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In Vitro Interactions between *Alternaria alternata*, *Fusarium equiseti* and *Glomus mosseae*

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Abstract

Germinated spores of the arbuscular mycorrhizas (AM) fungus *Glomus mosseae* did not affect growth of the saprophytic fungi *Alternaria alternata* and *Fusarium equiseti* on water agar. Both saprophytic fungi reduced spore germination of *G. mosseae* independently of pH changes in the medium. Soluble and volatile substances produced by the saprophytic fungi were involved in the inhibition of spore germination. Although *F. equiseti* inhibited the germination of *G. mosseae* spores, hyphal growth was markedly stimulated. The inoculation of maize plants with *A. alternata* and *F. equiseti* simultaneously with spores of *G. mosseae* significantly decreased the percentage of root length colonized by AM fungi, but there was no effect on AM colonization when *G. mosseae* was introduced before *A. alternata* and *F. equiseti*. The number of colony-forming units (CFU) of *A. alternata* decreased when *G. mosseae* was established in the root, but was not affected when both microorganisms were inoculated at the same time. The number of CFU of *F. equiseti* was not affected by the presence of *G. mosseae*.

Keywords: Arbuscular mycorrhizas, *Alternaria alternata*, *Fusarium equiseti*, *Glomus mosseae*, Saprophytic fungi

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1. Introduction

The activity of rhizosphere-inhabiting microorganisms exerts a significant effect upon arbuscular mycorrhizas, and thus influences plant health (Barea and Jeffries, 1995). Studies of the interactions between AM and other rhizosphere microorganisms have documented the qualitative effect of AM fungi on the population of these microorganisms (Meyer and Linderman, 1986). Many studies have focused on interactions between AM and pathogenic fungi, but information about the interactions between AM and saprophytic fungi is scarce (Bagyaraj, 1984). Some saprophytic fungi prefer to live in the rhizosphere and rhizoplane of plants, where they obtain greater nutritional benefit from organic and inorganic compounds released from living roots, together with sloughed cells (Alexander, 1977). Their activity produces substances that promote or inhibit the growth of other rhizosphere microorganisms (Curl and Truelove, 1986).

Alternaria alternata and *Fusarium equiseti* are common and ubiquitous species occurring in many plant-soil systems (Booth, 1971; Ellis, 1971). Antagonistic effects of *A. alternata* on numerous fungi and bacteria have been observed *in vitro* (Codignola and Gallino, 1975), and *F. equiseti* can produce several phytotoxic substances (Booth, 1971). Although AM fungi are not restricted to the rhizosphere as are other members of the soil microflora, they are influenced by soil microorganisms, especially before mycorrhiza formation (Curl and Truelove, 1986). A few studies mention the effect of some fungi, especially saprophytic fungi, on spore germination and hyphal growth of *G. mosseae* in axenic culture (Azcon-Aguilar et al., 1986; Calvet et al., 1992; McAllister et al., 1994).

The aim of this work was to study interaction between the saprophytic fungi *A. alternata, F. equiseti* and *G. mosseae,* and the effect of the saprophytes on spore germination and hyphal growth of the AM fungus.

2. Materials and Methods

Isolation of saprophytic fungi

The active fungi present in the rhizosphere soil and roots of maize cultivated in the Province of Buenos Aires (Argentina), were isolated by the particle washing method (Widden and Bisset, 1972) using a multichamber washing apparatus. Thirty washings were necessary to remove sclerotia, spores, and other fungal structures from soil particles and the roots of maize. Twenty soil particles (2 mm) were dried on sterilized filter paper and plated on 2% malt

extract agar (MEA) containing antibiotics (5 µg/l streptomycin and 10 µg/l tetracyclin). From the resulting colonies *Alternaria alternata* (Joly, 1964) and *Fusarium equiseti* (Booth, 1971) were selected and transferred to tubes of potato dextrose agar (PDA) and 2% malt extract at 4°C as stock cultures.

Effect of G. mosseae on spore germination of A. alternata and F. equiseti

The effect of *G. mosseae* on germination of spores of *A. alternata* and *F. equiseti in vitro* was tested on 1% sterile water agar, with pH adjusted to 7, with 10% KOH. Sporocarps of *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe were isolated by wet sieving and decanting (Gerdemann, 1955) from alfalfa pot cultures of a Rothamsted isolate of *G. mosseae* (BEG No. 12). Spores were obtained by dissecting the sporocarps, and were stored in water at 4°C and used within 1 month. Spores of *G. mosseae* were surface-sterilized as described by Mosse (1962). Twenty-five spores per plate were placed around the perimeter of the Petri dish and a thin streak with spores and mycelium of the saprophytic fungus to be assayed was placed in the center. Ten replicates were used and plates with the saprophytic fungus alone were used as controls. The plates were sealed to reduce the risks of dehydration and contamination, and were incubated in the dark at 25°C. The diameter of the saprophyte colony was checked after 2, 4, 6, 8, 10, 13 and 15 days.

Effect of A. alternata and F. equiseti on the development of G. mosseae

The effect of each saprophytic fungus on spore germination and hyphal growth of *G. mosseae* was tested in four different experiments conducted in 9 cm diameter plastic Petri dishes. In the first experiment, the effect of each saprophytic fungus on spore germination and mycelial length was tested *in vitro* on 1% sterile water agar (pH 7). Five surface-sterilized spores per plate were placed 1 cm from the edge of a Petri dish, and a thin streak with spores and mycelium of the saprophytic fungus was placed opposite and at least 7 cm away from them.

The second experiment was designed to test whether the saprophytic fungi affected *G. mosseae* spore germination indirectly due to pH modification. We added 10 mM 2-(N-morpholin) ethane sulfonic acid (MES) to 1% water agar to maintain the pH of the medium at 7 throughout the duration of the experiment. This buffer was previously shown not to affect germination of AM fungal spores *in vitro* (Carr and Hinkley, 1985; Calvet et al., 1992). Inocula of *G. mosseae* spores and of the saprophytic fungi were placed on water agar + MES, as described before.

The third experiment tested the effect of exudates from each saprophytic fungus on germination and hyphal growth of the AM fungus *in vitro*. Exudates were obtained by growing the fungus in 250 ml flasks containing 125 ml sterile potato dextrose liquid medium on a shaker at 28°C. After 72 h the culture medium was filtered through a disk of filter paper (Whatman No. 1) and sterilized twice by filtration through a 0.45 µm Millipore membrane. These exudates (2 ml) were added to 10 ml 1% water agar (pH 7) to a Petri dish. Five spores of *G. mosseae* were placed at the vertices of an imaginary pentagon inside the dish. In the control treatment, the same volume of potato dextrose liquid medium was substituted for the exudates.

In the fourth experiment the effect of volatile compounds released by the saprophytic fungi on spore germination and hyphal growth of *G. mosseae* was tested in divided plastic Petri dishes. In an initial assay the dishes contained 1% water agar (pH 7) on both sides. On one side, 5 AM fungus spores were placed near the edge of the plate, and the saprophytic fungus was inoculated in the other side. In a second assay the plates contained 1% water agar (pH 7) on one side and MEA medium on the other. Five AM spores were placed on the water agar, and one of the 2 saprophytic fungi was inoculated together on the nutrient agar.

In all four experiments ten replications of each treatment and ten controls were prepared with germinating *G. mosseae* spores on each culture medium. The plates were incubated at 25°C in the dark, and were sealed to reduce the risks of dehydration and contamination. Spore germination was determined after 2, 4, 6, 8, 10, 13 and 15 days under a light microscope. At the end of the experiment (16 d) hyphal length of the germinated *G. mosseae* spores was assessed using the gridline intersect method (Marsh, 1971).

Interaction between saprophytic fungi and G. mosseae, inoculated as spores, in the rhizosphere of maize under sterile conditions

This experiment was performed in 20 × 200 mm glass tubes filled with 25 g of a growth substrate (sand/vermiculite mixture, 1:1, v:v) and 12 ml diluted (1:2) Long Ashton nutrient solution (Hewitt, 1952) plus 50 µg/ml K_2HPO_4 (pH 7). The tubes were closed with cotton wool and autoclaved (120°C for 20 min). In each tube, 30 surface-sterilized spores of *G. mosseae* were placed under the seedlings. Seeds of maize (*Zea mays* L. cv. Calderon) were surface sterilized with $HgCl_2$ for 10 min and thoroughly rinsed with sterilized water. After germination, seedlings were selected for uniformity before planting. Plants were grown in a chamber with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 µE/m^2/s, 400–700 nm, with a 16/8 h day/night cycle at 25/19°C and 50% relative humidity.

The saprophytic fungi *A. alternata* and *F. equiseti* were obtained from rhizosphere and rhizoplane of maize plants as described before. An aqueous suspension in sterile distilled water, containing approximately 2×10^3 spores/ml, was prepared from cultures grown on PDA (1 week, 27°C).

Six treatments were used in all experiments: (1) uninoculated controls, (2) inoculated with *A. alternata* or *F. equiseti*, (3) inoculated with *G. mosseae*, and (4) inoculated with both *G. mosseae* and either *A. alternata* and *F. equiseti*. Plants were inoculated at the time of transplanting (after 2 weeks of growth). The saprophytic fungus was inoculated at the same time or 2 weeks after *G. mosseae*.

To evaluate the colony-forming units (CFU) of inoculated *A. alternata* or *F. equiseti* during the experiments, 1.4 g of growth substrate from 5 replicate tubes was collected 5 days and 10 weeks after the saprophytic fungus was inoculated, and the number of CFU was counted (Garcia-Garrido and Ocampo, 1988). Ten-fold serial dilutions were prepared for each sample. The CFU in suitable dilutions of such samples were counted on PDA medium. Growth substrate samples were quantified as follows (recovered from to 10^{-1} and 10^{-2} dilutions), dried at 105°C and weighed. The number of CFU was expressed per g of dry growth substrate. Plants were harvested after 10 weeks and shoot dry matter was weighed. After the plants were harvested, the root system in each of the 5 replicates per treatment was cleared and stained (Phillips and Hayman, 1970), and percentage root colonization was determined (Ocampo et al., 1980).

Statistical treatments

The data were analyzed by one-way analysis of variance. The orthogonal contrast comparisons and the standard errors of means are given.

3. Results

Of the 109 fungal strains isolated from the rhizosphere and rhizoplane of maize, 30% of the rhizosphere fungi were *A. alternata* and 25% of those in the rhizoplane were *F. equiseti*. These saprophytic fungi were not pathogenic to maize plants, even when plants were inoculated with a large number of fungal conidia (data not shown).

Germinated spores of *G. mosseae* did not affect the growth of saprophytic fungi cultivated on water agar (data not shown). Germination of *G. mosseae* spores cultivated on water agar (Fig. 1) was lower in the presence of *A. alternata*, but there was no effect on the hyphal length of *G. mosseae* (Table 1). *F. equiseti* decreased the percentage of spore germination of *G. mosseae* after 13

days (Fig. 1), and also increased hyphal length (Table 1) and shortened the time of production of vegetative spores after 10 and 13 days of incubation, respectively. The saprophytic fungi had similar effects on the percentage of spore germination and hyphal length of *G. mosseae* when grown on water agar buffered with MES.

Table 1. Hyphal length of *G. mosseae* in the presence of *A. alternata* and *F. equiseti* cultivated on water agar and water agar + MES; and in presence of exudates and volatile compounds produced by the saprophytic fungi grown in water agar and in MEA medium.

	Hyphal length (mm)				
Saprophytic fungus	Water agar	Water agar with MES	Exudates	Volatile compounds (water agar)	Volatile compounds (MEA)
Control	6.3 b	6.1 b	6.6 b	6.2 b	6.4 b
A. alternata	4.8 b	5.1 b	5.8 b	7.5 b	1.3 c
F. equiseti	12.3 a	11.8 a	11.1 a	8.1 b	2.3 c

The exudates of *A. alternata* significantly decreased the percentage of spore germination of *G. mosseae* in water agar after 6 days of incubation (Fig. 2). However, the exudates of *A. alternata* had no effect on the hyphal length produced by spores of *G. mosseae*. The exudates of *F. equiseti* did not affect the germination of *G. mosseae* spores (Fig. 2), but significantly increased the hyphal length (Table 1). After 15 days vegetative spores of *G. mosseae* were observed in the presence of exudates of *F. equiseti*, but not in the presence of exudates of *A. alternata* or in the control treatments.

Volatile compounds produced by *A. alternata* and *F. equiseti* significantly decreased the percentage of spore germination in *G. mosseae* (Fig. 3). However, the volatile compounds produced by both saprophytic fungi did not affect the hyphal length of *G. mosseae* (Table 1). Volatile compounds from *F. equiseti* shortened the time of production of vegetative spores, which appeared after 13 days. When MEA growth medium was used, the volatile compounds produced by both saprophytic fungi inhibited spore germination (Fig. 3), length of hyphae (Table 1), and the formation of vegetative spores in *G. mosseae* to a greater extent than when the fungi were grown on water agar.

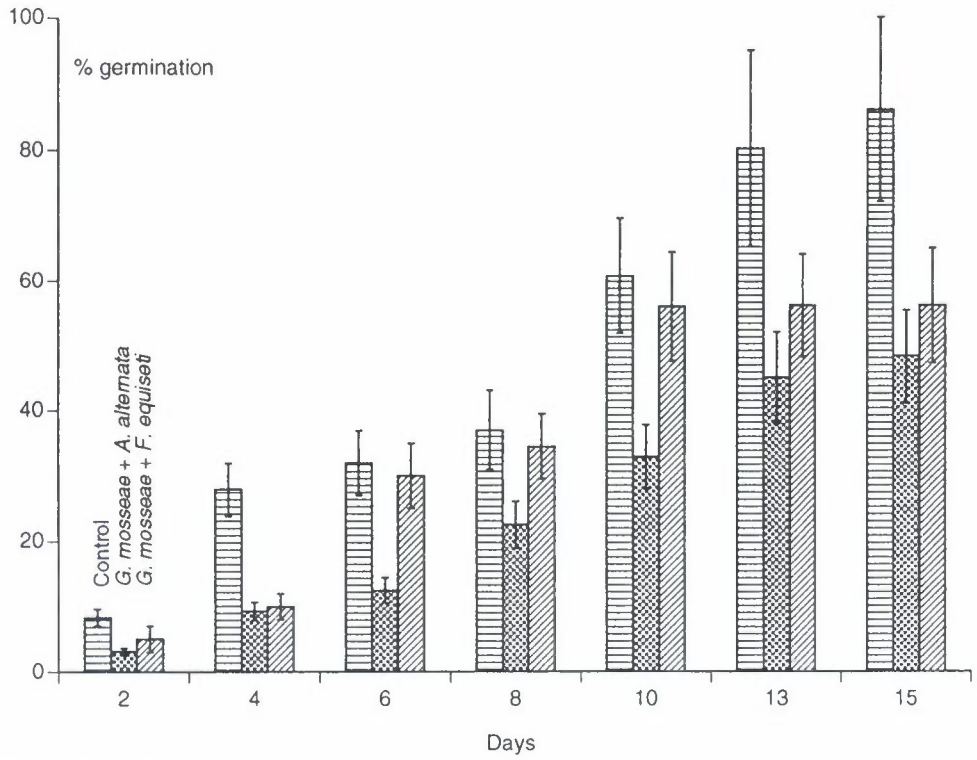

Figure 1. Effect of *Alternaria alternata* and *Fusarium equiseti* on the percentage of germination of *Glomus mosseae* cultivated on water agar. Standard errors of the means are given.

The dry weights of maize plants were not significantly different statistically in all treatments (data not shown). Inoculation of *A. alternata* and *F. equiseti* at the same time as *G. mosseae* significantly decreased the percentage of root length of maize plants colonized by AM, but no effect on AM colonization was observed when *A. alternata* and *F. equiseti* were inoculated two weeks after *G. mosseae* (Table 2). The number of CFU of *A. alternata* decreased when it was inoculated two weeks after *G. mosseae*, but were not affected when the saprophyte was inoculated at the same time as *G. mosseae* (Table 2). The number of CFU of *F. equiseti* was not affected by the presence of *G. mosseae* in any of the treatments tested (Table 2).

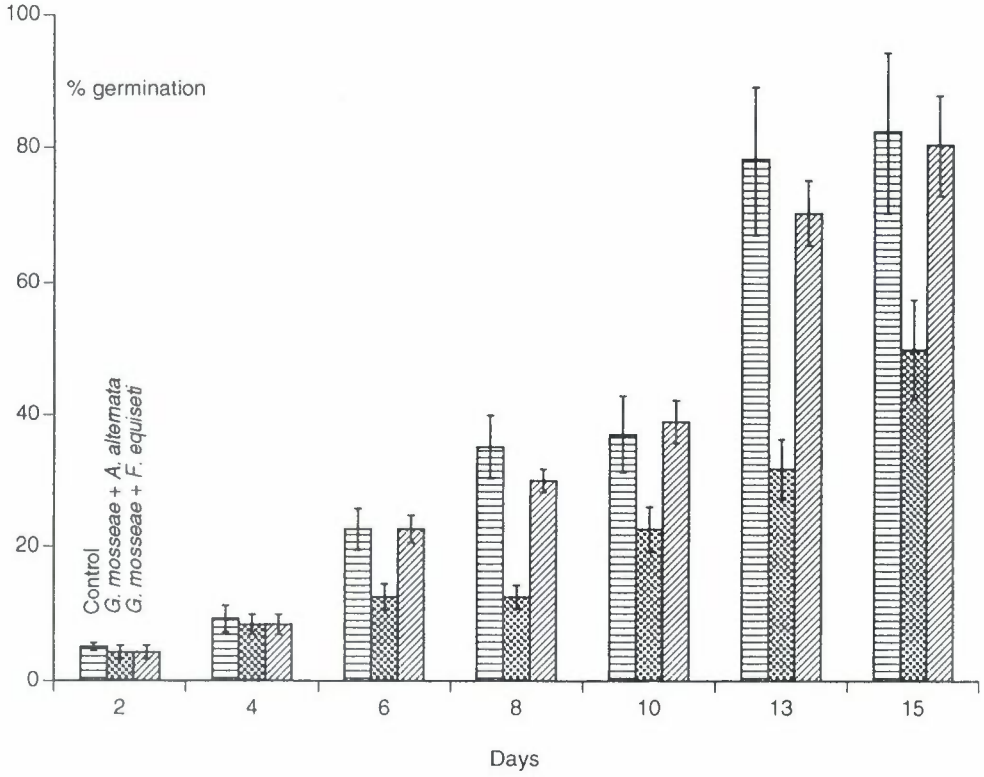

Figure 2. Effect of exudates of *Alternaria alternata* and *Fusarium equiseti* on the percentage of germination of *Glomus mosseae* cultivated on water agar. Standard errors of the means are given.

4. Discussion

A. alternata and *F. equiseti* inhibited the germination of *G. mosseae* spores independently of the pH of the medium, as has been observed with other saprophytic fungi (Calvet et al., 1992; McAllister et al., 1994). This effect may have been due to soluble or volatile substances produced by the saprophytic fungi. The production of antibiotics or CO_2 by *A. alternata* and *F. equiseti* may be involved in the decrease of the percentage of spore germination of *G. mosseae*. Moreover, our results show that soluble or volatile substances produced by the saprophytes did not inhibit hyphal growth from *G. mosseae* spores. Germination and hyphal growth are two separate processes that may be stimulated or inhibited by different compounds (Hepper, 1979). Carbon dioxide and ethylene are considered germination "modulators", stimulating or

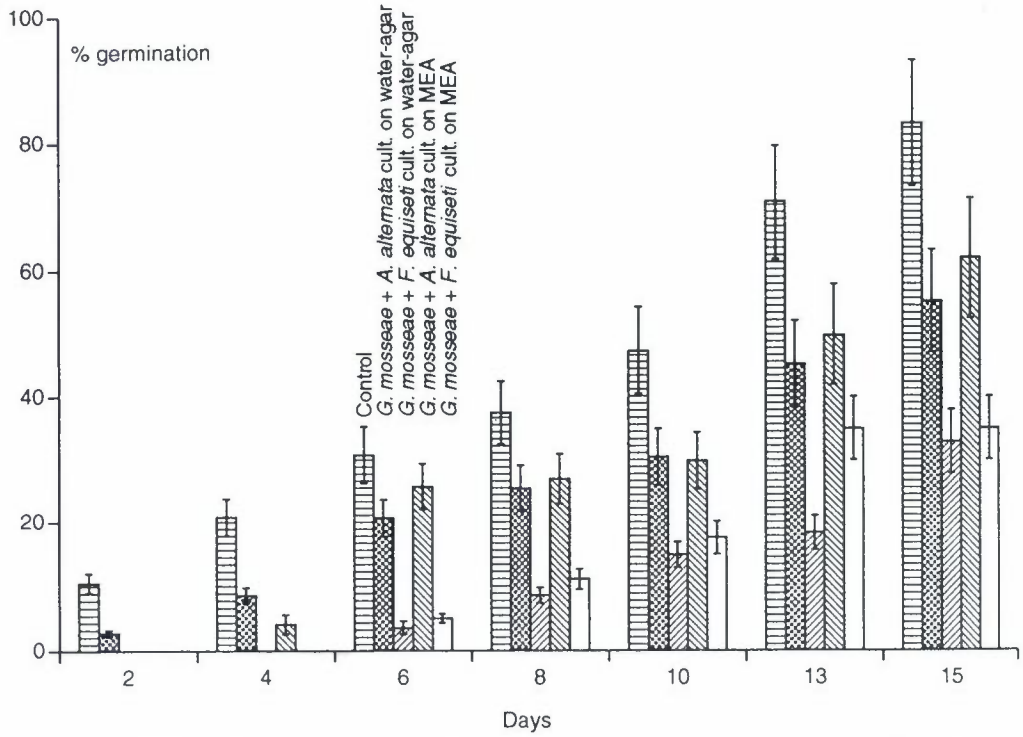

Figure 3. Effect of volatile compounds produced by *Alternaria alternata* and *Fusarium equiseti* on the percentage of germination of *Glomus mosseae* cultivated on water agar and on MEA. Standard errors of the means are given.

inhibiting germination and hyphal growth depending on their concentrations (Becard and Piche, 1989; Le Tacon et al., 1983; Vidal-Dominguez, 1991). The different results observed in divided plates when the saprophytes grew in rich or poor culture media suggest that stimulation or inhibition of hyphal growth of *G. mosseae* may have been due to the effect of substances with a similar "modulator" role.

The inhibition by *A. alternata* and *F. equiseti* of development of *G. mosseae* in roots of *Zea mays* when both fungi were inoculated at the same time suggest that there was a direct interaction between the mycorrhizal and the saprophytic fungi in the extramatrical phase of the former. Similar interactions have been proposed for other saprophytic fungi (McAllister et al., 1994) and other microorganisms (Caron et al., 1985; Garcia-Garrido and Ocampo, 1988). However, when the AM fungus was established in the root before in-

Table 2. Percentage of VAM root length and colony-forming units (CFU) of *A. alternata* and *F. equiseti* from the rhizosphere (per g dry weight of sand:vermiculite) of maize (*Z. mays*) plants grown under axenic conditions in tubes, inoculated or not with *G. mosseae* at different times.

Inoculation time	Treatment	Root length colonization %	CFU $\times 10\ g^{-1}$ soil after (weeks) 0	10
Saprophytic fungi inoculated at the same time as *G. mosseae*				
	Aa	0	88.2 a	3.1 a
	Fe	0	86.5 a	20.8 a
	M	64.3 a		
	M+Aa	14.1 b	88.2 a	6.2 a
	M+Fe	38.4 b	86.5 a	31.6 a
Saprophytic fungi inoculated two weeks after *G. mosseae*				
	Aa	0	87.4 a	5.2 a
	Fe	0	65.7 a	26.5 a
	M	56.4 a		
	M+Aa	46.1 a	87.4 a	0 b
	M+Fe	51.6 a	62.7 a	28.6 a

Aa = Plants inoculated with *A. alternata*; Fe = Plants inoculated with *F. equiseti*; M = Plants inoculated with *G. mosseae*. Each value is the mean for five pots. Within each saprophytic fungus and time of inoculation, column values followed by the same letter are not significantly different according to orthogonal contrast comparisons ($p = 0.05$).

oculation of the saprophyte, not only was it resistant to the negative effect of *A. alternata* and *F. equiseti*, but it also had the capacity to decrease the number of CFU of *A. alternata*, probably through its effect on plant physiology. Our *in vitro* assays suggest that these saprophytic fungi affect AM colonization partly because they inhibit the germination of *G. mosseae* chlamydospores. Although hyphal length in the AM endophytes was not affected by *A. alternata*, and was stimulated by *F. equiseti*, this was not reflected in the percentage of AM root colonization. It is known that during their presymbiotic phase, while growing from the propagule toward the root, mycorrhizal fungi can be influenced positively by some rhizosphere microorganisms, although this influence is not reflected in the percentage of AM root colonization (Fitter and

Garbaye, 1994). Hyphal development may have been too low to be observed, or may have occurred only in the first stages of hyphal development.

Further studies with *A. alternata, F. equiseti* and *G. mosseae,* in soil under greenhouse conditions, are needed to more fully elucidate the interactions between these microorganisms.

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