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Suppression of the Biocontrol Agent *Trichoderma harzianum* by Mycelium of the Arbuscular Mycorrhizal Fungus *Glomus intraradices* in Root-Free Soil

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***Trichoderma harzianum* is an effective biocontrol agent against several fungal soilborne plant pathogens. However, possible adverse effects of this fungus on arbuscular mycorrhizal fungi might be a drawback in its use in plant protection. The objective of the present work was to examine the interaction between *Glomus intraradices* and *T. harzianum* in soil. The use of a compartmented growth system with root-free soil compartments enabled us to study fungal interactions without the interfering effects of roots. Growth of the fungi was monitored by measuring hyphal length and population densities, while specific fatty acid signatures were used as indicators of living fungal biomass. Hyphal ^{33}P transport and β-glucuronidase (GUS) activity were used to monitor activity of *G. intraradices* and a GUS-transformed strain of *T. harzianum*, respectively. As growth and metabolism of *T. harzianum* are requirements for antagonism, the impact of wheat bran, added as an organic nutrient source for *T. harzianum*, was investigated. The presence of *T. harzianum* in root-free soil reduced root colonization by *G. intraradices*. The external hyphal length density of *G. intraradices* was reduced by the presence of *T. harzianum* in combination with wheat bran, but the living hyphal biomass, measured as the content of a membrane fatty acid, was not reduced. Hyphal ^{33}P transport by *G. intraradices* also was not affected by *T. harzianum*. This suggests that *T. harzianum* exploited the dead mycelium but not the living biomass of *G. intraradices*. The presence of external mycelium of *G. intraradices* suppressed *T. harzianum* population development and GUS activity. Stimulation of the hyphal biomass of *G. intraradices* by organic amendment suggests that nutrient competition is a likely means of interaction. In conclusion, it seemed that growth of and phosphorus uptake by the external mycelium of *G. intraradices* were not affected by the antagonistic fungus *T. harzianum*; in contrast, *T. harzianum* was adversely affected by *G. intraradices*.**

The antagonistic fungus *Trichoderma harzianum* is widely recognized as a potential biocontrol agent against several soilborne plant pathogens (16, 30). However, possible adverse effects of *T. harzianum* on plant-growth-promoting microorganisms, such as arbuscular mycorrhiza (AM) fungi, might be a drawback in the use of this biocontrol agent in plant protection. AM fungi are obligate biotrophic endosymbionts in roots of most herbaceous plants. These fungi grow from the roots out into the surrounding soil, forming an external hyphal network which increases uptake of mineral nutrients (37) and consequently promotes plant growth. However, an increasing number of reports support the concept that establishment and functioning of the AM symbioses are affected by a range of soil microorganisms that may act either supportively or detrimentally (21, 31).

AM fungi may also contribute to protection of the host plant against soilborne plant pathogens (15). Combinations of AM fungi and biocontrol agents like *T. harzianum* could, therefore, provide levels of disease control which are superior to the effects of the organisms when they are used alone (4, 21, 22), although previous results (3, 4, 25, 35) are contradictory. Naturally, the nature of the interactions between AM fungi and biocontrol agents is important for such additive or synergistic effects.

The effects of fungi belonging to the genus *Trichoderma* on spore germination and hyphal growth of *Glomus mosseae* have been examined in vitro, and contradictory results have been obtained (1, 2, 23). However, the results from pot experiments suggest that *Trichoderma* species suppress AM root colonization (24, 35, 46), although this depends on the timing of inoculation (24) and the host plant species (5). On the other hand, adverse effects of AM fungi on the population density of *Trichoderma koningii* have also been observed (24).

So far, most of the pot experiments dealing with interactions between saprotrophic fungi (e.g., *Trichoderma* spp.) and AM fungi have been carried out in soil containing roots. Under these conditions possible effects of the saprophytes on AM spore germination and root colonization cannot be clearly distinguished from effects on the outgrowth and functioning of the external mycelium. In addition, the majority of these studies have focused on the effect on the host plant rather than on measuring the biomass and specific activity of the organisms involved. Consequently, specific interactions between the external mycelia of AM fungi and saprotrophic microorganisms are poorly understood.

The present work was carried out in order to test the hy-

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potheses that *T. harzianum* and the external hyphal network of *Glomus intraradices* interact and that the interactions affect growth and activity. The use of a compartmented growth system with root-free soil compartments (RFSC) allowed us to study interactions between *G. intraradices* and *T. harzianum* without direct interference from roots. As growth and metabolism of *T. harzianum* are prerequisites for antagonism, the impact of wheat bran, added as an organic nutrient source for *T. harzianum*, was investigated. Fungal growth was measured by using specific fatty acid signatures in combination with hyphal length or population size measurements. Hyphal phosphorus transport was used to monitor activity of *G. intraradices*. The use of a β-glucuronidase (GUS)-transformed strain of *T. harzianum* enabled us to monitor the metabolic activity of this organism by quantifying GUS activity.

MATERIALS AND METHODS

Fungi, plants, and soil. *T. harzianum* Rifai isolate T3a was originally isolated from a *Pythium*-suppressive peat (45) and was transformed with the *Escherichia coli* GUS gene and the hygromycin B resistance gene (40). The transformant resembles the wild type in terms of phenotypic characters (40) and ecological fitness (12). For inoculum production, T3a was grown on peat-bran (36) for 2 weeks. The preparation was suspended in water and filtered through four layers of cheesecloth to remove the peat-bran. The conidia were washed three times in water and resuspended, and the concentration of conidia was determined with a hemocytometer. A defined amount of conidia was sprayed on top of either sterile quartz sand or wheat bran and left to dry for 16 h before these preparations were thoroughly mixed with the soil (see below).

Cucumber (*Cucumis sativus* L. cv. Aminex; F1 hybrid; Novartis Seeds A/S, Hedehusene, Denmark) was used as the host plant for the AM fungus *G. intraradices* Schenck & Smith (BEG 87). A crude inoculum of the AM fungus containing soil, roots, and spores was obtained from a *Trifolium subterraneum* L. pot culture.

The soil was a 1:1 (wt/wt) mixture of sandy loam and quartz sand. It contained 8 mg of 0.5 M $NaHCO_3$-extractable P per g of soil (26), had a pH(H_2O) of 6.1, and was partially sterilized by irradiation (10 kGy; 10-MeV electron beam) to eliminate any indigenous fungi. The following nutrients were mixed into the soil: NH_4NO_3 (86 mg kg^{-1}), KH_2PO_4 (44 mg kg^{-1}), K_2SO_4 (70 mg kg^{-1}), $CaCl_2$ (70 mg kg^{-1}), $CuSO_4 \cdot 5H_2O$ (22 mg kg^{-1}), $ZnSO_4 \cdot 7H_2O$ (5 mg kg^{-1}), $MnSO_4 \cdot 7H_2O$ (10 mg kg^{-1}), $CoSO_4 \cdot 7H_2$ (0.33 mg kg^{-1}), $NaMoO_4 \cdot 2H_2O$ (0.2 mg kg^{-1}), and $MgSO_4 \cdot 7H_2O$ (20 mg kg^{-1}).

Experimental setup and growth conditions. Cucumber plants were grown in compartmented growth units made of polyvinyl chloride tubes (internal diameter, 4.5 cm). Each unit consisted of a 32.5-cm-long central root compartment separated from two lateral 7-cm-long RFSC by a 37-μm-pore-size nylon mesh (20). Each root compartment was filled with 740 g of soil; in the *G. intraradices* treatments 300 g of this soil was replaced with an inoculum-soil mixture (1:2, wt/wt). This inoculum-soil mixture was placed in the central compartment between the two RFSC, each of which contained 50 g of soil. In order to establish similar initial microflora communities in all treatments, all units received 10 ml of a soil suspension obtained by wet sieving (20-μm-pore-size nylon mesh) 100 g of inoculum in 1 liter of water. Finally, water was added to the soil in each unit to 60% of the water-holding capacity, and the soil was kept at room temperature for 4 days.

Two surface-sterilized, pregerminated cucumber seeds were sown in each unit and thinned to one after seedling emergence. After another 2 weeks the soil in both RFSC was replaced by a similar amount of soil containing combinations of *T. harzianum* and wheat bran.

The initial population densities of *T. harzianum* were 5×10^4 CFU g of $soil^{-1}$ in treatments with wheat bran and 10^7 CFU g of $soil^{-1}$ in treatments without wheat bran. The differences in the initial population densities of *T. harzianum* used were due to expected increases in the population densities in treatments with wheat bran; thus, our goal was to have approximately the same density in all treatments at the end of the experiment independent of the nutrient source. Wheat bran was added at a concentration of 0.5% (wt/wt). In addition to *T. harzianum* and wheat bran, $H_3{}^{33}PO_4$ (4 kBq g of $soil^{-1}$) was mixed homogeneously into the soil (20) in one of the RFSC in half of the replicate growth units.

Plants were maintained in a growth chamber equipped with Osram daylight lamps providing photosynthetically active radiation equivalent to 500 to 550 μmol m^{-2} s^{-1} for 16 h per day. The day and night temperatures were 21 and 16°C, respectively. Initially, the growth units were arranged randomly, and then they were rearranged daily, so that each unit was in a new position every day. The growth units were watered daily to maintain 60% of the water-holding capacity (by weight). Nitrogen was supplied weekly as a NH_4NO_3 solution; a total of 155 mg of N per plant was added during the growth period.

Harvest and plant analysis. Two randomly chosen replicate units for each treatment were harvested 5 and 10 days after the soil in the RFSC was replaced. The remaining four replicate units for each treatment were harvested after 20 days. On the day of harvesting, the shoot of each plant was separated from the roots, dried for 24 h at 80°C, and weighed. The soil core was removed from the central root compartment, and the root system of each plant was washed, dried for 48 h at 80°C, and weighed. Colonization of the root systems by *G. intraradices* was analyzed by the method of Kormanik and McGraw (18), except that trypan blue was used instead of acid fuchsin. The contents of the RFSC without ^{33}P were emptied into plastic bags, and the soil was thoroughly mixed. The mixed soil was subsampled by weight (see below). Dilution plating of soil samples to determine the population density of *T. harzianum* was carried out on the day of harvesting. The soil samples used for the GUS assay were stored at −80°C and analyzed the following day. All other soil samples were stored at −18°C until they were analyzed. The soil dry weight for each RFSC was determined after drying at 86°C.

Hyphal length density of and phosphorus transport by *G. intraradices*. The hyphal length densities in RFSC soil from all units were determined by a membrane filter technique (14). The background values were subtracted, and the results were expressed in meters of hyphae per gram (dry weight) of soil.

To determine AM hyphal phosphorus transport, dried plant materials (shoots and roots) from the third harvest were ground and digested in a nitric acid-perchloric acid solution (4:1, vol/vol). Three milliliters of the diluted digest was mixed with 15 ml of scintillation fluid (Ultima gold; Packard Instrument Co., Meriden, Conn.) and β-emission was counted with a Packard TR1900 liquid scintillation counter in order to determine the ^{33}P contents. The counts were corrected for background values and were expressed in total counts per minute (cpm) per plant.

Population density of *T. harzianum*. Two grams of each soil sample was suspended in 100 ml of sterile water and homogenized with an Ultra-turrax T 25 (IKA-Labortechnic, Staufen, Germany) for 2 min at 13,500 rpm. Serial dilutions were prepared, and aliquots were plated onto the *Trichoderma*-selective medium mTSM (12). The plates were incubated for 4 days at root temperature, before the colonies were counted. To verify that the colonies derived from the transformant, a ring was cut in each colony with a cork borer (diameter, 6 mm), and 7 μl of a 1-mg/ml solution of 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-Gluc) (Sigma Chemical Co., St. Louis, Mo.) in extraction buffer (50 mM phosphate, pH 7.0) containing 0.05% Triton X-100 (Bie & Berntsen, Rødovre, Denmark), 1 mM *N*-lauroyl-sarcosine (Sigma), and 1 mM EDTA (Sigma) was added to each ring. The plates were incubated in the dark at 37°C for 2 h, and the number of blue colonies was counted. Approximately 100 randomly chosen colonies were tested per treatment.

GUS assay. Two grams of each soil sample was frozen at −80°C for 1 day, homogenized in liquid nitrogen, and suspended in 30 ml of extraction buffer (see above). The suspensions were incubated for 1 to 2 h on ice before they were centrifugated at 13,000 × *g* for 12 min at 4°C to pellet the soil particles. One-milliliter portions of the supernatants were transferred to test tubes containing 1.0 ml of 2 mM 4-methylumbelliferyl-β-D-glucuronide (Sigma) in extraction buffer. After incubation for 20 min at 37°C, the enzyme activity was quenched by transferring 50-μl portions of the assay solutions to 1.95 ml of 0.1 M carbonate stop buffer. The fluorescence emitted by the enzymatically released 4-methylbelliferylone (MU) moiety was monitored with a luminescence spectrometer (LS50B; Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) by excitation at 365 nm and reading at 455 nm. The values obtained were corrected for nonenzymatic hydrolysis of 4-methylumbelliferyl-β-D-glucuronide and were converted to nanomoles of MU per minute per gram of soil. The GUS activity was used as an indicator for the general metabolic activity of the transformant in soil (12).

Fatty acid analysis. Three grams of each soil sample from the second and third harvests was freeze-dried, placed in Teflon tubes with two tungsten mill balls, and ground on a rotary shaker for 5 min. Lipid extraction was carried out by the method of Frostegård et al. (8). The lipids were extracted from the soil in 10 ml of one-phase chloroform-methanol-citrate buffer (1:2:0.8, vol/vol/vol; pH 4.0). After centrifugation for 10 min at 750 × *g*, the pellets were washed with 5 ml of the one-phase mixture, and the supernatants were combined. The extract was split into two phases by adding 4 ml of chloroform and 4 ml of 0.15 M sodium citrate buffer (pH 4.0). The extracted lipids were fractionated on silicic acid columns (100/200 mesh; Unisil) into neutral, intermediate, and polar lipids by elution with 5 ml of chloroform, 20 ml of acetone, and 5 ml of methanol, respectively. The polar phospholipids (PLFAs) and neutral lipids (NLFAs) were dried under nitrogen along with 23 μg of nonadecanoate (fatty acid methyl ester 19:0) per ml, which was added as an internal standard. Lipids in both fractions were then transformed into free fatty acid methyl esters by mild alkaline methanolysis (7). These were then analyzed on an HP-5890 gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with a flame ionization detector and a 50-m HP5 capillary column (9). The retention times relative to the internal standard were used to identify the fatty acids. The background values were subtracted, and the results were expressed in nanomoles of fatty acid per gram of soil. The nomenclature for the fatty acids follows that used by Tunlid and White (42). The following 11 PLFAs (15:0, i16:0, 10Me16:0, i17:0, a17:0, 17:1ω8, cy17:0, 17:0, 10Me17:0, 10Me18:0, and cy19:0) were used as indicators of bacterial biomass (10). Fatty acid 16:1ω5 was used as an indicator of AM fungal biomass (27). While PLFAs mainly represent membrane structures, NLFAs represent storage lipids associated with spore structures (28). Thus, the NLFA/PLFA ratios

TABLE 1. Shoot and root dry weights, percentages of the root systems colonized by *G. intraradices*, and AM-mediated ^{33}P uptake from root-free soil as affected by inoculation with *G. intraradices* in the root compartment and *T. harzianum* in the RFSC with and without wheat bran amendment[a]

Treatment			Shoot dry wt (g)	Root dry wt (g)	% Colonization	Uptake of ^{33}P (10^3 cpm dry plant^{-1})
G. intraradices	*T. harzianum*	Wheat bran				
−	−	−	3.59 ± 0.39	0.38 ± 0.18	0	151 ± 53
−	+	−	3.71 ± 0.24	0.31 ± 0.05	0	333 ± 412
−	−	+	3.45 ± 0.51	0.29 ± 0.04	0	139 ± 40
−	+	+	3.46 ± 0.38	0.29 ± 0.02	0	151 ± 53
+	−	−	3.22 ± 0.19	0.38 ± 0.03	58.5 ± 2.4	1,013 ± 70
+	+	−	3.46 ± 0.19	0.42 ± 0.05	44.5 ± 12.7	1,168 ± 21
+	−	+	3.41 ± 0.11	0.44 ± 0.06	55.5 ± 7.0	1,037 ± 87
+	+	+	3.22 ± 0.32	0.36 ± 0.04	50.8 ± 7.5	921 ± 199

[a] Only data from the third harvest (30 days after seedling emergence) are presented. The statistical analysis was based on data from all three harvest times. *G. intraradices P* values for shoot dry weight, root dry weight, and uptake of ^{33}P were 0.016, 0.002, and <0.001, respectively. *T. harzianum P* values for shoot dry weight, root dry weight, percent colonization, and uptake of ^{33}P were 0.636, 0.400, 0.024, and 0.210, respectively. Wheat bran *P* values for shoot dry weight, root dry weight, percent colonization, and uptake of ^{33}P were 0.274, 0.110, 0.162, and 0.479, respectively.

of AM fungi may indicate carbon allocation to storage structures. The PLFA 18:2ω6,9 was used as a biomass indicator for dikaryotic fungi (i.e., *Ascomycota* and *Basidiomycota*), which in soil basically means the saprotrophic fungi (10, 19). As *G. intraradices* is known to produce minor amounts of 18:2ω6,9 (19), the proportions of 18:2ω6,9 relative to the amount of 16:1ω5 were subtracted from the total and the remaining quantity of 18:2ω6,9 was considered to represent saprotrophic fungi.

Statistics. The experiment had a complete factorial design with eight main treatments (see Table 1), and each main treatment had eight replicates (i.e., a total of 64 plants). Each replicate growth unit had two RFSC, which obviously were not true replicates but were treated as such after a test for independence. The data for the last harvest were based on four true replicates. A preliminary experiment was conducted by using the wild-type *T. harzianum* strain T3 and *G. intraradices* in a similar experimental setup.

Data for PLFAs 16:1ω5 and 18:2ω6,9 were subjected to square root transformation, while data for the bacterium-specific PLFAs were transformed logarithmically to obtain variance homogeneity before analysis. Levels of significance for the main treatments and their interactions were calculated by using the General Linear Models Procedure (PROC GLM; SAS Institute, Cary, N.C.). The affiliation of the RFSC was included as a factor in each analysis. Correlations between data were determined by regression analysis (PROC REG).

RESULTS

Mycorrhizal root colonization and plant dry weight. Only data based on the four true replicate plants from the third harvest are shown in Table 1. Plants inoculated with *G. intraradices* became mycorrhizal, while uninoculated plants remained nonmycorrhizal. The presence of *T. harzianum* in root-free soil reduced root colonization by *G. intraradices* ($P = 0.024$) independent of the presence of wheat bran. *G. intraradices* had a negative effect on the shoot dry weight ($P = 0.016$) and a positive effect on the root dry weight ($P = 0.002$) (Table 1). The average shoot dry weights were 3.55 g in the absence of *G. intraradices* and 3.33 g in the presence of *G. intraradices*, while the average root dry weights were 0.32 g in the absence of *G. intraradices* and 0.40 g in the presence of *G. intraradices*. Neither *T. harzianum* nor wheat bran had any effect on the plant dry weight.

Hyphal length density of and phosphorus transport by *G. intraradices*. The background values for the hyphal length density in root-free soil without *G. intraradices* were not affected by the presence of *T. harzianum*. In the absence of wheat bran and *G. intraradices*, the average hyphal length density was 2.0 m g of soil^{-1} and did not differ between harvests. In treatments without *G. intraradices* but with wheat bran added, the average hyphal length density increased from 3.1 m g of soil^{-1} at the first harvest to 5.3 m g of soil^{-1} at the third harvest. These background values for nonmycorrhizal treatments were subtracted from the hyphal length densities in the mycorrhizal treatments and were used to calculate the hyphal length density of *G. intraradices*. In this way, outgrowth of the external mycelium of *G. intraradices* could be detected at the first harvest (i.e., 5 days after replacement of the soil in the RFSC). In general, the density increased throughout the experiment ($P < 0.001$), but the increase was greater ($P < 0.001$) for the treatments with wheat bran (Fig. 1). In addition, there was an interaction ($P < 0.001$) between *T. harzianum* and wheat bran in the sense that *T. harzianum* reduced the hyphal length density of *G. intraradices* only in the presence of wheat bran. There were also interactions between harvest time and wheat bran ($P < 0.001$) and between harvest time and *T. harzianum* ($P < 0.001$).

Mycorrhizal plants contained 5.4 times as much ^{33}P as nonmycorrhizal plants (Table 1). *G. intraradices*-mediated ^{33}P uptake was not affected by the presence of either *T. harzianum* or wheat bran, and the average ^{33}P content of plants was 8.41×10^5 cpm after subtraction of the values obtained for the corresponding nonmycorrhizal controls.

Population density and GUS activity of *T. harzianum*. No indigenous *Trichoderma* spp. were detected in the soil, and *T.*

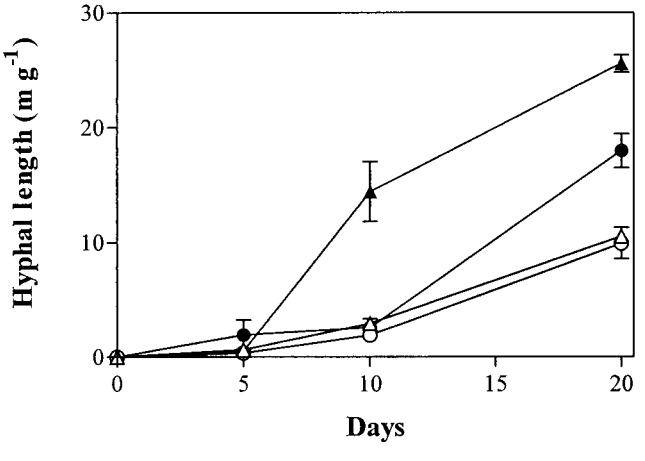

FIG. 1. Hyphal length density of *G. intraradices* in root-free soil as influenced by the presence of wheat bran and/or *T. harzianum* T3a. The background values for non-AM treatments were subtracted. The bars indicate standard errors. Symbols: △, treatment without both wheat bran and *T. harzianum*; ▲, treatment with wheat bran but without *T. harzianum*; ○, treatment with *T. harzianum* but without wheat bran; ●, treatment with both wheat bran and *T. harzianum*.

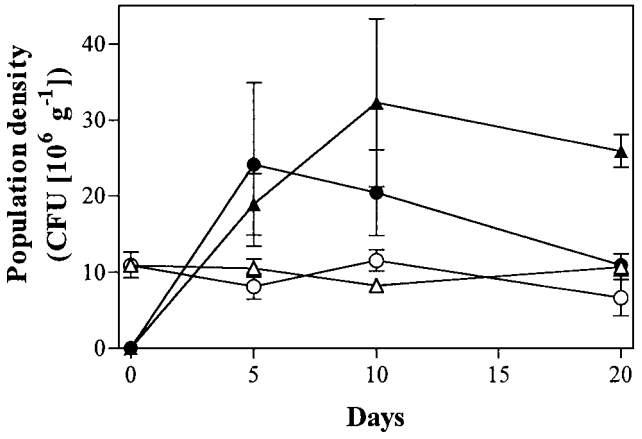

FIG. 2. Population development of *T. harzianum* T3a in root-free soil as influenced by the presence of wheat bran and/or the outgrowth of external mycelium of *G. intraradices*. The bars indicate standard errors. Symbols: △, treatment without both wheat bran and *G. intraradices*; ▲, treatment with wheat bran but without *G. intraradices*; ○, treatment with *G. intraradices* but without wheat bran; ●, treatment with both wheat bran and *G. intraradices*.

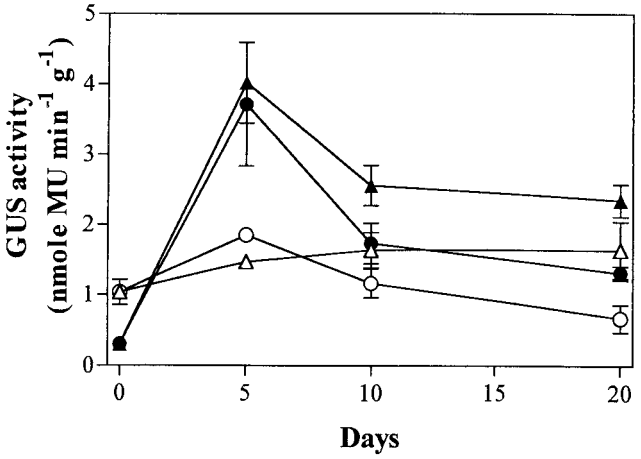

FIG. 3. GUS activity of *T. harzianum* T3a in root-free soil as affected by the presence of wheat bran and/or the outgrowth of external mycelium of *G. intraradices*. Background values obtained for treatments that did not include *T. harzianum* were subtracted. The bars indicate standard errors. Symbols: △, treatment without both wheat bran and *G. intraradices*; ▲, treatment with wheat bran but without *G. intraradices*; ○, treatment with *G. intraradices* but without wheat bran; ●, treatment with both wheat bran and *G. intraradices*.

harzianum was absent in the treatments to which it was not added. The development of the *T. harzianum* population in root-free soil responded positively ($P = 0.002$) to the wheat bran amendment (Fig. 2). After 20 days, the population density of *T. harzianum* was significantly reduced in soil containing both *G. intraradices* and wheat bran. *G. intraradices* had no effect in the absence of wheat bran. The statistical significance of the effect of *G. intraradices* was $P = 0.002$, while the significance of the interaction with wheat bran was $P = 0.031$.

The background GUS activity in extracts from root-free soil without *T. harzianum* was not affected by *G. intraradices* and wheat bran and appeared to be almost constant throughout the experiment, with an average value of 1.52 nmol of MU min^{-1} g of soil^{-1}. The GUS activity in extracts from root-free soil containing *T. harzianum* was always higher than the GUS activity in the corresponding soil from treatments not containing *T. harzianum*. The data presented below are corrected for the corresponding background values. In soil from treatments containing *T. harzianum*, the GUS activity responded positively ($P < 0.001$) to the wheat bran amendment (Fig. 3). In soil from treatments containing neither *G. intraradices* nor wheat bran, the activity increased until day 10. The maximum activity for all other treatments was reached at day 5, after which the activity decreased. From day 10 to day 20, *G. intraradices* reduced the GUS activity both in the absence and in the presence of wheat bran. The levels of significance for the effect of *G. intraradices* in root-free soil were $P = 0.026$ at day 10 and $P = 0.003$ at day 20.

Content of fatty acid 16:1ω5. Fatty acids were extracted only on days 10 and 20. The nonmycorrhizal background values for PLFA 16:1ω5 were 0.41 and 0.71 nmol g of soil^{-1} for treatments with and without wheat bran, respectively. The average NLFA 16:1ω5 background value was 2.14 nmol g of soil^{-1}.

After the corresponding background values for treatments without *G. intraradices* were subtracted, the quantity of PLFA 16:1ω5 in root-free soil increased with time ($P < 0.001$), but the increase was greater ($P < 0.001$) in soil amended with wheat bran than in nonamended soil (Fig. 4). *T. harzianum* had no effect on PLFA 16:1ω5. The ratio of NLFA 16:1ω5 to PLFA 16:1ω5 also increased with time ($P < 0.001$). On the last harvest (day 20), the ratio was higher ($P = 0.033$) for treatments without wheat bran than for treatments with wheat bran (Fig. 5). *T. harzianum* had no effect on the NLFA/PLFA ratio for 16:1ω5.

Content of fatty acid 18:2ω6,9. The background values for PLFA 18:2ω6,9 for treatments without *T. harzianum* were 2.87 and 0.28 nmol g of soil^{-1} with and without wheat bran, respectively. On average, inoculation with *T. harzianum* increased the PLFA 18:2ω6,9 content ($P < 0.001$) in root-free soil 1.6-fold at day 10 and 1.2-fold at day 20. The background-corrected amount of PLFA 18:2ω6,9 in root-free soil was higher ($P = 0.002$) on day 10 than on day 20 (Fig. 6). The presence of wheat bran had a positive ($P = 0.009$) effect on PLFA 18:2ω6,9 at day 10. *G. intraradices* had no effect on PLFA 18:2ω6,9.

No correlations were found between PLFA 18:2ω6,9 data and data for the GUS activity or population density of *T. harzianum*. Likewise, PLFA 18:2ω6,9 was not correlated with the AM-specific PLFA signature 16:1ω5.

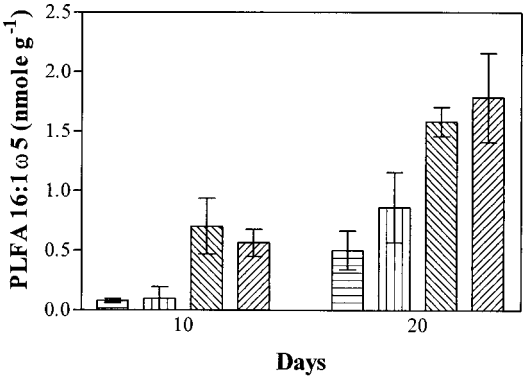

FIG. 4. Quantification of the AM-specific PLFA signature 16:1ω5 in root-free soil as affected by wheat bran and/or *T. harzianum* T3a. The background values for nonmycorrhiza treatments were subtracted. The bars indicate standard errors. ▭, treatment without both wheat bran and *T. harzianum*; ▥, treatment with *T. harzianum* but without wheat bran; ▨, treatment with wheat bran but without *T. harzianum*; ▨, treatment with both wheat bran and *T. harzianum*.

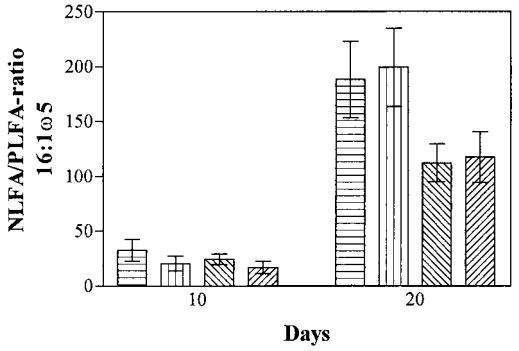

FIG. 5. Ratio of NLFA to PLFA for the AM-specific signature 16:1ω5 in root-free soil as influenced by the presence of wheat bran and/or *T. harzianum* T3a. The bars indicate standard errors. ▭, treatment without both wheat bran and *T. harzianum*; ▥, treatment with *T. harzianum* but without wheat bran; ▧, treatment with wheat bran but without *T. harzianum*; ▨, treatment with both wheat bran and *T. harzianum*.

Content of bacterium-specific PLFAs. The total amount of bacterium-specific PLFAs in root-free soil was evaluated only on day 20 (Table 2). There was a positive effect ($P < 0.001$) of the wheat bran amendment. Neither *T. harzianum* nor external mycelium of *G. intraradices* had any effect on the bacterial PLFAs.

A regression analysis based on data obtained for the *G. intraradices* treatments alone revealed significant positive relationships between the total bacterial PLFAs and the AM-specific PLFA signature 16:1ω5. For treatments with and without wheat bran the correlation coefficient (r^2) was 0.76 ($P < 0.001$) (Fig. 7). For treatments with wheat bran only the r^2 value was 0.69 ($P = 0.011$). No correlation was found between the sum of bacterium-specific PLFA signatures and PLFA 18:2ω6,9 ($r^2 = 0.03$; $P = 0.517$).

DISCUSSION

The present work demonstrated that there is a clear interaction between the antagonistic fungus *T. harzianum* and the external mycelium of the AM fungus *G. intraradices*. The interaction was in favor of *G. intraradices*, which suppressed both the population density and the activity of *T. harzianum*.

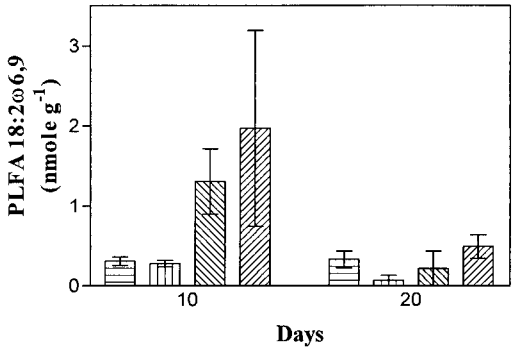

FIG. 6. Biomass of *T. harzianum* T3a estimated by using PLFA 18:2ω6,9 in root-free soil as affected by wheat bran and/or *G. intraradices*. Contributions from *G. intraradices* were subtracted, as were background values obtained from treatments without *T. harzianum*. The bars indicate standard errors. ▭, treatment without both wheat bran and *G. intraradices*; ▥, treatment with *G. intraradices* but without wheat bran; ▧, treatment with wheat bran but without *G. intraradices*; ▨, trreatment with both wheat bran and *G. intraradices*.

TABLE 2. Total amounts of bacterium-specific PLFAs in root-free soil as affected by external mycelium of *G. intraradices*, *T. harzianum* T3a, and wheat bran[a]

Treatment			Bacterial PLFAs (nmol g of soil^{-1})
G. intraradices	*T. harzianum*	Wheat bran	
−	−	−	11.95 ± 0.84
−	+	−	12.21 ± 0.55
−	−	+	26.34 ± 6.92
−	+	+	22.10 ± 4.63
+	−	−	8.95 ± 0.92
+	+	−	10.85 ± 0.82
+	−	+	20.14 ± 2.56
+	+	+	26.10 ± 4.67

[a] PLFAs were quantified after 20 days. *P* values for *G. intraradices*, *T. harzianum*, and wheat bran were 0.069, 0.243, and <0.001, respectively.

The antagonist had no adverse effect on the AM-specific biomass indicator PLFA 16:1ω5 (Fig. 4), the AM sporulation index, expressed as the 16:1ω5 NLFA/PLFA ratio (Fig. 5), or the activity, expressed as AM-mediated ^{33}P uptake (Table 1). This was the case even when the soil was amended with an organic nutrient source, which supported a considerable level of metabolic activity of the antagonist. Only the length density of the external mycelium of *G. intraradices* was reduced by *T. harzianum* in combination with wheat bran. As the staining technique used to quantify hyphae in soil does not discriminate between living and dead hyphae, these observations suggest that *T. harzianum* may exploit dead *G. intraradices* mycelium but not its living biomass. This is in contrast to the results of Rousseau et al. (33), who reported that *T. harzianum* can be an aggressive mycoparasite on hyphae of *G. intraradices*. The discrepancy in these findings could be due to differences between isolates, but it is also possible that the in vitro method based on Ri T-DNA-transformed pea roots used by Rousseau et al. resulted in the formation of an external mycelium which, compared to the mycelium formed in a soil environment, had altered physiological properties and therefore was more vulnerable to antagonism. In addition, *T. koningii* has been shown to adversely affect the succinate dehydrogenase activity of *G. mosseae* inside the roots of the host plant (24). Although these results describe interactions between different species of *Glomus* and *Trichoderma*, respectively, we cannot rule out the possibility that different activity measurements can give considerably different results. In the present experiment, *T. harzianum* had a negative impact on AM root colonization. As the antagonist was added only to the RFSC and at the time when

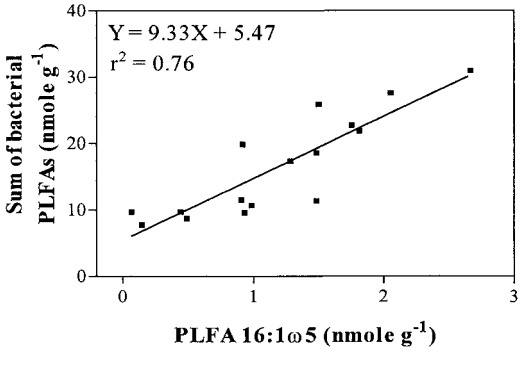

FIG. 7. Correlation between the sum of bacterium-specific PLFA signatures and the AM-specific PLFA signature 16:1ω5.

the symbiotic relationship had been established (i.e., 10 days after seedling emergence), this adverse effect was most likely mediated through an effect on the external mycelium of *G. intraradices*, although the mechanism is not clear.

This study confirmed that addition of wheat bran stimulates mycelial growth of *G. intraradices*. While PLFA 16:1ω5 mainly represents membrane structures, NLFA 16:1ω5 represents storage lipids associated with spores (28). This implies that the NLFA/PLFA ratio for 16:1ω5 can be used as an index for the growth strategy of the AM fungus. The decrease in the NLFA/PLFA ratio in the presence of wheat bran (Fig. 5) indicated that the organic amendment stimulated vegetative growth of *G. intraradices*. This was also illustrated by the fact that both the hyphal length density (Fig. 1) and the quantity of PLFA 16:1ω5 (Fig. 4) were enhanced in the presence of wheat bran. These findings are in agreement with the results of others (17, 20, 32, 38) which indicated that growth of external mycelia of AM fungi in general can be stimulated by external organic nutrient sources. Vancura et al. (43) have shown that specific associations are formed between the external mycelium of *Glomus fasciculatum* and selected bacteria. Since AM fungi probably lack enzymes that degrade organic matter, the stimulatory effects of organic amendments on hyphal biomass could be due to uptake of essential resources (e.g., amino acids [13]) released by decomposition of organic matter by associated saprotrophic microorganisms. In this case, the increase in AM hyphal biomass should depend on the biomass and activity of the saprotrophic microorganisms. The correlations between the sum of bacterium-specific PLFA signatures and the AM-specific signature 16:1ω5 found in present study (Fig. 7) support this hypothesis. In agreement with this, the biomass of external hyphae of AM fungi increased in response to addition of a nonsterile soil leachate to pasteurized soil (39). In the present study, slight variations in the amounts of available external nutrient sources could have resulted in correlations between coexisting microorganisms in the soil. However, the lack of correlation between the sum of bacterium-specific PLFA signatures and PLFA 18:2ω6,9 ($r^2 = 0.03$; $P = 0.517$) suggests that this was not the case. The lack of correlation between the PLFA 18:2ω6,9 and the AM-specific PLFA 16:1ω5 ($r^2 < 0.01$; $P = 0.985$) indicates that saprotrophic fungi have a different functional relationship with *G. intraradices* than saprotrophic bacteria have.

The increase in growth of the external mycelium of *G. intraradices* in response to the wheat bran amendment did not result in a similar increase in the AM-mediated ^{33}P uptake. This lack of correspondence was probably caused by immobilization of ^{33}P by saprotrophic microorganisms, which were also stimulated by the organic amendment. However, it is also possible that the ^{33}P was taken up by the external mycelium but not transferred to the host plant.

In the present study, external mycelium of *G. intraradices* suppressed both the population development of *T. harzianum* (Fig. 2) and the metabolic activity of this organism (Fig. 3). The stimulatory effect of the wheat bran amendment on the growth of *G. intraradices*, as discussed above, makes it interesting to speculate on nutrient competition as a likely mechanism of interaction. When *G. intraradices* also reduced the GUS activity in the absence of wheat bran, this could have been due to competition for the organic nutrient sources generated through general microbial turnover in the soil or it could have been caused by an antagonistic effect of *G. intraradices* or its associated bacterial microflora directly on the resting conidia, which do have detectable GUS activity (40). The absence of an effect of *G. intraradices* on the population development in treatments without wheat bran (Fig. 2) could have been due to the low sensitivity of the dilution plating method.

The fact that *T. harzianum* was allowed to colonize the wheat bran-amended soil prior to the invasion by the external mycelium of *G. intraradices* indicates that *G. intraradices* and/or its associated microflora has a combative strategy which allows it to gain access to the soil and organic matter and to have a restrictive influence on *T. harzianum*. Similarly, the external mycelium of *G. intraradices* was able to invade soil and organic matter already colonized by the saprotrophic fungus *Fusarium culmorum* (19). In both cases, it seems that the external mycelium of *G. intraradices* was not affected by the wide range of hydrolytic enzymes and secondary metabolites which are produced by *T. harzianum* (6, 11, 34) and *F. culmorum* (41, 44).

PLFA 18:2ω6,9 has been used to estimate the biomass of saprotropic fungi in the presence of AM fungi (19, 29). In the present work, treatments with *T. harzianum* contained only 1.2 to 1.6 times as much PLFA 18:2ω6,9 as the corresponding treatments without *T. harzianum*. Estimates of *T. harzianum* biomass based on this signature were therefore less reliable due to variations both in the background values and in the effects of the treatments. In any case, the background-corrected data for PLFA 18:2ω6,9 (Fig. 6) revealed the same tendencies as data for the population density and GUS activity. Thus, the biomass of *T. harzianum*, as expressed by PLFA 18:2ω6,9, responded positively to the wheat bran amendment and was higher on day 10 than on day 20. A decrease in the PLFA 18:2ω6,9 content due to the presence of *G. intraradices* would have been expected, as was observed for the population development and activity of *T. harzianum*. However, such a decrease was not observed, possibly because of the great variation in the data for PLFA 18:2ω6,9.

A GUS-transformed strain of *T. harzianum* was used in the present study to facilitate monitoring of its metabolic activity. The transformant used resembles the wild type in terms of phenotypic characters (40) and ecological fitness (12). The results of a preliminary experiment in which the wild-type *T. harzianum* strain T3 and *G. intraradices* were used strongly support the results presented here. We therefore believe that the conclusions drawn from the present data apply equally well to the transformant and the wild-type strain of *T. harzianum*.

In conclusion, *T. harzianum* did not affect the growth and activity of the external mycelium of *G. intraradices*, while the AM fungus had an adverse effect on the population development and activity of *T. harzianum*. The stimulatory effect of the wheat bran amendment on the growth of *G. intraradices* suggests that nutrient competition could be a mechanism of interaction. However, this hypothesis has to be investigated further. Additional research is also necessary to clarify whether other strains of the organisms behave in the same way and how the adverse effect of *G. intraradices* influences the biocontrol efficacy of *T. harzianum*.

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