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Effects of *Pseudomonas fluorescens* DF57 on growth and P uptake of two arbuscular mycorrhizal fungi in symbiosis with cucumber

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Abstract The effect of *Pseudomonas fluorescens* DF57 on growth and P uptake of two arbuscular mycorrhizal (AM) fungi in symbiosis with cucumber plants was studied in compartmentalised growth systems. Hyphae of *Glomus intraradices* Schenck & Smith (BEG87) or *G. caledonium* (Nicol. & Gerd.) Trappe & Gerdeman (BEG15) grew into lateral root-free compartments. Non-mycorrhizal plants served as control. The soil in half of the growth units of each mycorrhizal treatment was inoculated with *P. fluorescens* DF57. *P. fluorescens* DF57 enhanced hyphal length density of one of the AM fungi, *G. caledonium,* but this was not reflected in a higher hyphal transport of P from the root-free soil to the plant. The total P content was higher in plants grown in symbiosis with *G. intraradices* than in plants in the other treatments. *G. caledonium* and *P. fluorescens* DF57 had a synergistic effect in that total P content in plants inoculated with *G. caledonium* was higher in the presence than in the absence of *P. fluorescens* DF57.

Key words Arbuscular mycorrhizas · *Pseudomonas fluorescens* · Phosphorus transport

Introduction

Arbuscular mycorrhizal (AM) fungi are an integral part of the root system of most plants and constitute an important group of organisms in the soil microbial community. The AM fungi play a key role in nutrient cycling by transport of nutrients, mainly phosphorus (P), from the soil to the plant. Hyphal growth and P uptake are influenced by a range of biotic and abiotic factors including soil bacteria. Plant-growth-promoting rhizobacteria (PGPR) can influence growth of hyphae from germinating arbuscular mycorrhizal spores (Burla et al. 1996; Barea et al. 1998), colonisation of plant roots by AM fungi (Meyer and Linderman 1986), and growth of external AM hyphae and dehydrogenase activity of the AM fungus (Gryndler and Vosátka 1996). However, both stimulatory and inhibitory effects of PGPR on AM fungal growth have been reported. This variation may depend on the isolates of bacteria used (Burla et al. 1996; Requena et al. 1997; Walley and Germida 1997), the time of inoculation (Krishna et al. 1982) or time of harvest (Staley et al. 1992).

Experiments in compartmentalised growth systems, where either external hyphae alone or roots together with associated hyphae were allowed to grow into identical soil volumes, have shown that the AM hyphal P uptake can be higher than the combined P uptake by roots and hyphae (Jakobsen 1995; Ravnskov and Jakobsen 1995). The microbial activity measured by different parameters was markedly higher in soil with both root and hyphae than in soil with hyphae alone (Olsson et al. 1996). These results led us to hypothesise that AM fungi compete for P with the microbial population associated with roots.

Pseudomonas fluorescens is a common bacterium in the rhizosphere and is considered as a PGPR (Höfte et al. 1991). For possible commercial use of *P. fluorescens* as a biocontrol agent it is important to know how it influences other growth-promoting microorganisms, such as the AM fungi. The objective of the present study was to examine the effect of *P. fluorescens* DF57 on the growth and P uptake capacity of two AM fungi, *Glomus intraradices* and *G. caledonium*.

Materials and methods

Biological materials and treatments

The mycorrhizal fungi *G. intraradices* Schenck & Smith (BEG87) and *G. caledonium* (Nicol. & Gerd.) Trappe & Gerdemann

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(BEG15) were each grown in symbiosis with cucumber, *Cucumis sativus* L. (Aminex, F1 hybrid). The fungal inoculum consisted of soil, roots of *Trifolium subterraneum* L. colonised by the AM fungi and AM fungal spores. The bacterial inoculant *P. fluorescens* DF57, which is resistant to ampicillin and chloramphenicol, was originally isolated from the rhizosphere of cucumber and is antagonistic against *Pythium* but not higher fungi. The strain has been used for several studies of the fate and activity of bacteria released into the soil environment (Binnerup et al. 1993; Kragelund et al. 1997; Jensen et al. 1998). *P. fluorescens* DF57 was grown overnight in Luria broth (0.4% glucose, 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.2) on a rotary shaker (200 rpm) at 30 °C. Cells were harvested by centrifugation for 10 min at 7000 rpm at 20 °C and resuspended in 0.9% NaCl. Following two washes in 0.9% NaCl, they were resuspended in sterile distilled water and the resultant cell suspension was used as inoculum.

The experiment was carried out as a two-factorial (mycorrhiza × *P. fluorescens* DF57) experiment with six treatments. The mycorrhizal treatments included plants associated with *G. intraradices* or *G. caledonium* as well as non-mycorrhizal plants. The soil of all mycorrhizal treatments was either inoculated with *P. fluorescens* DF57 or not. All treatment had four replicates.

Experimental setup

Plants were grown in compartmentalised growth units, consisting of a central root compartment and two lateral root-free compartments (Larsen and Jakobsen 1996). The compartments were separated by a mesh with 20-μm openings. The central root compartment was filled with 740 g of an irradiated (10 kGy, 10 MeV electron beam) 1:1 (w:w) sandy loam (Jakobsen and Nielsen 1983) and sand mixture containing 8 mg P kg^{-1} soil of 0.5 M $NaHCO_3$-extractable P (Olsen et al. 1954). The soil for half of the growth units was watered to 30% of water holding capacity with the cell suspension of *P. fluorescens* DF57, whereupon the bacteria were uniformly mixed into the soil to a density of approximately 2×10^8 cells g^{-1} soil. Soil for the rest of the growth units was supplied with sterile distilled water only. The root compartments contained a bottom layer of 300 g soil, a middle layer of 245 g soil plus 75 g fungal inoculum and a top layer of 120 g soil. The fungal inoculum was replaced by the soil:sand mixture in control plants without mycorrhizas. The lateral root-free compartments were filled with 60 g of the same soil but in a 1:4 soil:sand mixture containing 3 mg P kg^{-1} soil of 0.5 M $NaHCO_3$-extractable P. Nutrients were mixed into all soil volumes before the soil was filled into the growth units (mg kg^{-1} dry soil): K_2SO_4, 71.0; $CaCl_2.5H_2O$, 71.0; $CuSO_4.5H_2O$, 2.0; $ZnSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 10.0; $CoSO_4.7H_2O$, 0.35; $Na_2MoO_4.2H_2O$, 0.18; $MgSO_4.7H_2O$, 20.0.

Ten ml of a filtrate of the fungal inoculum was added to all growth units in order to obtain similar microbial populations apart from AM fungi and *P. fluorescens* DF57. The filtrate was obtained by mixing 100 g of each fungal inoculum, suspending it in 400 ml water and sieving it through a 20-μm mesh.

An aqueous solution of carrier-free $H_3{}^{33}PO_4$ was mixed into the soil at 4 kBq g^{-1} soil in one of the root-free compartments of each growth system (Larsen and Jakobsen 1996). All growth units were watered to 65% of water holding capacity and incubated for 5 days before sowing.

Two pregerminated seeds of cucumber were sown in each growth unit and plants were thinned to one per unit after emergence. The growth units were placed in a growth chamber with a 16/8 h light/dark cycle, a photosynthetic photon flux density of approximately 500 μmole $m^{-2}\,s^{-1}$ (Osram HQI-T 250 W/D) and day/night temperatures of 20/16 °C. The growth units were watered daily to 65% of water holding capacity. Nitrogen was supplied as an NH_4NO_3 solution at 25 mg N per plant once a week. Four replicates of each treatment were harvested 33 days after planting.

Plant analyses and mycorrhiza measurements

Dry weights of shoots and roots were determined after drying at 80 °C for 24 h. Ground samples of shoots and roots were digested in a 4:1 nitric acid:perchloric mixture and the ^{33}P content in the digests was measured by liquid scintillation counting (Packard TR 1900). Total P content of the digests was determined by a molybdate blue method (Murphy and Riley 1962) on a Technicon Autoanalyzer II.

Fresh root subsamples were cleared and stained as in Kormanik and McGraw (1982), except that trypan blue was used instead of acid fuchsin, and the fraction of root lengths colonised by the AM fungus were determined. Root lengths were measured in accordance with the technique described by Newman (1966). Hyphal length density of the AM fungi in root-free soil was measured by a gridline-intersect method as in Jakobsen et al. (1992).

Detection of bacteria

The number of *P. fluorescens* DF57 was determined by immunofluorescense microscopy (IFM). Soil samples (1 g) were suspended in 10 ml of a 2% formaldehyde solution and stored at 5 °C for a maximum of 2 weeks. Soil suspensions were then sonicated for 5 min (Ultrasons, P Selecta), shaken for 20 min at 300 rpm and diluted 100-fold in a 2% formaldehyde solution. The suspensions were filtered through a black 0.2-μm-pore-size polycarbonate filter (Nuclepore; Costar, Cambridge, Mass.). The immunostaining procedure was modified after Kragelund and Nybroe (1996) with a 20-min blocking step, a 24-h incubation with primary antibody (1:1000, Hansen et al. 1997) and a 3 h incubation with FITC-conjugated secondary antibody (Dako, F0205, 1:20). Filters were mounted on a microscope slide in No Fade medium (Johnson and de Noguira Araujo 1981) and the number of *P. fluorescens* DF57 cells was counted at × 1000 magnification using a Zeiss Axiovert 35M fluorescence microscope.

Total numbers of bacteria in root and root-free compartments were determined by direct counting of bacteria after staining with acridine orange (AODC). Bacteria were prepared for staining as described for immunodetection above. The polycarbonate filters were placed on a 0.04% AODC solution diluted in 2% formaldehyde for 5 min and the total number of bacteria then counted by fluorescence microscopy as described above.

Results

Effects of *P. fluorescens* DF57 on growth of the mycorrhizal fungi

The root length colonised by each AM fungus was unaffected by the presence of *P. fluorescens* DF57, but a greater length of root was colonised by *G. intraradices* than by *G. caledonium* (Table 1).

The hyphal length density of *G. intraradices* was about 23 m hyphae g^{-1} dry soil and was also unaffected by *P. fluorescens* DF57, whereas the hyphal length density of *G. caledonium* was significantly higher in soil with *P. fluorescens* DF57 than in soil without the bacteria (Fig. 1). The average hyphal length density in soil of non-mycorrhizal plants was 3.08 ± 0.22SE m g^{-1} dry soil. Hyphal measurements were corrected for this background value. The stimulating effect of *P. fluorescens* DF57 on hyphal growth of *G. caledonium* was also observed in a preliminary experiment (data not shown).

Table 1 Dry weights, root lengths and colonised root lengths of cucumber plants within three different mycorrhizal treatments. Letters after figures refers to a multiple range test ($LSD_{0.05}$). The *No AM fungus* treatment was not included in statistical analysis of root length colonised by AM

Mycorrhizal treatment	*Pseudomonas fluorescens* DF57	Shoot dry weight (g)	Root dry weight (g)	Total root length (m)	Root length colonised by AM (m)
No AM fungus	−	2.47	0.40a	15.3abc	0
	+	2.49	0.42a	13.1a	0
Glomus intraradices	−	2.18	0.57b	17.7c	16.9b
	+	2.46	0.74c	16.0bc	15.0b
Glomus caledonium	−	2.26	0.47ab	13.6ab	10.2a
	+	2.53	0.47ab	15.1abc	10.8a
Two-way Anova					
Mycorrhiza		*P* 0.622	***	*	***
P. fluorescense DF57		*P* 0.153	*P* 0.111	*P* 0.309	*P* 0.560
Interaction		*P* 0.674	*P* 0.179	*P* 0.129	*P* 0.265

* $P<0.05$, $n=4$
*** $P<0.001$, $n=4$

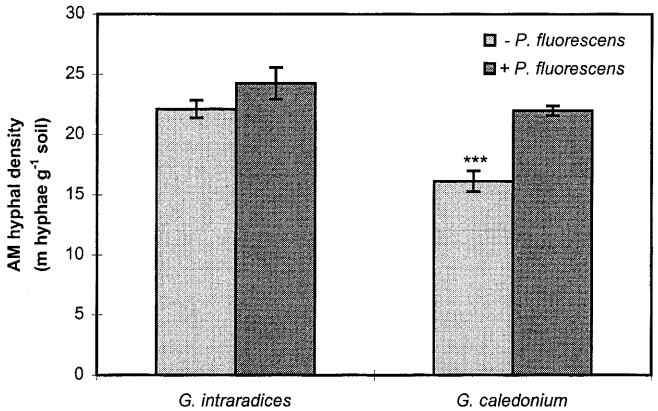
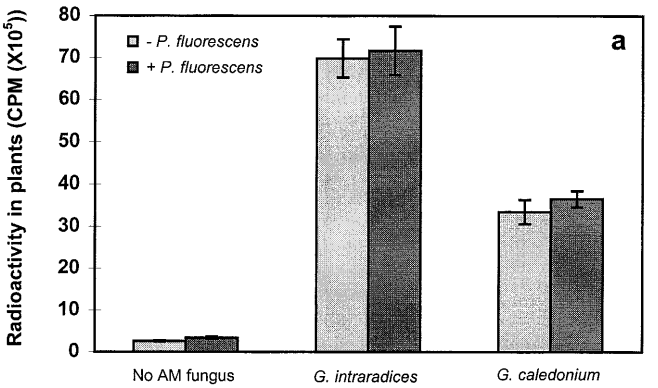


Fig. 1 Hyphal length density of *Glomus intraradices* and *G. caledonium* in root-free soil without and with *Pseudomonas fluorescens* DF57. $LSD_{0.05}$ value for the data set was 2.8 m hyphae g^{-1} soil. *Small bars* represents SEM. *Asterisks* above the bars refer to a one-way analysis of variance between treatments without *P. fluorescens* DF57 and with *P. fluorescens* DF57 (***$P<0.001$, $n=4$)



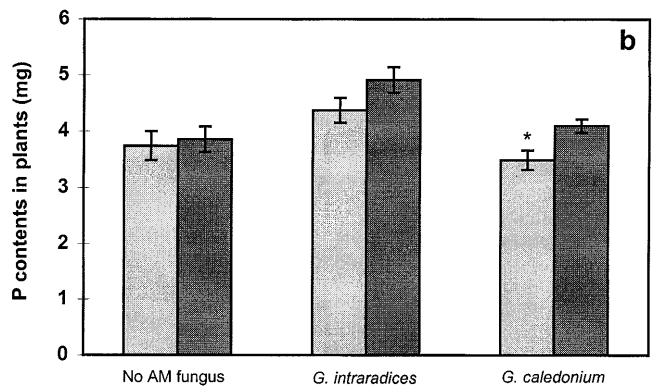


Fig. 2 Contents of ^{33}P (**a**) and total P (**b**) in cucumber plants with three different mycorrhizal treatments: none, *Glomus intraradices* and *G. caledonium*. The two bars within each mycorrhizal treatment represent two bacterial treatments, one without and one with *Pseudomonas fluorescens* DF57. $LSD_{0.05}$ values for the data were 10^6 cpm and 0.67 mg in **a** and **b**, respectively. *Bars* represent SEM. The *asterisk* above the bar refers to a one-way analysis of variance between treatments without *P. fluorescens* DF57 and with *P. fluorescens* DF57 (*$P<0.05$, $n=4$)

Effects of *P. fluorescens* DF57 on AM fungal P uptake and total P content in plants

The AM fungal transport of ^{33}P from root-free compartments to cucumber plants was unaffected by the presence of *P. fluorescens* DF57 (Fig. 2a). The ^{33}P uptake from root-free compartments was, however, markedly higher with *G. intraradices* than with *G. caledonium* (Fig 2a).

The total P content was significantly higher in cucumber inoculated with *G. intraradices* than in plants without mycorrhizas or in plants colonised by *G. caledonium* (Fig. 2b). The P content in plants was not affected by *P. fluorescens* DF57 only, but plants with dual inoculation of *P. fluorescens* DF57 and *G. caledonium*

had a higher P content than plants inoculated with *G. caledonium* alone (Fig. 2b). Shoot P concentrations and P contents (data not shown) responded to mycorrhizas and to *P. fluorescens* DF57 similar to total plant P contents.

Effects of AM fungi and *P. fluorescens* DF57 on plant growth

Neither the AM fungi nor *P. fluorescens* DF57 affected shoot dry weights, but *G. intraradices* enhanced root dry weights compared with control plants (Table 1). Root dry weights of plants with dual inoculation of *G. intraradices* and *P. fluorescens* DF57 were significantly higher than those of plants in other treatments (Table 1). Root lengths of non-mycorrhizal plants with *P. fluorescens* DF57 were significantly lower than those of plants colonised by *G. intraradices*. Root lengths of plants inoculated with *G. caledonium* alone were significantly lower than those of plants inoculated with *G. intraradices* alone (Table 1).

Effects of AM fungi on number of bacteria

The number (IFM) of *P. fluorescens* DF57 was significantly higher in root-free soil with mycorrhizal hyphae than in soil containing no AM fungal hyphae (Table 2). The mean background value of IFM counts in soil without *P. fluorescens* DF57 was 1.1×10^6 cells g^{-1} soil. The total number of bacteria estimated by AODC was generally higher in soil with mycorrhizal fungi than in soil without the fungi, both in the root-free and the root compartments. In root-free soil, the total number of bacteria estimated by AODC was higher with *G. caledonium* and *P. fluorescens* DF57 than in the other treatments (Table 2).

Discussion

Pseudomonas fluorescens DF57 did not affect AM fungal colonisation of plants in the present experiment. This is in accordance with results of Burla et al. (1996), who showed that two different strains of *P. fluorescens* did not affect colonisation of cotton by *G. mosseae*. However, another plant growth-promoting bacterium, *P. putida,* which is taxonomically closely related to *P. fluorescens* (Palleroni 1984), increased colonisation of roots by AM fungi (Meyer and Linderman 1986; Gryndler and Vosátka 1996). *P. fluorescens* DF57 did stimulate hyphal length density of external hyphae of *G. caledonium* in the present experiment. This is in accordance with results of Gryndler and Vosátka (1996), who found a stimulating effect of *P. putida* not only on hyphal growth but also on dehydrogenase activity of *G. fistulosum*. They tested the effect of whole bacteria cells and also effects of different culture fractions of *P. putida* on AM fungal growth. Their results indicate that the stimulating effect of the bacterium on the AM fungus was due to release of biologically active molecules from the bacteria.

Stimulated AM hyphal growth should result in an increase in hyphal P uptake from soil, but the increased hyphal growth of *G. caledonium* observed in this study did not produce a higher uptake of ^{33}P from the hyphosphere soil. This might be explained by immobilisation of P in other microorganisms, as the total number of bacteria in the hyphosphere soil was significantly higher in the treatment with both *G. caledonium* and *P. fluorescens* DF57 than in other treatments. However, since *P. fluorescens* DF57 did not influence AM fungal P uptake from hyphosphere soil in the present experiment, there appeared to be no competition for P between this particular bacterium and the two AM fungi. A further explanation is that the fungus used more P for its own growth and metabolism or that the stimu-

Table 2 The influence of *Glomus intraradices* on the number of *P. fluorescens* DF57 estimated by immunofluorescens microscopy (IFM) in root-free compartments and on total number of bacteria stained with acridine organe (AODC) in root-free and root compartments, respectively. Letters after figures refers to a multiple range test ($LSD_{0.05}$)

Mycorrhizal treatment	*Pseudomonas fluorescens* DF57	IFM: root-free soil (cells g^{-1} soil)	AODC: root-free soil (cells g^{-1} soil)	AODC: soil with roots (cells g^{-1} soil)
No AM fungus	−	−	9.00 ± 10^8a	7.43×10^8a
	+	1.55×10^7a	1.09×10^9ab	7.77×10^8a
Glomus intraradices	−	−	1.11×10^9b	1.18×10^9bc
	+	2.16×10^7b	1.16×10^9b	1.34×10^9c
Glomus caledonium	−	−	1.19×10^9b	1.20×10^9bc
	+	2.04×10^7b	2.02×10^9c	1.06×10^9b
Two-way Anova				
Mycorrhiza		**	***	***
P. fluorescens DF57		−	***	*P* 0.792
Interaction		−	***	*P* 0.181

** $P < 0.01$, $n = 4$
*** $P < 0.001$, $n = 4$

lated growth of the external mycelium of the fungus caused a delay in P transport to the plant.

Pseudomonas fluorescens DF57 did not affect plant growth but increased the total P content in plants colonised by *G. caledonium*. A synergistic effect of dual inoculation with a mix of different species of AM fungi and *P. putida* on P concentrations in plants was also observed by Meyer and Linderman (1986). However, dual inoculation with *P. fluorescens* DF57 and *G. intraradices* led to no synergistic effect on plant P uptake in the present work. These contrasting results might be related to the use of different AM species. Andrade et al. (1997) showed that six AM fungal isolates had different effects on the composition of the microbial community in the rhizosphere. Similarly, Secilia and Bagyaraj (1987) found that the effect of four species of AM fungi on the microbial community in soil varied. They suggested that different AM fungi affect the release of root exudates differently and that this is responsible for the different effects of the AM fungi on the microbial community. However, a change in the microbial community responsible for decomposition of organic matter could also lead to changes in the amounts of nutrients available to the plants and, thereby, the release of inorganic nutrients in soil.

The AM fungi generally increased the number of bacteria both in rhizosphere and hyphosphere soil in the present experiment. These results are in contrast to those of Christensen and Jakobsen (1993), who reported an adverse effect of AM on number of bacteria in the rhizosphere. Olsson et al. (1996) used two different AM fungal species and found either no effect or a stimulating effect of AM on total number of bacteria in the rhizosphere, and either no effect or an adverse effect of AM fungi in hyphosphere soil. These three studies are similar in their use of AODC to obtain the total number of bacteria, and cucumber was grown in the same field soil in all experiments. However, it can not be excluded that the use of different soil:sand ratios influenced the results, as the results were similar when the same AM fungus and the same substrate was used as in the present experiment and in the study of Olsson et al. (1996). Both experiments showed a stimulating effect of AM on the total number of bacteria in the 1:1 (soil:sand) rhizosphere of cucumber in symbiosis with *G. caledonium*. Thus the influence of AM on the number of bacteria in soil may depend on both the combination of species in the symbiosis and the composition of the substrate.

Overall, these results show that the effect of *P. fluorescens* DF57 on growth of the AM fungi varies with the AM fungal species and that a stimulating effect on growth of external mycelium of the fungus is not necessarily reflected in a higher P uptake of the AM fungus. This emphasises that a quantification of fungal structures can be a poor measure of mycorrhizal functioning. The absence of increased hyphal P uptake in response to the hyphal growth increase could be due to competition for P between the AM fungus and microorganisms other than *P. fluorescens* DF57, but this needs further investigation.

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