

IN VITRO INTERACTIONS BETWEEN THE VESICULAR–ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS MOSSEAE* AND SOME SAPROPHYTIC FUNGI ISOLATED FROM ORGANIC SUBSTRATES

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Summary—Six saprophytic fungi belonging to different genera and isolated from three organic substrates were tested under controlled *in vitro* conditions for their effect on the germination of *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe resting spores and on the development of vesicular–arbuscular mycorrhizal fungal (VAMF) mycelium. The development of *G. mosseae* was stimulated by sterile water extracts from organic substrates and by the presence of *Trichoderma* spp isolated from these substrates. Microbial inoculation did not affect the percentage of spore germination, which reached 90–95% after 26 days' incubation, but the germination rate was hastened and the development of VAM mycelium from germinated spores was enhanced by the presence of *Trichoderma* spp. Stimulation caused by *Trichoderma* might be attributed to the production of volatile compounds more than to non-volatile compounds released to the growing media. No hyphal contact between both types of fungi was necessary to detect stimulation in dual inoculation treatments. The other saprophytes tested inhibited spore germination.

INTRODUCTION

The use of organic soilless media in horticultural practice is widespread. Peat and composted organic materials are the most popular and have been studied for their suppressive and conducive properties involved in biological control of soil-borne plant pathogens.

These media usually lack vesicular–arbuscular mycorrhizal fungal (VAMF) propagules, therefore the root system of plants grown in such conditions remains non-mycorrhizal unless appropriately inoculated. Mycorrhizas can enhance nutrient uptake, promote plant uniformity and reduce transplanting damage (Biermann and Linderman, 1983), however, the establishment of effective mycorrhizas in organic media often fails because of the high organic matter content and phosphorus availability of these substrates (Graham, 1984). Moderate VAM colonization levels and plant responses have been recorded when P availability is reduced by the use of rock phosphate (Graham and Timmer, 1984).

In addition, substrates showing suppressiveness against soil-borne plant pathogenic fungi could inhibit VAM as well. For instance, a study regarding the influence of the substrate on the interaction between the VAMF *Glomus intraradices* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the rhizosphere of tomato plants, showed that the tested substrates affected both pathogen development and mycorrhizal colonization of plant roots (Caron *et al.*, 1985). We have been unable to locate any infor-

mation published, to date, on the specific interactions between microbiota and VAMF in organic substrates. These microorganisms can be linked to suppressive and conducive properties and have an active role as antagonistic agents. Kuter and Hoitink (1985) found that microorganisms associated with biological control in soils, i.e. *Trichoderma* spp and *Gliocladium* spp, were isolated from organic composts showing suppressiveness against “damping-off” caused by *Rhizoctonia solani*. Also bacterial antagonistic agents have been isolated from bark composts, but they were not tested for their effect on VAMF of most horticultural and ornamental crops.

In this paper, six saprophytic fungi belonging to different genera and isolated from three organic substrates were tested under *in vitro* conditions for their effect on the germination of *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe resting spores and on the development of VAMF mycelium.

MATERIALS AND METHODS

Organic substrates

Three organic substrates were used to isolate saprophytic fungi: a sphagnum peat (Floratorf-500) and two composts suppressive for fusarium wilt of carnation (Pera and Calvet, 1989); a pine bark compost (IMC-2, Bark Products Ltd), and a composted olive pumice from raw olive pumice (Calvet *et al.*, 1985).

Isolation of saprophytic fungi

The fungi present in the substrates were isolated by the dilution plate method. 10 ml of each organic substrate were vigorously shaken in 100 ml sterile distilled water for 30 min, successive 10-fold dilutions were prepared from each suspension and 0.1 ml of each dilution were plated on substrate extract water agar (SEWA). The substrate extracts were prepared by shaking 100 ml of each organic substrate in 500 ml distilled water for 30 min at room temperature, and successively filtered for sterilization through a 0.22 μm pore membrane filter. The sterile filtrates were added to 1.5% water agar (WA) before solidifying at 1:1 (v/v). The final pH was 6.8 ± 0.1 for all extract-media, after the pH of sphagnum peat was adjusted to 7 with CaCO_3 prior to extraction. Petri dishes were kept for 5 days' at 25°C in the dark, and fungal populations were counted as colony forming units ml^{-1} for each substrate using appropriate dilutions.

From a total of 4.09×10^6 cfu ml^{-1} , four fungal isolates were selected as the most frequent colony forming units in composted olive pumice: two *Trichoderma aureoviride* (isolates T₂ and T₃), one *T. harzianum* (T₁) and one *Aspergillus fumigatus* (A). In composted pine bark only a *Trichoderma harzianum* (T₄) was isolated (9×10^4 cfu ml^{-1}), and in sphagnum peat, a *Penicillium decumbens* (P) isolate was the only fungus detected in the plate dilution test (1×10^5 cfu ml^{-1}).

All these saprophytes were cultivated on 2% malt extract (Difco) agar (MEA) and potato dextrose agar (PDA, Difco), and then preserved in silica gel tubes at 4°C as stock cultures (Smith and Onions, 1983). Before using them in dual inoculation tests, they were inoculated from these tubes to plating media and grown for 4 days at 25°C.

Axenic spore germination and germ tube growth of *G. mosseae*

Sporocarps of *G. mosseae* were extracted from rhizosphere soil in pot culture containing a high inoculum density through a 250 μm mesh by wet sieving and decanting. Resting spores were excised from the sporocarps and placed on a moistened Millipore (8 μm pore) membrane filter of 45 mm dia in a glass Petri dish at 4°C for 4 days' before sterilization.

80–100 spores per filter were surface sterilized with the solution used by Mosse (1962) containing 2% Chloramine T, 300 μg streptomycin sulfate ml^{-1} and Tween 20 (2–5 drops). The sterilization was always done in Millipore "sterilet" units by first filtering the solution through 0.45 μm and leaving it in contact with the spores for 14 min. Spores were then rinsed three times with sterile distilled water and carefully placed on the culture medium (either WA or SEWA), six spores per Petri dish were inoculated, located on the vertices of an imaginary hexagon with about

3.5 cm sides. Plates were sealed with parafilm and kept at 25°C in the dark for 28 days. Each experiment was repeated at least twice under identical experimental conditions.

Effect of sterile substrate extracts on axenic germination and mycelial development of *G. mosseae*

The presence in the organic substrates of any water-soluble chemical compound able to inhibit or stimulate the germination of *G. mosseae* resting spores and the development of VAMF mycelium *in vitro* was tested using SEA as culture medium. Substrate extract agar plates of each organic substrate were prepared as for the isolation of saprophytic fungi. In the control treatment, the extract was replaced with the same volume of sterile distilled water.

Spore germination rate, hyphal growth from each germinated resting spore and the number of vegetative spores formed on the mycelium were assessed under a light microscope, after 3, 5, 7, 10, 12, 14 and 28 days' incubation. Spores damaged during the surface sterilization process were not considered.

There were six Petri dishes per treatment (substrate extract) and six spores per Petri dish. Spore germination data were analyzed by a chi-square test. Data on vegetative spore production after 28 days' growth were processed by ANOVA and Tukey's multiple range test ($P \leq 0.01$).

Effect of saprophytic fungi on *G. mosseae* resting spores

The effect of saprophytic fungi on spore germination and mycelial growth *in vitro* was also tested. The growing medium was 0.75% water agar, pH adjusted to 7.0 and autoclaved (20 min at 120°C). The number and placement of the *G. mosseae* spores in the plate were as before, and the saprophytic fungi were inoculated in the center of the plate. The number of germinated resting spores, as well as the number of vegetative spores formed on the mycelium developed from the germ tube, were assessed during 28 days' at 25°C in the dark. This experiment was repeated twice in 9-cm-dia Petri dishes and also in 14-cm-dia dishes where *G. mosseae* spores were inoculated only in one half of the plate at 6 cm from the saprophytic fungus to check any chemotaxic interaction between it and the *G. mosseae* germinating spores.

Effect of saprophytic fungi on *G. mosseae* spore germination and mycelium development in 10 mM MES culture medium

To check whether the effect of the saprophytic fungi on *G. mosseae* spore germination was directly caused by fungal metabolites or if it was an indirect effect due to acidification, an efficient buffer, 10 mM 2-(*N*-morfolin) ethane sulfonic acid (MES), was added to water agar. MES had been used before (Carr and Hinckley, 1985), and did not affect *in vitro* spore germination of *G. caledonium*. The final pH

of MES 10 mm–water agar was 6.8 on the plate surface.

Three saprophytic fungi were chosen for this experiment: *Trichoderma aureoviride* (T₃), which strongly stimulated germination rate and production of vegetative spores, and the two fungi showing an inhibitory effect on the germination of *G. mosseae* *in vitro*, i.e. *Aspergillus fumigatus*, isolated from composted olive pumice, and *Penicillium decumbens*, isolated from sphagnum peat. Saprophytic fungi were inoculated on the center of the plates equidistantly from *G. mosseae* resting spores and incubated in the experimental conditions already described.

Effect of non-volatile compounds released by *Trichoderma* spp on axenic growth of *G. mosseae*

1.5% Water agar was used in this experiment. Solidified medium in each Petri dish was covered by a sterilized cellophane disk. A 5-mm-dia mycelial piece of *Trichoderma* spp from PDA 4 day-old cultures was placed on the center of the disk and kept for 72 h at 25°C in the dark to allow the fungus to grow on the cellophane. Once the disk was removed from the plate under sterile conditions, *G. mosseae* surface-sterilized spores were inoculated on the water agar, and incubated for 28 days. The pH on the surface of the medium was measured upon growth of the saprophytes alone.

RESULTS

Effect of sterile substrate extracts on axenic germination and mycelial development of *G. mosseae*

Sterile water extracts from the organic substrates tested did not inhibit the axenic germination of *G. mosseae* resting spores [Fig. 1(A)], which was not different from the control after 28 days' incubation. Moreover, VAMF mycelial growth, measured as

the production of vegetative spores, was significantly ($P \leq 0.01$) stimulated by the extracts [Fig. 1(B)].

Effect of saprophytic fungi on *G. mosseae* resting spores

The number of germinated *G. mosseae* resting spores in cocultures with saprophytic fungi is recorded in Fig. 2(A). Figure 2(B) shows results of the production of vegetative spores on VAM mycelium *in vitro* after 28 days' incubation.

After 5 and 7 days' incubation, respectively, the mycelia of *T. aureoviride* (T₃ and T₄) and *T. harzianum* (T₁ and T₂) had grown over the entire agar surface [Fig. 2(A)] and the first vegetative spores appeared on the VAM hyphae.

Inoculation with *P. decumbens* or *A. fumigatus* strongly inhibited *G. mosseae* spore germination *in vitro* and a limited development of VAMF mycelium from germ tube was observed in these treatments. Furthermore, no vegetative spores appeared on VA mycelium in the presence of *A. fumigatus*, and only after 28 days' incubation were these structures detected in control and *P. decumbens* treatments. Growth of VAMF hyphae was independent of the development of the mycelium of the saprophyte on the plates.

Effect of saprophytic fungi on *G. mosseae* spore germination and mycelium development in 10 mM MES buffered agar medium

The former experiment confirmed that *P. decumbens* and *A. fumigatus* had a direct inhibitory effect on the germination of *G. mosseae* resting spores, and that the effect was not mediated by changes induced on the pH of the medium [Fig. 3(A)]. These saprophytic fungi did not significantly affect the production of vegetative spores in the VA mycelium over the control [Fig. 3(B)].

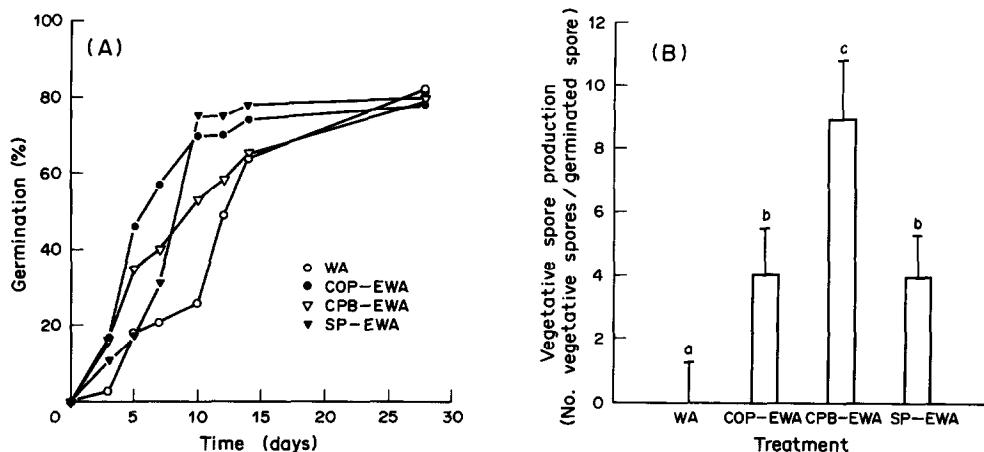


Fig. 1. Effect of substrate extracts on the axenic germination of *G. mosseae* (A) and on the production of vegetative spores after 28 days' incubation (B). WA: water agar; COP-EWA: composted olive pumice extract water agar; CPB-EWA: composted pine bark extract water agar; SP-EWA: sphagnum peat extract water agar. Bars with identical letters are not significantly different after Tukey's multiple range test ($P \leq 0.01$).

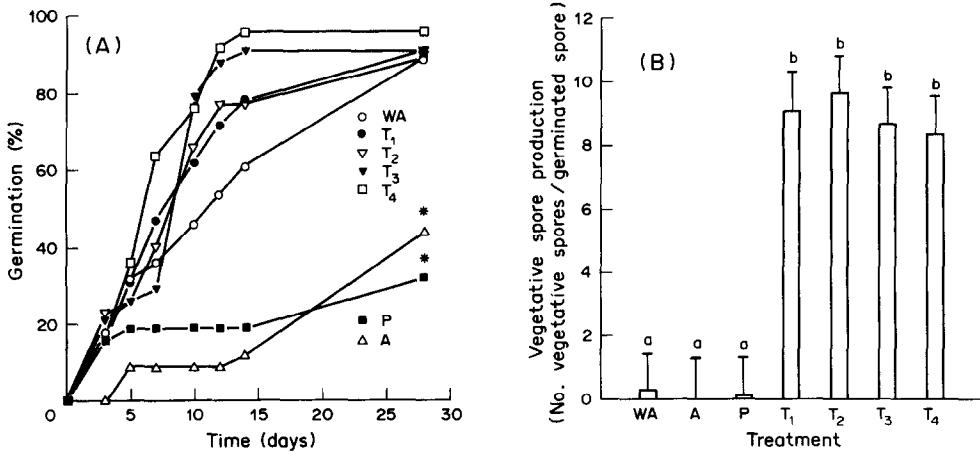


Fig. 2. Axenic germination of *G. mosseae* resting spores (A) and production of vegetative spores after 28 days' incubation (B) in the presence of saprophytic fungi. WA: water agar; T₁ and T₄: *T. harzianum*; T₂ and T₃: *T. aureoviride*; P: *P. decumbens*; A: *A. fumigatus*. *Germination (%) significantly different from control after a non-parametric chi-square test. Bars with identical letters are not significantly different after Tukey's multiple range test ($P \leq 0.01$).

When *T. aureoviride* was inoculated in the center of the plate, *G. mosseae* germination rate was enhanced and the production of vegetative spores was stimulated after 28 days' incubation. These secondary spores appeared on the mycelium after 7 days' incubation. There were no vegetative spores on VAM mycelium grown in plates inoculated with *A. fumigatus* and only very few in plates inoculated with *P. decumbens*.

Effect of non-volatile compounds released by Trichoderma spp on axenic growth of G. mosseae

Non-volatile compounds as released by *Trichoderma* spp to the growing medium [Fig. 4(A)] did not produce inhibitory effects on spore germination. The number of vegetative spores produced on VAM mycelium was not different from the number

recorded in non-inoculated water agar [Fig. 4(B)]. The pH on the agar surface did not change after *Trichoderma* spp growth on the cellophane disks.

Axenic mycelial growth from spore germ tubes was very limited as was the production of vegetative spores. They appeared only after 28 days' incubation in control plates and in plates inoculated with *T. aureoviride* (T₂ and T₃) and always in a very low number.

DISCUSSION

The stimulation of VAMF *in vitro* growth by other rhizosphere microorganisms has been reported (Azcón-Aguilar *et al.*, 1986; Mayo *et al.*, 1986; Mugnier and Mosse, 1987; Azcón, 1987, 1989). Our results confirm these findings and also show that

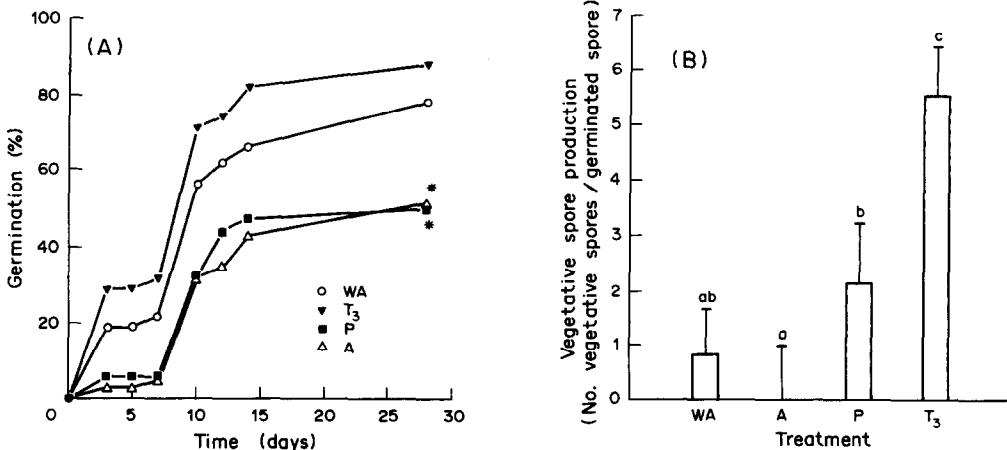


Fig. 3. Axenic germination of *G. mosseae* (A) and production of vegetative spores after 28 days' incubation (B) in the presence of saprophytic fungi on water agar buffered with 10 mM MES. WA: water agar; T₃: *T. aureoviride*; P: *P. decumbens*; A: *A. fumigatus*. *Germination (%) significantly different from control after a non-parametric chi-square test. Bars with identical letters are not significantly different after Tukey's multiple range test ($P \leq 0.01$).

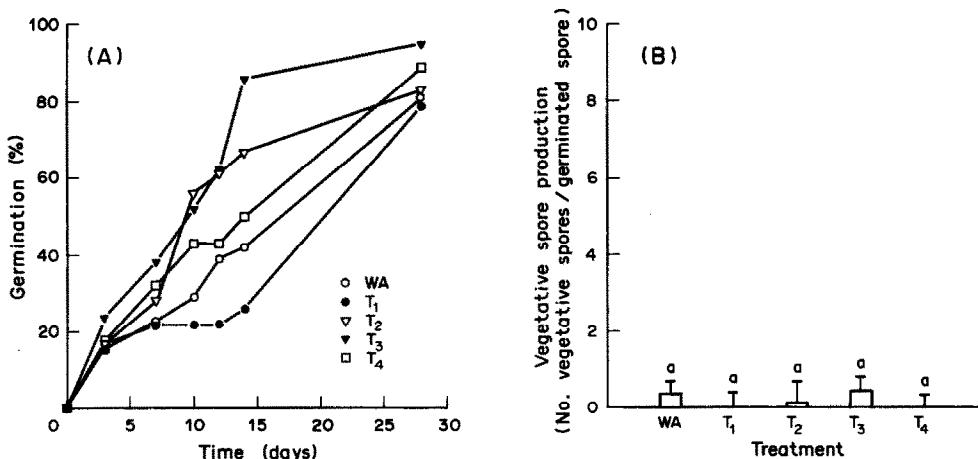


Fig. 4. Effect of non-volatile compounds released by *Trichoderma* spp on axenic germination of *G. mosseae* (A) and vegetative spore production after 28 days' incubation (B). WA: water agar; T₁ and T₄: *T. harzianum*; T₂ and T₃: *T. aureoviride*. Bars with identical letters are not significantly different after Tukey's multiple range test ($P \leq 0.01$).

G. mosseae germination is stimulated by sterile water extracts from organic substrates and moreover by the presence of *Trichoderma* spp isolated from these substrates. Filter-sterilized substrate extracts and saprophytic fungi had a similar effect on germination and mycelial growth of *G. mosseae* resting spores. Although the stimulation caused by substrate extracts added to the culture medium can be attributed to a nutrient supply which enhances the development of VAM mycelium (Hepper, 1979), metabolites present in the filter-sterilized substrate extracts, produced by the substrates microbiota, could also be involved.

Microbial inoculation did not affect the amount of spore germination, which reached 90–95% after 26 days' at 25°C, but the germination rate was hastened and the development of VAM mycelium from germinated spores was enhanced by the presence of *Trichoderma* spp. Stimulation caused by *Trichoderma* might be attributed to the production of volatile compounds in the monoxenic culture, more than to non-volatile compounds released to the growing media through cellophane disks. No hyphal contact between both types of fungi was observed under the light microscope in dual inoculation treatments. This agrees with the observations of Azcón-Aguilar *et al.* (1986) for certain unidentified fungi that stimulated germination and growth of *G. mosseae* spores under axenic conditions.

The effect of the saprophytes inhibiting *G. mosseae* spore germination was independent from a pH effect because it was still evident in MES-buffered water agar at pH 7. Hepper (1979) reported that applying Actinomycine D, a specific inhibitor of mRNA, resulted in limited growth of VAM mycelium *in vitro* from germ tubes. The effect of *P. decumbens* and *A. fumigatus* was similar to that described by Hepper, as the mycelial development of VAMF was very

sparse and almost non-existent when these fungi were inoculated onto plates with *G. mosseae* resting spores. Both saprophytes might produce antibiotic-like substances affecting *G. mosseae* spore germination under axenic conditions.

Azcón (1989) has shown that there is a selective influence of rhizosphere bacteria on VAM stimulation for different *Glomus* species. Such a selective influence seems to be exerted as well *in vitro* by saprophytic fungi isolated from organic substrates. In this paper, stimulatory and inhibitory effects due to saprophytic fungi on *G. mosseae* spore germination and VAM *in vitro* growth have been detected. Our results obtained *in vitro* should obviously not be considered as equivalent to those *in planta*. We assume that the interaction between saprophytic fungi and VAM fungi can be different in the rhizosphere of a soil environment from the result of *in vitro* plate assays. The mechanisms of action of these fungi and their influence in a growing medium for plants with mycorrhizal inoculation is receiving current interest in this laboratory.

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