



IN VITRO INTERACTIONS BETWEEN *TRICHODERMA KONINGII*, *FUSARIUM SOLANI* AND *GLOMUS MOSSEAE*

C. B. McALLISTER,¹ I. GARCÍA-ROMERA,² A. GODEAS¹ and J. A. OCAMPO^{2*}

¹Departamento de Ciencias Biológicas, 4° II Pabellón, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina

²Departamento de Microbiología, Estación Experimental del Zaidín, C.S.I.C., Profesor Albareda 1, E-18008 Granada, Spain

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Summary—The inoculation of maize plants with spores of *G. mosseae* decreased the populations of the saprophytic fungi *Trichoderma koningii* and *Fusarium solani*. However, *F. solani* had no effect on mycorrhizal development, whereas *G. mosseae* was inhibited in its extramatrical stage by *T. koningii*.

Germinated spores of *G. mosseae* did not affect growth of the saprophytic fungi on water-agar. *T. koningii* inhibited the germination, but not mycelial development of *G. mosseae*; this inhibition was independent of the change in pH of the medium. *F. solani* did not inhibit germination of *G. mosseae* spores, and endophyte hyphal development was markedly stimulated. The influence of soluble and volatile substances produced by the saprophytic fungi on these effects is discussed.

INTRODUCTION

Studies of *T. koningii* and *F. solani* showed that the colonization of plant root with arbuscular mycorrhizal (AM) fungi may decrease or remain unaffected, depending on the time of inoculation of one microorganism with respect to the other (McAllister *et al.*, 1994). Because AM fungi live partly within the host root, and obtain energy from the plant, AM fungi have a competitive advantage over other rhizosphere microorganisms (Read *et al.*, 1985). Thus, saprophytic microorganisms were unable to affect the amount of mycorrhizal colonization of the host root when the inoculated arbuscular endophyte had previously colonized the host plant. However, when the saprophytic microorganisms were inoculated onto maize and lettuce plants after *G. mosseae* the proportional amount of AM colonization of roots decreased considerably (McAllister *et al.*, 1994). The inhibition of root mycorrhizal colonization by saprophytic fungi may be due not only to a direct interaction between the two fungi, but also to induction of plant defense mechanisms (Wyss *et al.*, 1992).

Our aim was to study the interaction between the saprophytic fungi *T. koningii* and *F. solani* and the AM fungus *G. mosseae*, and its effect on spore germination and mycelial development.

MATERIALS AND METHODS

Interaction between saprophytic fungi and G. mosseae in the rhizosphere of maize

This experiment was done in 20 × 200 mm glass tubes filled with 25 g of a sand-vermiculite mixture (1:1, V/V) and 12 ml of half-strength Hewitt's nutrient solution plus 50 µg K₂HPO₄ ml⁻¹ (pH 7), plugged with cotton wool and autoclaved at 120°C for 20 min. Sporocarps of *G. mosseae* were obtained from rhizosphere soil in pot culture containing a high inoculum density by wet sieving (250 µm) soil and decanting. Resting spores freshly isolated from these sporocarps by excision were placed on damp filter paper in a glass Petri dish and stored at 4°C for 4 days before sterilization. The spores were surface-sterilized as described by Mosse (1962). In each tube, 30 surface-sterilized spores of *G. mosseae* were placed under the seedlings. Seeds of maize (*Zea mays* cv. Calderon) were surface-sterilized with HgCl₂ 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 UE m⁻² s⁻¹, 400–700 nm, with a 16–8 h day–night cycle at 25–19°C and 50% r.h.

The saprophytic fungi *T. koningii* and *F. solani* were obtained from the rhizosphere and rhizoplane of maize plants (McAllister *et al.*, 1994). An aqueous

*Author for correspondence.

suspension in sterile distilled water containing $ca 2 \times 10^3$ spores ml^{-1} was prepared from cultures grown in potato dextrose agar (PDA) for 1 week at 27°C.

Four treatments were used in all experiments: (1) uninoculated controls; (2) inoculated with *T. koningii* or *F. solani*; (3) inoculated with *G. mosseae*; and (4) inoculated with both *G. mosseae* and either *T. koningii* or *F. solani*. Plants were inoculated at the time of transplanting or after 2 weeks of growth. The saprophytic fungi were inoculated at the same time as and 2 weeks after *G. mosseae*.

To evaluate the population of inoculated *T. koningii* or *F. solani* during the experiments, the sand-vermiculite mixture from five replicate tubes was collected 5 days and also at 10 weeks after inoculation of saprophytic fungi, and saprophytic colony-forming units (CFUs) were counted (McAllister *et al.*, 1994). Ten replicate tubes per treatment were used. Plants were harvested after 10 weeks and shoot dry matter wt was determined. The root system was harvested in each of the five replicates per treatment, then cleared and stained (Phillips and Hayman, 1970), and percentage root colonization was measured (Ocampo *et al.*, 1980).

Effect of G. mosseae on saprophytic fungi

The effect of *G. mosseae* on the germination of each saprophytic fungus *in vitro* was tested on sterile water-agar 1%, pH 7. Twenty-five surface-sterilized spores per plate were placed around the perimeter of the Petri dish and a thin streak with spores and mycelium of the saprophytic fungi to be assayed was inoculated in the center. Ten replicates were used and plates with the saprophytic fungus alone were used as controls. The plates were sealed to reduce dehydration and contamination risks, and were kept in the dark at 25°C. Periodically, the diameter of the colony of the saprophyte was examined for 15 days.

Effect of saprophytic fungi on G. mosseae spores

The effect of each saprophytic fungus on spore germination and hyphal growth of *G. mosseae* was tested in four different experiments using 9-cm dia plastic Petri dishes.

In the first expt the effect of each saprophytic fungus on spore germination and mycelial growth *in vitro* was tested on sterile water-agar 1%, pH 7. Five surface-sterilized spores per plate were placed near (1 cm) the edge of a Petri dish, and at least 7 cm away from spores, a thin streak with spores and mycelium of the saprophytic fungus to be assayed was inoculated.

A second expt was carried out to check whether the effect of saprophytic fungi on *G. mosseae* spore germination and mycelium development was an indirect effect due to pH modification caused by the saprophytic fungus, 10 mM 2-(*N*-morpholin) ethane sulfonic acid (MES) was added to water-agar 1%. Thus, the pH of the medium was 7 and remained at this value at the end of the experiment after its

measurement on the surface of the medium. This buffer did not affect *in vitro* germination of AM fungus spores (Carr and Hinkley, 1985; Calvet *et al.*, 1992). *G. mosseae* spores and the saprophytic fungus inoculum were placed on water-agar + MES, as described above.

The production by the saprophytic fungi of any water-soluble compounds able to affect the germination and mycelial development of AM fungus *in vitro* was tested in a third expt. Exudates of each saprophytic fungus were obtained by growing them in 250 ml flasks containing 125 ml of sterile PDA liquid medium in a shaker at 28°C. After 72 h the culture medium was filtered through a disk of filter paper and sterilized twice through a 0.45 µm Millipore membrane. These exudates (2 ml) were added to 10 ml water-agar 1% (pH 7) in a Petri dish. Five spores of *G. mosseae* were placed at the vertices of an imaginary pentagon in each Petri dish. In the control, the same volume of sterile distilled water was substituted for the exudates.

In the fourth expt the effect of volatile compounds released by saprophytic fungi on germination and hyphal growth of *G. mosseae* spores was tested using divided plastic Petri dishes. In an initial assay we used water-agar 1% adjusted to pH 7 in both halves of the Petri dish. On one side, five AM fungus spores were placed near one edge of the plate, and the saprophytic fungus was inoculated on the other side of the plate. In a second assay we used water-agar 1% (pH 7) on one side of the plate and malt extract agar (MEA) in the other. Five AM spores were placed on the water-agar, and the saprophytic fungus was inoculated on the nutrient agar.

In the four expts, 10 replicates of each treatment were used, and 10 plates were used as controls, with *G. mosseae* spores growing axenically on each culture medium. The plates were sealed to reduce dehydration and contamination risks, and were kept in the dark at 25°C. Spore germination rate was periodically determined using a light microscope until the mycelium of *G. mosseae* contacted the mycelium of the saprophytic fungus (expts 1, 2 and 3), or for 20 days (expt 4). The experiments were then ended and hyphal growth of germinated spores of *G. mosseae* was assessed using the gridline intersect method (Marsh, 1971).

Effect of mycorrhizal and non-mycorrhizal root extracts on development of saprophytic fungi

Maize and lettuce plants were grown in pots with steam-sterilized soil mixed with sterilized quartz sand and with *G. mosseae* as AM inoculum (McAllister *et al.*, 1994). After 10 weeks of growth root extracts of mycorrhizal and non-mycorrhizal maize and lettuce plants were obtained with two different procedures:

(1) 2 g of root were ground in 3 ml Tris-HCl buffer (0.1 M, pH 7) in a mortar, filtered and centrifuged at 20,000 g for 10 min.

(2) 1 g of root was ground in 25 ml 70% methanol with a mortar, and centrifuged at 10,000 g for 10 min. The supernatant was dried in a vacuum in a rotary evaporator at 40°C and redissolved in 2 ml 100% methanol. After evaporation to dryness, the extract was again redissolved in 2 ml 100% methanol.

Each saprophytic fungus was grown in a Petri dish containing 20 ml of one-tenth strength PDA medium. When the saprophytic colony was about 3 cm dia, five 5-mm-dia pits were cut in the PDA medium around the colony, about 5 mm away from its edge. Root extract (40 µl), Tris-HCl buffer or methanol (as a control) was poured into the pits.

Plates were kept for 48 h at 28°C, and the appearance of halos of inhibition of the saprophytic colony around the pits was observed.

Five replicates were prepared for each extract.

Statistical treatments

Percentage AM root length and CFUs of saprophytic fungus were analyzed by the Duncan multiple range test ($P = 0.05$). Spore germination data were analyzed by a normal distribution test (5% significance), and hyphal length was compared with Student's *t*-test for a significance of 5%.

RESULTS

The dry wt of maize plants were similar in all treatments tested (data not shown). Inoculation of *T. koningii* at the same time as *G. mosseae* significantly decreased the percentage of the root length of maize plants colonized by AM, but no effect on AM colonization was observed when *T. koningii* was inoculated 2 weeks after *G. mosseae* (Table 1). The inoculation of *F. solani* did not affect the percentage of the root length of maize plants colonized by AM in any of the treatments tested (Table 1).

Table 1. Percentage of root length colonized by AM and colony-forming units (CFU) of *T. koningii* and *F. solani* from the rhizosphere (g^{-1} dry wt 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 $\times 10^6 g^{-1}$ soil after (weeks)	
			0	10
Saprophytic fungi inoculated at the same time as <i>G. mosseae</i>	Tk.		110 ^a	41.5 ^a
	Fs.		66.2 ^a	23.6 ^a
	M	64.3 ^a		
	M + Tk.	7.5 ^b	110 ^a	32.8 ^a
Saprophytic fungi inoculated 2 weeks after <i>G. mosseae</i>	M + Fs.	62.9 ^a	66.2 ^a	29.2 ^a
	Tk.		98.8 ^a	29.4 ^b
	Fs.		62.7 ^a	18.5 ^b
	M	56.4 ^a		
	M + Tk.	45.8 ^a	98.8 ^a	8.7 ^c
	M + Fs.	55.7 ^a	62.7 ^a	9.8 ^c

Tk = Plants inoculated with *T. koningii*; Fs = PLANTS inoculated with *F. solani*. M = Plants inoculated with *G. mosseae*. Each value is the mean for five pots. Within each saprophytic fungus column, values followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

As Table 1 shows, the population of *T. koningii* and *F. solani* decreased when they were inoculated 2 weeks after *G. mosseae*, but they were not affected when inoculated at the same time as *G. mosseae*.

Germinated spores of *G. mosseae* did not affect the growth of saprophytic fungi cultivated on water-agar (data not shown).

The percentage germination of *G. mosseae* spores cultivated in water-agar [Fig. 1(a)] decreased significantly in the presence of *T. koningii* after 13 days of incubation, but no effect on the hyphal length of *G. mosseae*, measured as number of intersections (Marsh, 1971), was observed [Fig. 1(b)]. After 15 days of incubation the mycelia of the saprophytic fungi were intermingled with those of *G. mosseae*; however, no hyperparasitism of the *T. koningii* hyphae on *G. mosseae* hyphae was observed. *F. solani* did not affect the percentage spore germination of *G. mosseae*, but in fact significantly increased hyphal length and vegetative spore formation after 10 and 13 days of incubation, respectively. Similar effects of the saprophytic fungi on the percentage of germination of spores and on hyphal length of *G. mosseae* when grown on water-agar buffered with MES was observed. Identical results were obtained when *G. mosseae* was grown in the presence of exudates of the saprophytic fungi.

Volatile compounds produced by *T. koningii* significantly decreased percentage of spore germination of *G. mosseae* after 20 days of incubation. However, the volatile compounds produced by *F. solani* did not affect percentage germination of the AM spores. Volatile compounds from *T. koningii* did not affect hyphal length in *G. mosseae*, but those from *F. solani* significantly increased the length of the mycelium of the AM endophyte. Volatile compounds from *T. koningii* and *F. solani* shortened the time of production of vegetative spores, which appeared after 10 days. When the growth medium used was MEA [Figs 2(a) and (b)] the volatile compounds produced by both of the saprophytic fungi inhibited spore germination, length of mycelia and the formation of vegetative spores in *G. mosseae*.

Under our experimental conditions the extracts of mycorrhizal roots had no clear inhibitory effect on saprophytic fungus development *in vitro*.

DISCUSSION

Our results show that *G. mosseae* decreases the saprophytic fungal population through its effect on the plant. However, *F. solani* had no effect on mycorrhizal development in any of the treatments tested, whereas *T. koningii* inhibited *G. mosseae* in its extramatrical stage. These results are similar to those observed by McAllister *et al.* (1994) in which the inoculum consisted of spores, mycelia and pieces of colonized root, suggesting that the effect of the interaction between the two microorganisms does not

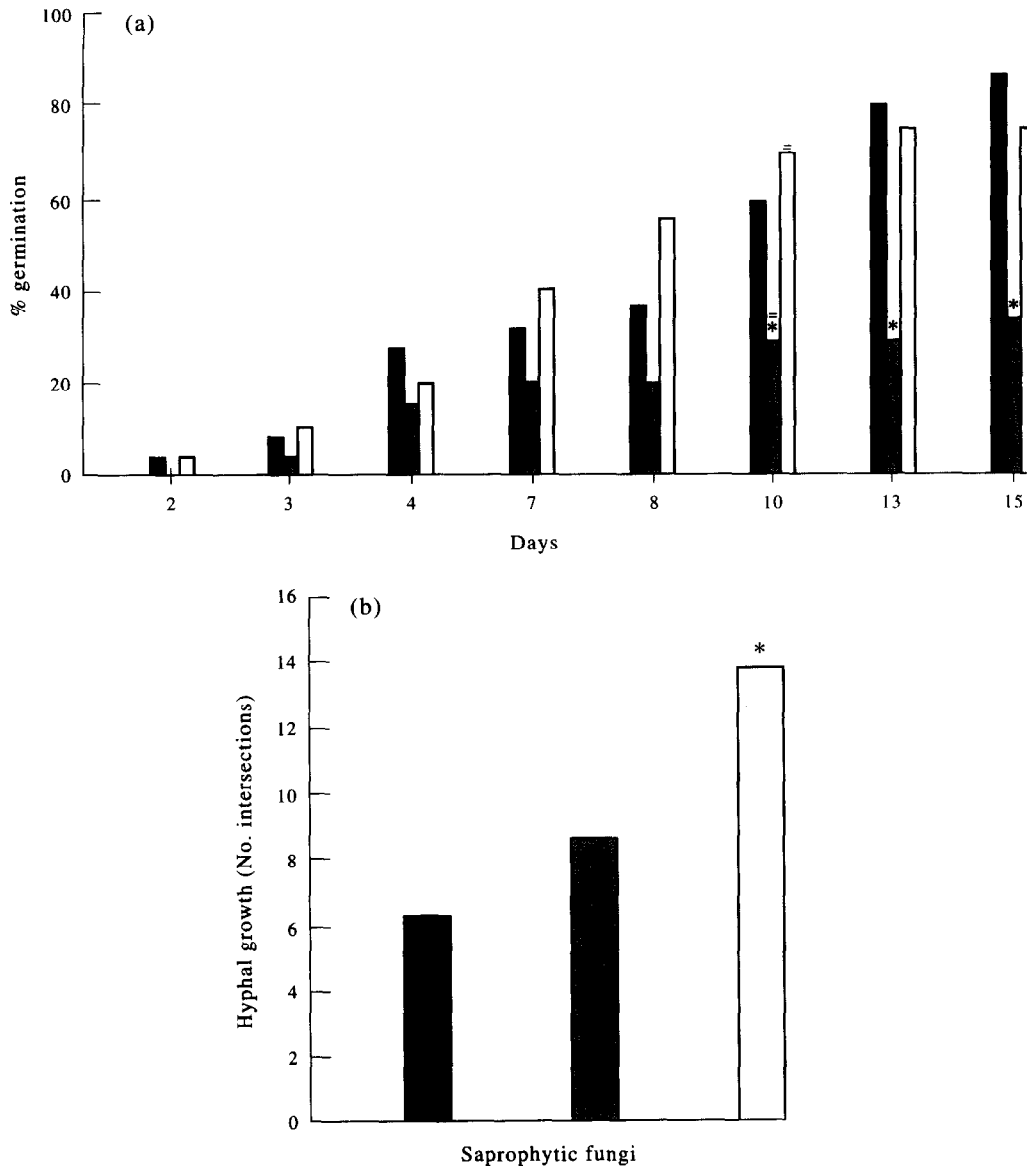


Fig. 1. Effect of *T. koningii* and *F. solani* on percentage germination (a) and hyphal length and production of vegetative spores (b) of *G. mosseae* cultivated in water-agar. (=) = Time of appearance of vegetative spores. (*) = Significantly different from control (see Materials and Methods). (■) Control; (▨) *G. mosseae* plus *T. koningii*; (□) *G. mosseae* plus *F. solani*.

depend on the potential or kind of *G. mosseae* inoculum used.

A direct effect of *T. koningii* on the germination or presymbiotic development of *G. mosseae* was observed in *in vitro* experiments in which the saprophytic fungus was paired with spores of *G. mosseae*. Inhibition due to a change in pH of the medium as a consequence of the growth and metabolic activity of saprophytic fungi can be ruled out as the results obtained with MES-buffered water-agar were similar to those observed in water-agar. It is reasonable to assume that any soluble or volatile substance produced by the saprophyte is able to inhibit the germination of *G. mosseae* spores, but not hyphal growth

from these spores. Germination and hyphal growth are two separate processes that may be stimulated or inhibited by different compounds (Hepper, 1979). Although mycoparasitism has often been described as the mechanism of antagonistic action of *Trichoderma* species (Chu and Wu, 1981; Camporota, 1985), this was not the mechanism involved in this case. Coiling of *Trichoderma* hypha around the hypha of *G. mosseae* was not observed in the plate assays, even when the plates were incubated after the two fungi made contact. Although mycoparasitism phenomena were not observed, our results are not surprising, as they were not observed in interactions between *G. mosseae* and *T. aureoviride* (Calvet *et al.*, 1993).

However, Calvet *et al.* reported stimulation by this saprophyte of *in vitro* germination and hyphal growth of *G. mosseae*. Their result contrasts with the one we observed with *T. koningii*, confirming the idea that the effects of the interaction may be completely different, even considering different species of the same genus. Some *Trichoderma* species produce water-soluble or volatile antibiotics (Cutler and Lefiles, 1978) that may have a fungistatic effect on other fungal species (Pavlica *et al.*, 1978; Chu and Wu, 1981). Particularly, *T. koningii* is known to produce peptide antibiotics (Dennis and Webster, 1971).

Nevertheless, the effect of *T. koningii* on the germination of *G. mosseae* spores is likely to be a consequence of the non-additive action of the volatile and

soluble compounds produced, as the percentages of spore germination observed were similar in divided plates and in water-agar plates to which fungal exudates of *T. koningii* were added.

In vitro assays with *F. solani* show that this saprophytic fungus does not affect AM colonization, partly because it does not inhibit the germination of *G. mosseae* chlamydo spores. Although endophyte hyphal development was strongly stimulated, this was not reflected in the percentage of AM root length colonized. Hyphal development may have been too low to be observed, or stimulation may have occurred only in the first stages of hyphal development. Soluble and volatile substances seem to be responsible for this effect.

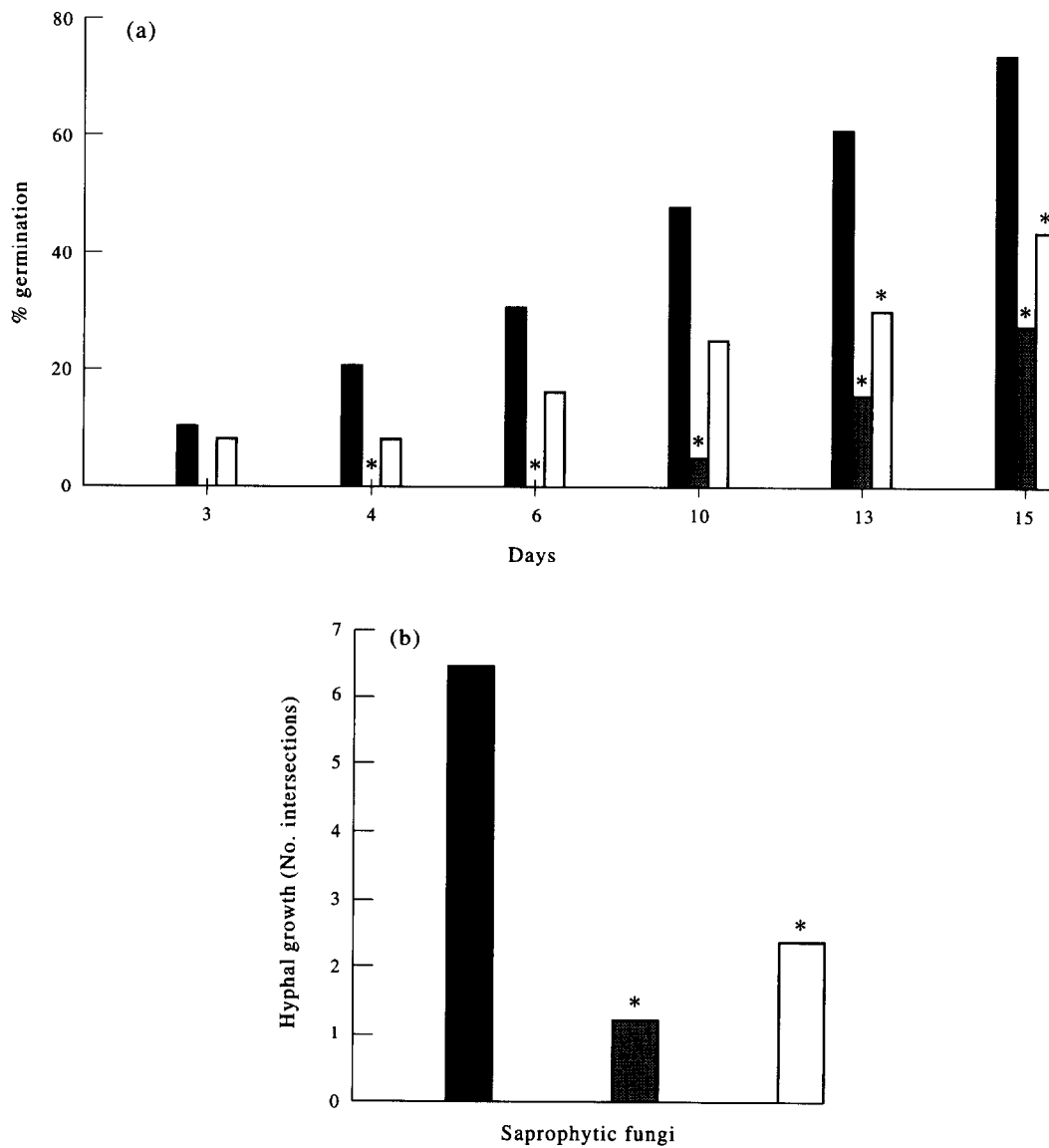


Fig. 2. Effect of volatile compounds produced by *T. koningii* and *F. solani*, cultivated in MEA, on percentage germination (a) and hyphal length and production of vegetative spores (b) of *G. mosseae* cultivated in water-agar. (=) = Time of appearance of vegetative spores. (*) = Significantly different from control (see Materials and Methods). (■) Control; (▨) *G. mosseae* plus *T. koningii*; (□) *G. mosseae* plus *F. solani*.

A few studies mention the positive effect of some soil fungi on the germination and saprophytic growth of *G. mosseae* spores in axenic culture (Azcon-Aguilar *et al.*, 1986; Calvet *et al.*, 1992). This stimulation has usually been explained as an effect of a combination of moderate concentrations of CO₂ and fungal exudates.

Carbon dioxide is considered a germination "modulator", stimulating or inhibiting germination depending on its concentration. The different results observed in divided plates, when the saprophyte grew in rich or poor culture media, suggest that the stimulation of hyphal growth of *G. mosseae* is due to the effect of this modulator.

The absence of a clear effect of extracts of mycorrhizal plant roots on the development of saprophytic fungi may be due to the extraction procedure used in our experiments. Further studies will be designed with different methods of plant root extraction to investigate these effects.

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