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Interactions between *Trichoderma pseudokoningii* strains and the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora rosea*

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Abstract The interaction between *Trichoderma pseudokoningii* (Rifai) 511, 2212, 741A, 741B and 453 and the arbuscular mycorrhizal fungi *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe BEG12 and *Gigaspora rosea* Nicolson & Schenck BEG9 were studied in vitro and in greenhouse experiments. All *T. pseudokoningii* strains inhibited the germination of *G. mosseae* and *Gi. rosea* except the strain 453, which did not affect the germination of *Gi. rosea*. Soluble exudates and volatile substances produced by all *T. pseudokoningii* strains inhibited the spore germination of *G. mosseae*. The germination of *Gi. rosea* spores was inhibited by the soluble exudates produced by *T. pseudokoningii* 2212 and 511, whereas *T. pseudokoningii* 714A and 714B inhibited the germination of *Gi. rosea* spores by the production of volatile substances. The strains of *T. pseudokoningii* did not affect dry matter and percentage of root length colonization of soybean inoculated with *G. mosseae*, except *T. pseudokoningii* 2212, which inhibited both parameters. However, all *T. pseudokoningii* strains decreased the shoot dry matter and the percentage of AM root length colonization of soybean inoculated with *Gi. rosea*. The saprotrophic fungi tested seem to affect AM colonization of root by effects on the presymbiotic phase of the AM fungi. No influence of AM fungi on the number of CFUs of *T. pseudokoningii* was found. The effect of saprotrophic fungi on AM fungal development and function varied with the strain of the saprotrophic species tested.

Keywords Arbuscular mycorrhiza · *Glomus mosseae* · *Gigaspora rosea* · *Glycine max* · Saprotrophic fungi

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Introduction

The beneficial effect of arbuscular mycorrhizal (AM) fungi on plant growth has been show repeatedly by many researchers. As AM fungi are partially outside of the host, external factors such as soil rhizosphere microorganisms affect the development and function of the symbiosis (Gryndler 2000). Saprotrophic fungi are important and common components of the soil rhizosphere (Dix and Webster 1995). They are important because of the large amount of microbial biomass they supply to soil. Hyphae extend out into the mineral soil producing very fine mycelial networks that facilitate substrate collection (Wainwright 1992). Species of *Trichoderma* are found in all soils, including the forest humus layer as well as agricultural and orchard soils (Samuels 1996). They are involved in complex interactions such as antibiosis (Cook and Baker 1983), fungistasis (Pavlica et al. 1978) and mycoparasitism (Elad 1986). Several experimental results indicate interactions between AM and saprotrophic fungi in the soil rhizosphere and in plant root colonization (Gryndler 2000). Saprotrophic fungi mainly influence AM fungi when the latter are in the presymbiotic phase of the symbiosis development (McAllister et al. 1994, 1995; Fracchia et al. 1998; Garcia-Romera et al. 1998). In fact, the presence of saprotrophic fungi affects both positively and negatively spore germination of AM fungi. Volatile and soluble exudates produced by saprotrophic fungi are involved in these effects (McAllister et al. 1994, 1995; Fracchia et al. 1998). However, results from pots experiments also indicate that saprotrophic fungi can affect AM development and function when AM fungi are

inside the root (McAllister et al. 1994, 1995). Nevertheless, results of research on the interactions between soil saprophytic and AM fungi differ widely, even when the same species of saprotrophic fungi are involved. For example, *Trichoderma harzianum* have been found to have antagonistic, neutral and stimulating effects on AM (Rouseau et al. 1996; Siddiqui and Mohmood 1996; Fracchia et al. 1998; Godeas et al. 1999; Green et al. 1999). On the other hand, adverse, neutral and positive effects of AM fungi on the population density of *Trichoderma* have also been observed (Fracchia et al. 1998; Green et al. 1999; Godeas et al. 1999). Thus variation in the interaction between AM and saprotrophic fungi may be due to the different strains of saprotrophic fungi species.

The present work was carried out to test the effects of several *T. pseudokoningii* strains on spore germination of the AM fungi *Glomus mosseae* and *Gigaspora rosea* and on the root colonization of soybean by these fungi, as well as the effect of AM colonized plants on the number of CFU of *Trichoderma* in soil.

Material and methods

Experiment 1: isolation of saprotrophic fungi

The active strains of *T. pseudokoningii* Rifai, anamorph of *Hypocrea pseudokoningii* Samuels & Petrini (Samuels et al. 1998), present in maize rhizosphere soil and roots from Pergamino, Buenos Aires province were isolated by the particle washing method (Widden and Bisset 1973) using a multichamber washing apparatus. Thirty washings were necessary to remove fungal sclerotia, spores, etc, from the soil particles and roots of maize. Twenty soil particles (2 mm diameter) were dried on sterilized filter paper and planted in 2% malt extract agar with antibiotics (5 μg l^{-1} streptomycin and 10 μg l^{-1} tetracycline). From the resulting colonies, strains 511, 2212, 741A, 741B and 453 of *T. pseudokoningii* were selected and transferred to tubes of potato dextrose agar (PDA) and 2% malt extract at 4°C as stock cultures. Strains are kept at the fungal culture collection of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.

Experiment 2: effect of T. pseudokoningii on spore germination of G. mosseae and Gi. rosea

The effect of *T. pseudokoningii* on spore germination of *G. mosseae* (BEG12) and *Gi. rosea* (BEG9) was tested in three different experiments conducted in 9-cm-diameter plastic Petri dishes. In the first experiment, the effect of *T. pseudokoningii* on spores in vitro was tested on 1% sterile water agar pH 7. Spores of *Gi. rosea* and sporocarps of *G. mosseae* were isolated by wet sieving soil from clover pot cultures (Gerdemann 1955), stored in water at 4°C and used within 1 month. Spores of *G. mosseae* were obtained by dissecting sporocarps. All spores were surface sterilized as described by Mosse (1962). Five surface-sterilized spores of AM fungi per plate were placed near (1 cm) the edge of a Petri dish; a thin streak of *T. pseudokoningii* was inoculated opposite and at least 7 cm distant.

The second experiment tested the effect of exudates from *T. pseudokoningii* on germination of AM fungus in vitro. Exudates were obtained by growing the fungus 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 by filtration through a 0.45-μm Millipore membrane. Aliquots (2 ml) of these exudates were added to 10 ml of 1% water agar pH 7 in a Petri dish. Five spores of *G. mosseae* or *Gi. rosea* were placed at the vertices of an imaginary pentagon inside the dish. In the control treatment, the same volume of sterile PDA liquid medium was substituted for the exudates.

In the third experiment, the effect of volatile compounds released by *T. pseudokoningii* on germination of *G. mosseae* and *Gi. rosea* spores was tested in divided plastic Petri dishes. In an initial assay, the dishes contained 1% water agar (pH 7) on both sides. In one section, five AM fungus spores were placed near the edge of the plate and *T. pseudokoningii* was inoculated in the other half. In a second assay, the plates contained 1% water agar (pH 7) in one section and asparagine medium (Galvagno 1976) in the other. The standard asparagine medium (GA) consisted of $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; glucose, 1 g; asparagine, 4 g; distilled water to 1 l. Five AM spores were placed on the water agar and the saprotrophic fungus was inoculated onto the nutrient agar.

In the three experiments, 10 replications of each treatment were prepared with germinating *G. mosseae* or *Gi. rosea* spores on each culture medium. The plates were incubated at 25°C in the dark and were sealed to reduce dehydration and contamination. Spore germination rate was periodically examined under a light microscope for 12 days.

Interaction between AM and saprotrophic fungi with soybean

Plants were grown in 250-ml-capacity open pots filled with soil collected from Pergamino in the Province of Buenos Aires. This soil was of the Argiudol type with a pH of 7.1, and was steam sterilized and mixed with sterilized perlite in the proportions 4:1 (v/v). Seeds of soybean (*Glycine max* cv. Nidera) were surface sterilized with $HgCl_2$ for 10 min, thoroughly rinsed with sterilized water and sown in moistened sand. After germination, uniform seedlings were planted and grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 E m^{-2} s^{-1}, 400–700 nm, with a 16/8 h day/night cycle at 25/19°C and 50% relative humidity. Plants were watered from below and fed with a nutrient solution (Hewitt 1952).

The AM inoculum consisted of 5 g of rhizosphere soil from an alfalfa pot culture of *G. mosseae* and *Gi. rosea* containing spores, mycelium and colonized root fragments. The presence of *Trichoderma* was checked by the procedure described below for evaluation of saprotrophic populations in soil pot. Soil filtrate (Whatman No. 1 filter paper) from the rhizosphere of mycorrhizal plants inoculated with *G. mosseae* or with *Gi. rosea* was added to the AM noninoculated treatment. The filtrate contained common soil microorganisms but no propagules of AM fungi.

An aqueous suspension in sterile distilled water of each saprotrophic fungal strain was prepared from cultures grown in PDA for 1 week at 27°C. The number of spores in the suspension was determined using a Neubauer chamber and the suspension diluted to give approximately 10^8 spores ml^{-1}. Each saprotrophic fungal strain was inoculated to pots in 2.5 ml of this suspension.

Four treatments were used: (1) noninoculated controls, (2) inoculated with *T. pseudokoningii* (3) inoculated with *G. mosseae* or *Gi. rosea*, and (4) inoculated with *T. pseudokoningii* and either *G. mosseae* or *Gi. rosea*. Plants were inoculated with the mycorrhizal fungus at the time of transplanting and the saprophytic fungus 2 weeks later. The pot experiment was designed as a 3×6 factorial with *G. mosseae* and *Gi. rosea* inoculation and saprotrophic fungi inoculation as factors. The experiments were repeated three times; each data set is the average of five replicate pots from one representative experiment.

To evaluate the population of inoculated saprotrophic fungi, rhizosphere soils were sampled after 1, 15, 30 and 45 days, as described by McAllister et al. (1994). Sampled soil was replaced by autoclaved soil. About 5 g of rhizosphere soil was taken from each of the experimental pots and 10-fold aqueous dilution series (from 10^{-1} to 10^{-4}) prepared. The numbers of saprotrophic CFUs in 10^{-3} dilutions of such samples, taken from the five replicate pots of each treatment, were counted on malt extract agar medium. Rhizosphere soil was quantified by recovering soil from dilutions of 10^{-1} and 10^{-2},

drying at 105°C and weighing. The number of CFUs was expressed per g of dry rhizosphere soil.

Plants were harvested after 8 weeks and dry matter weight determined. Part of the root system was cleared and stained (Phillips and Hayman 1970), and the percentage of root colonization measured by the grid line intersect method (Giovannetti and Mosse 1980).

The percentage values were arcsine transformed for statistical analysis. The data obtained for percentage of spore germination, hyphal length, plant dry weight, percentage of AM colonization and number of CFU of saprotrophic fungi were subjected to ANOVA. Comparisons of means were made by the Duncan's multiple range test (P <0.05) and by LSD (Tukey multiple range test, P <0.05).

Results

The percentage germination of *G. mosseae* spores decreased significantly in presence of all *T. pseudokoningii* strains tested (Fig. 1a). Strains 2212, 741A, 741B and 511 decreased the percentage germination of *Gi. rosea* spores, but no effect of *T. pseudokoningii* 453 on spore germination was observed (Fig. 1b). The percentage germination of *G. mosseae* spores decreased significantly in presence of exudates from all *T. pseudokoningii* strains tested (Fig. 2a). However, soluble exudates of strains 2212 and 511 inhibited spore germination of *Gi. rosea* (Fig. 2b). Volatile compounds produced by *T. pseudokoningii* strains did not affect the percentage germination of *G. mosseae* or *Gi. rosea* spores. However, when the GA growth medium used the volatile compounds produced by the saprotrophic fungi inhibited spore germination of *G. mosseae* (Fig. 3a). In contrast, volatile compounds produced by strains 741A and 741B grown in GA medium inhibited spore germination of *Gi. rosea* (Fig. 3b).

None of the *T. pseudokoningii* strains affected shoot or root dry weights of noncolonized plants (Table 1 and Table 2). The shoot dry weight and percentage AM root length of soybean colonized with *G. mosseae* was decreased by the presence of *T. pseudokoningii* 2212 (Table 1) but not strains 511, 741A, 741B or 453. The shoot and root dry weights and the percentage of AM root length of soybean colonized by *Gi. rosea* was decreased by the presence of all *T. pseudokoningii* strains tested (Table 2).

The population of the different *T. pseudokoningii* strains in the rhizosphere of soybean decreased during the assay (result not shown), but the number of CFUs was not affected by the presence of *G. mosseae* (Table 1) or *Gi.*

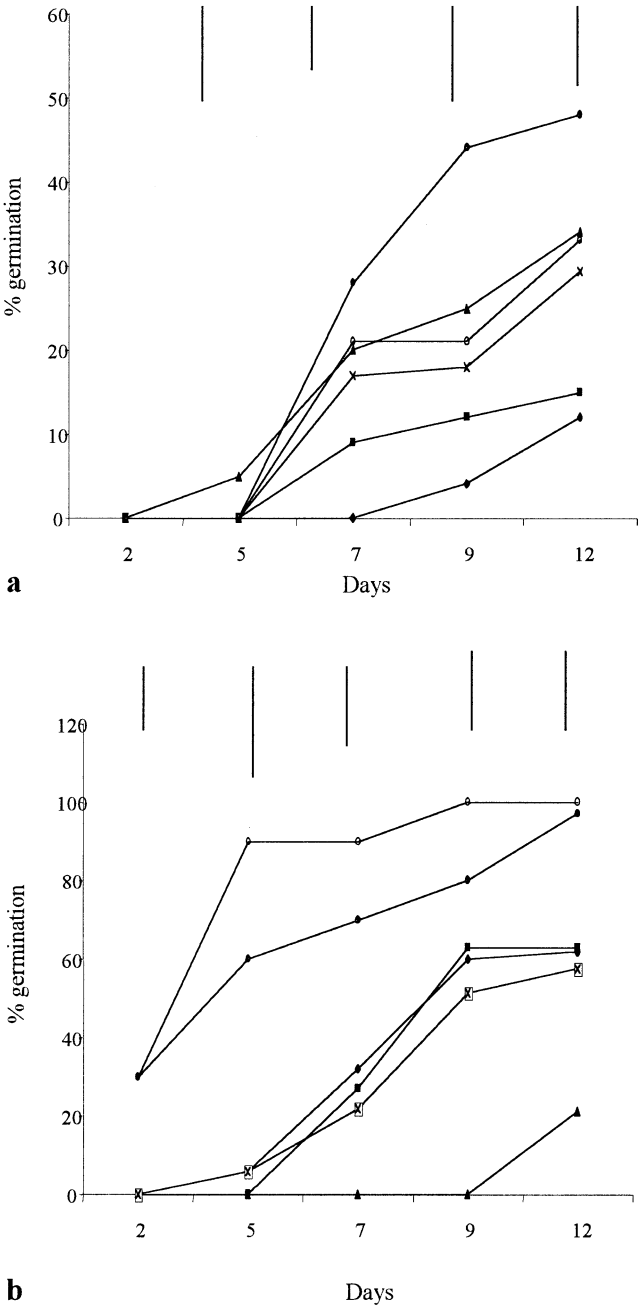
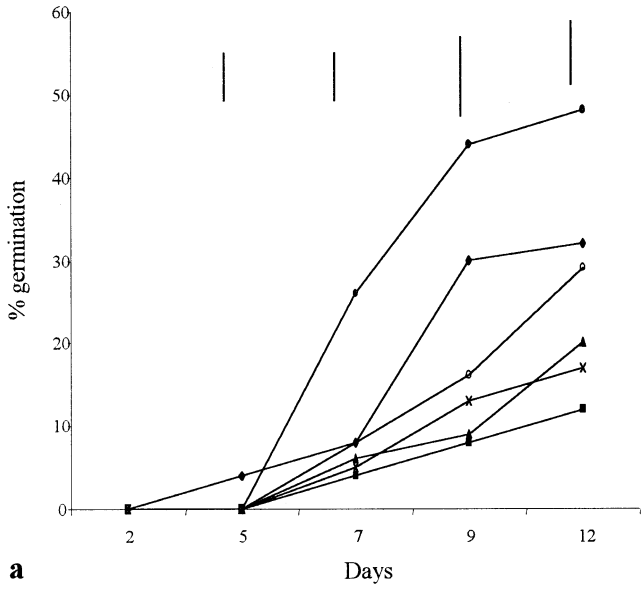
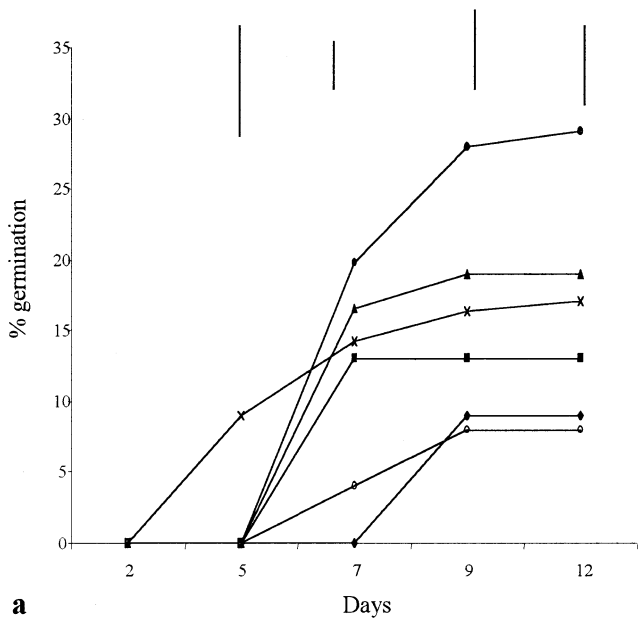


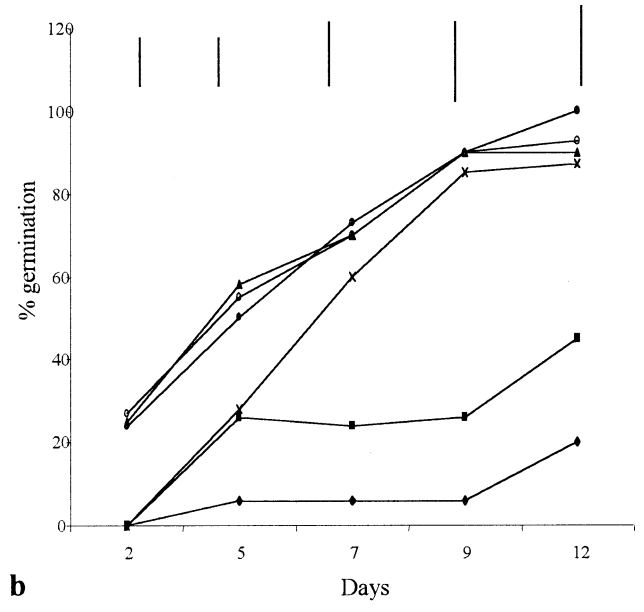
Fig. 1 Effect of *Trichoderma pseudokoningii* strains on the germination of *Glomus mosseae* (**a**) and *Gigaspora rosea* (**b**) spores. Vertical lines correspond to LSD values (Tukey multiple range test, P <0.05). ● Control; ◆ 511; ■ 2212; ▲ 741A; × 741B; ◯ 453

Table 1 Shoot and root dry weights (mg), number of CFUs (10^3 per g soil) of *Trichoderma pseudokoningii* strains and the mycorrhizal colonization of soybean (*Glycine max*) plants (% root length) inoculated (+) or not (−) with *Glomus mosseae*. Values for each parameter followed by the same letter are not significantly different according to Duncań s multiple range test (P 0.05)

Trichoderma	Shoot dry weight		Root dry weight		CFUs		Colonization
	−	+	−	+	−	+	
Control	570a	670b	290a	210a	0	0	26b
511	512a	602b	240a	270a	24b	24b	26b
2212	507a	550a	270a	230a	20ab	22ab	13a
741A	580a	690b	240a	290a	30b	29b	24b
741B	521a	623b	260a	230a	35b	31b	25b
453	550a	632b	240a	271a	15a	15a	27b







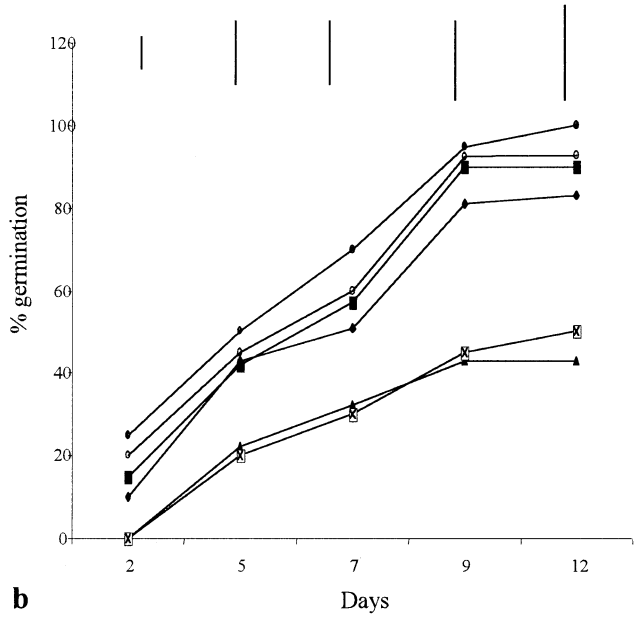


Fig. 2 Effect of exudates of *T. pseudokoningii* strains on the germination of *G.. mosseae* (**a**) and *Gi. rosea* (**b**) spores. Vertical lines correspond to LSD values (Tukey multiple range test, $P < 0.05$). ● Control; ◆ 511; ■ 2212; ▲ 741A; × 741B; ◯ 453

Fig. 3 Effect of volatile compounds of *T. pseudokoningii* strains grown in GA on the germination of *G. mosseae* (**a**) and *Gi. rosea* (**b**) spores. Vertical lines correspond to LSD values (Tukey multiple range test, $P < 0.05$). ● Control; ◆ 511; ■ 2212; ▲ 741A; × 741B; ◯ 453

Table 2 Shoot and root dry weights (mg), number of CFUs (10^3 per g soil) of *T. pseudokoningii* strains and mycorrhizal colonization of soybean plants (% root length) inoculated (+) or not (–) with *Gigaspora rosea*. Values for each parameter followed by the same letter are not significantly different according to Duncań multiple range test (P 0.05)

Trichoderma	Shoot dry weight		Root dry weight		CFUs		Colonization
	–	+	–	+	–	+	
Control	251a	418b	148a	209b	0	0	45c
511	279a	275a	156a	156a	28b	25ab	20ab
2212	223a	376a	141a	142a	21ab	24ab	25b
741A	258a	325a	156a	146a	32b	28b	24b
741B	268a	369a	143a	154a	37b	35b	16ab
453	271a	295a	158a	149a	19a	19a	11a

rosea (Table 2). *Trichoderma* were not found in the rhizosphere of mycorrhizal and nonmycorrhizal soybeans that were not inoculated with the saprotrophic fungi (Table 2).

Discussion

In vitro experiments in which saprotrophic fungi were paired with spores of *G. mosseae* or *Gi. rosea* showed a direct effect of *T. pseudokoningii* on the germination of spores of both AM fungi. The results suggest a direct interaction between the mycorrhizal fungus and the saprotrophic fungi in the presymbiotic phase of the former. Similar interactions have been proposed for other saprotrophic fungi (McAllister et al. 1994). It is known that effects of the genus *Trichoderma* on AM spore germination may differ with the species used (Rouseau et al. 1996; Siddiqui and Mohmood 1996; Fracchia et al. 1998; Godeas et al. 1999; Green et al. 1999). However, our results show that the effect of *T. pseudokoningii* also depends on the strain of the saprotrophic fungus. All *T. pseudokoningii* strains inhibited the germination of *G. mosseae* and *Gi. rosea* except strain 453, which did not affect the germination of *Gi. rosea*. The importance of soluble exudates and volatile substances produced by saprotrophic fungi in interactions with AM fungi has been demonstrated (McAllister et al. 1994, 1995). Thus, it is reasonable to assume that soluble or volatile substances produced by the *T. pseudokoningii* strains could have inhibited the germination of *G. mosseae* and *Gi. rosea* spores. Soluble exudates and volatile substances prepared from all *T. pseudokoningii* strains in these experiments inhibited spore germination of *G. mosseae*. The effect of *T. pseudokoningii* on the germination of *G. mosseae* spores is likely to be a consequence of the nonadditive action of the volatile and soluble compounds produced, as the percentages of spore germination observed were similar in the presence of volatiles and soluble exudates. Moreover, the germination of *Gi. rosea* spores was inhibited by soluble exudates from *T. pseudokoningii* 2212 and 511, whereas *T. pseudokoningii* 714A and 714B inhibited the germination of *Gi. rosea* spores by the production of volatile substances. These results indicate that volatile and soluble exudates have different modes of action on AM spore germination. The volatile substances produced by *T. pseudokoningii* only inhibited germination of AM fungal spores as a result of increased metabolic activity when the saprotrophic fungus grew in a richer medium. The nature of these volatile inhibitors is unknown.

The AM colonization level of soybean varied with the AM endophyte. Relatively low percentages of root colonization were observed in soybean inoculated with *G. mosseae*. Similar results with some *G. mosseae* strains have been observed previously (Schreiner and Bethlenfalvay 1997; Venedikian et al. 1999). The effects of *T. pseudokoningii* on plant dry weight and AM root length colonization differed with the saprotrophic strain used and the genus of AM fungi. The strains of *T. pseudokoningii* did not affect dry matter or percentage of root length colonization of soybean inoculated with *G. mosseae*, except *T. pseudokoningii* 2212, which inhibited both parameters. However, all *T. pseudokoningii* strains decreased shoot dry matter and percentage of AM root length colonization of soybean inoculated with *Gi. rosea*. It has been suggested that saprotrophic fungi can affect AM colonization of root by effects on the presymbiotic phase of the AM fungi (McAllister et al. 1994). However, the absence of an effect on AM root colonization by *T. pseudokoningii* 511, 741A, 741B and 453, which were able to decrease the spore germination of *G. mosseae*, indicates an effect of these saprotrophic fungi on the presymbiotic phase of *G. mosseae* insufficient to decrease mycorrhizal colonization of plant roots. On the other hand, *T. pseudokoningii* 453 did not affect the spore germination of *Gi. rosea* but decreased AM colonization.

In spite of the antagonistic or neutral effects of some *T. pseudokoningii* strains on the colonization of soybean root by AM fungi, no influence of AM fungi on number of CFUs of *T. pseudokoningii* was found. This lack of an effect has been observed for several saprotrophic fungi co-inoculated with *G. mosseae* (McAllister et al. 1996, 1997; Garcia-Romera et al. 1998). In conclusion, the action of saprotrophic fungi on AM fungal development and function may be different with different strains of the same saprotrophic species.

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