculate products till now (Owen *et al.*, 2015). The results are varied, inconsistent and contradictory, which may discourage companies to allow their products to undergo a rigorous scientific examination (Owen *et al.*, 2015).

Conclusion

The concentration of spores in *ENTRIVIT*® was much lower than that described by the manufacturer. Hence, the

expected effect of *ENTRIVIT*® was not achieved using the manufacturer's recommended application. Either the concentration of spores in *ENTRIVIT*® should be increased, or the recommended application dosage should be changed; for example, we would recommend using 3−4× the current standard dosage. However, the effects of both options should be explored. In addition the manufacturer should correct the description of the product's AMF contents.

Cultivation and plant soils are commonly used for the cultivation of vegetables and ornamental plants, in greenhouses and gardens. These soil types contain high concentrations of added nutrients, including P; this may have resulted in the failure of mycorrhizal formation in these soils. Hence, the expected effect of *ENTRIVIT*® was not achieved. We consider that the manufacturer should give different dosage recommendations, depending on the nutrient concentrations of the substrate. Tests to identify the appropriate dosage recommendations need to be carried out. Finally, the way that *ENTRIVIT*® should be applied needs to be reconsidered by the manufacturer and is likely to be dependent on the substrate type.

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