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Milan Gryndler · Miroslav Vosátka

The response of *Glomus fistulosum–maize* mycorrhiza to treatments with culture fractions from *Pseudomonas putida*

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Abstract This study examined which culture fraction of the plant-growth-promoting bacterium *Pseudomonas putida* (Trevisan) Migula has an effect on growth and mycorrhiza formation of maize (*Zea mays* L.). Shoot dry weight and total leaf area of plants did not increase after inoculation with *Glomus fistulosum* but were significantly higher than the controls when the plants were dual inoculated with *G. fistulosum* and living cells of *P. putida*. Mycorrhizal infection of the roots was significantly higher when plants were inoculated with *G. fistulosum* together with living cells of *P. putida* or with *G. fistulosum* and dialysed cell extracts of *P. putida* than with *G. fistulosum* alone. Development of arbuscular mycorrhizal (AM) extraradical hyphae and the proportion of extraradical hyphae showing NADH diaphorase activity were significantly enhanced by inoculation of plants with living cells of *P. putida* or dialysed cell extracts of *P. putida*. No stimulation of extraradical hyphae proliferation from in vitro incubated mycorrhizal root segments was observed after application of culture fractions of *P. putida*. However, the percentage contamination of the root segments by extraneous filamentous fungi significantly decreased in the presence of living cells of *P. putida*.

Key words Arbuscular mycorrhizal fungi (AMF) · Bacterial culture fractions · Dehydrogenase vital staining · Extraradical mycelium

Introduction

The saprophytic microflora of the soil transforms organic matter, utilises root exudates and decomposes plant residues; soil bacteria are an important part of the soil microflora. Interactions between various groups of soil bacteria and arbuscular mycorrhizal fungi (AMF) have often been observed (e.g. Barea and Azcón-Aguilar 1982; Meyer and Linderman 1986a), but the mechanism of interaction is still not completely understood. There are some reports of stimulatory effects of bacteria that produce plant growth regulators (Azcón et al. 1978). Knowledge of interactions could be important for the practical application of mycorrhizal inocula because bacterisation (inoculation with bacterial cells) together with inoculation with AMF has been proposed, not only in soil or in soil-based cultivation substrata but also in completely artificial materials (Linderman 1992). Fluorescent pseudomonads are often used in the biological control of plant diseases caused by fungi (Howel and Stipanovic 1980) and for stimulation of plant growth (Vancura 1989), and could be combined with AMF in plant protection preparations. Studies of the interactions between fluorescent pseudomonads and two AMF species by Paulitz and Linderman (1989) indicated that antibiotic-producing strains might delay the germination of spores of mycorrhizal fungi, but that the colonization of the root remained unaffected. The effect of inoculation with the plant-growth-promoting bacterium *Pseudomonas putida* on the development of mycorrhizal infection was reported to be time dependent (Meyer and Linderman 1986b), the stimulatory effect being observed during the early stages of infection. Vosátka et al. (1992) observed a synergistic positive effect of *P. putida* and five different mycorrhizal fungi on the growth and yield of micropropagated strawberry. The fluorescent pseudomonads are potentially pathogenic for animals and man. Thus, if the beneficial effects of these biocontrol agents are due to the production and release of biologically active compounds, such cultural products could be applied instead of living bacterial cells.

In this present study we investigated the effect of various culture fractions of *P. putida* on the growth and formation of mycorrhiza of maize (*Zea mays* L.) grown in an artificial Perlite-sand based substratum.

M. Gryndler (✉)
Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnská 1083, CZ-14220 Praha 4, Czech Republic

M. Vosátka
Institute of Botany, Academy of Sciences of the Czech Republic, CZ-25243 Pruhonice, Czech Republic

Materials and methods

Fractionation of bacterial cultures

Bacterial cells of *P. putida*, strain K11 (Institute of Microbiology, Prague) were cultivated in 500-ml flasks in medium T3 (Taylor 1951) shaken at 28 °C for 48 h. The culture was then centrifuged at 20 000 *g* for 30 min. The supernatant was filtered through a nitrocellulose filter (0.22 μm pore diameter) and then dialysed against 0.1% (w/v) $MgSO_4.7H_2O$ for 20 h using dialysis tubing which retains macromolecules with a molecular weight greater than 12 000 (fraction S, dialysed culture liquid). The sedimented cells in centrifuge tubes were washed three times in 0.1% (w/v) $MgSO_4.7H_2O$. One-third of the cells was collected and designated fraction L (washed living cells). A further third of the cell suspension was heated at 100 °C for 60 min to give fraction H (cells killed by heat). The remaining third portion of cells were disintegrated by passing three times through a French press (Aminco, SLM Instruments, 103 bar), centrifuged (see above), filtered through a 0.22-μm pore nitrocellulose filter, and divided into two parts. One part was designated fraction C (the cell extract), and the second portion was dialysed against 0.1% (w/v) $MgSO_4.7H_2O$ for 20 h to give fraction CD (the dialysed cell extract). The oxidisable carbon in all fractions (dialysed culture liquid, living cells, heated cells and crude and dialysed cell extracts) was estimated using a dichromate oxidation method with anhydrous glucose as the reference standard (Bragdou 1951). All preparations were diluted to give an equal carbon content equivalent to 302 mg l^{-1} glucose.

Experiment 1

Maize plants (CE 240) were cultivated in a hydroponic cultivation system. Plastic tubes (20 cm in length, 5 cm in diameter) were sealed with gauze at the bottom and filled with 150 ml of Perlite overlaid by 50 ml of a mixture of Perlite and steamed soil (5/1, v/v). The sandy-loam soil was steamed three times for 6 h at 80 °C. This layer was inoculated with 300 surface-disinfected (2% w/v Chloramine T for 4 min) spores of *Glomus fistulosum* (Skou and Jacobsen) and supplied with 1 ml of the bacterial preparations. Control treatments and the treatment with the mycorrhizal fungus alone included by 1 ml of 0.1% $MgSO_4.7H_2O$. A pregerminated maize seed was then put into each tube and covered by 50 ml of Perlite, with 14 plants cultivated per treatment under constant environmental conditions in a growth chamber: 16 h photoperiod (330 μE PAR), 23 °C/70% RH humidity by day, 18 °C/75% RH by night. The plastic tubes were kept in a plastic tub with 2 l of nutrient solution containing (mg l^{-1}): $MgSO_4.7H_2O$ 720, KH_2PO_4 12.2, $Ca(NO_3)_2.4H_2O$ 295, KNO_3 240, $MnCl_2.4H_2O$ 0.75, KI 0.75, $ZnSO_2.H_2O$ 0.75, H_3BO_3 1.5, $CuSO_4.5H_2O$ 0.001, FeNaEDTA 4.3 and $Na_2MoO_4.2H_2O$ 0.00017. The solution was renewed twice a week. The plants were harvested after 7 weeks cultivation and a part of each root system was washed, cleared in 10% KOH and stained using trypan blue (Phillips and Hayman 1970) to estimate the percentage of root length infected using the grid-line intersect method (Giovannetti and Mosse 1980). The percentage root length with developed root hairs was estimated using the same method. Maize leaf area was measured by a photo-area-meter (LI3100; LI-Cor Inc.).

The length and dehydrogenase (NADH diaphorase) activity of the AMF extraradical hyphae attached to the root surface and the hyphae in the substratum were determined using the method of iodonitrotetrazolium (INT) staining (Sylvia 1988). Root segments (1 cm) were incubated in 10-ml glass tubes with INT solution for 14 h at 28 °C. The segments were then stained with 0.5% trypan blue in lactophenol for 2 h and washed in distilled H_2O, and 40 randomly selected microscopic fields were evaluated along each segment. The lengths of active (stained red and containing granules of reduced INT stain) and inactive hyphae (blue, counterstained by trypan blue) per 1 mm of root were measured using the grid-line intersect method (Giovannetti and Mosse 1980). Aliquots (5 g) of substratum were weighed, mixed in a 500-ml beaker with tap water and poured through a 250-μm sieve into a blender. The sample was blended for 20 s and a 5-ml subsample of the supernatant was pipetted onto a membrane filter (4.5 cm diameter, 0.6 μm pore size). The lengths of active and inactive hyphae were measured on 40 randomly selected microscopic fields within each filter and expressed as the length per gram of dry substrate.

Experiment 2

Bacterial fractions (diluted as described above) or 0.1% (w/v) $MgSO_4.7H_2O$ as a control were used as media for incubation of mycorrhizal root fragments. Fine roots of 7-week-old maize heavily colonized with *G. fistulosum* were surface disinfected with sodium hypochlorite (2% w/v, 2 min), cut into approximately 2-mm segments and incubated in Petri dishes in hanging drops of bacterial preparation at pH 7.0. The dialysed supernatant of the bacterial culture and both cell extracts were filter sterilised before use. Sixteen root segments were cultivated per Petri dish with 5 replicate dishes per treatment. After 8 days of incubation, the proliferation of coarse aseptate hyphae (considered as AMF hyphae) growing out from the root segments was evaluated. Contamination of root segments by other fungi was also recorded. The results were expressed as the mean percentage of segments showing proliferation or fungal contamination.

Data analysis

The data were analysed by one-way ANOVA and Duncan's multiple range test.

Results

Experiment 1

Root systems in all treatments inoculated with the mycorrhizal fungus were mycorrhizal, whereas no mycorrhizas were found in the control treatment (Table 1). Mycorrhizal infection was significantly stimulated only by living bacterial cells, compared with the treatment

Table 1 Effect of culture fractions of *Pseudomonas putida* (*L* living cells, *H* cells killed by heat, *C* cell extract, *CD* dialysed cell extract, *S* dialysed culture liquid) on mycorrhizal root colonisation, sporulation and the abundance of root hairs. Means followed by the same letters are not significantly different ($P = 0.05$). For percentage of infection n = 14, number of spores and root hairs n = 7

Treatment	Mycorrhizal infection (%)	Number of spores (per g of substratum)	Root hairs (%)
Control	0	4*	20 bc
Glomus fistulosum	19.5 bc	65 ab	19 b
G. fistulosum + L	30.7 a	72 a	33 ab
G. fistulosum + H	17.6 c	48 b	30 ab
G. fistulosum + C	15.9 c	56 ab	23 ab
G. fistulosum + CD	25.4 ab	66 ab	24 ab
G. fistulosum + S	16.1 c	56 ab	37 a

* Residual spores from the sterile soil added to the cultivation medium

Table 2 Effect of culture fractions of *P. putida* on the length and dehydrogenase (DH) activity of extraradical mycelium of *Glomus fistulosum* in the substratum and attached to the root surface. Abbreviations as in Table 1. Means followed by the same letters are not significantly different ($P=0.05$, n = 7)

Treatment	Mycelium in substratum (m/g)		Mycelium on the root surface (mm/mm)	
	Total	DH active	Total	DH active
Control	0.07	0.01	0.006	0.001
G. fistulosum	1.38 c	0.44 c	0.095 ab	0.031 b
G. fistulosum + L	2.44 ab	1.34 ab	0.139 ab	0.088 a
G. fistulosum + H	1.58 bc	0.88 abc	0.078 ab	0.039 b
G. fistulosum + C	1.24 c	0.56 c	0.062 b	0.021 b
G. fistulosum + CD	2.62 a	1.46 a	0.154 a	0.092 a
G. fistulosum + S	1.65 bc	0.82 bc	0.080 ab	0.035 b

Table 3 Effect of *G. fistulosum* inoculation and culture fractions of *P. putida* on shoot dry weight, shoot/root ratio and total leaf area of maize. Abbreviations as in Table 1. Means followed by the same letters are not significantly different ($P=0.05$, n = 14)

Treatment	Shoot dry weight (g)	Shoot/ root ratio	Total leaf area (cm^2)
Control	3.00 b	2.57 a	626 b
G. fistulosum	3.53 ab	2.62 a	690 ab
G. fistulosum + L	4.44 a	2.89 a	789 a
G. fistulosum + H	4.05 ab	2.75 a	701 ab
G. fistulosum + C	4.01 ab	2.64 a	691 ab
G. fistulosum + CD	3.99 ab	2.76 a	730 ab
G. fistulosum + S	3.78 ab	2.46 a	656 b

Table 4 Effect of culture fractions of *P. putida* on the proliferation of extraradical mycelium growing from root segments colonised by the arbuscular mycorrhizal fungus *G. fistulosum* and on the frequency of segments showing contamination by other fungi. Abbreviations as in Table 1. Means followed by the same letters are not significantly different ($P=0.05$, n = 5)

Treatment	Proliferation (%)	Contamination (%)
G. fistulosum	11.2 ab	28.8 b
G. fistulosum + L	13.7 ab	2.5 c
G. fistulosum + H	13.5 ab	40.0 ab
G. fistulosum + C	1.2 b	35.0 ab
G. fistulosum + CD	21.2 a	36.3 ab
G. fistulosum + S	13.7 ab	46.3 a

with mycorrhizal fungus alone, and no inhibitory effects of culture fractions on the mycorrhizal infection were found. The highest number of root hairs was found when plants received the dialysed culture liquid. The amounts of both total and active AMF extraradical mycelium in the substratum (Table 2) were significantly stimulated by addition of living bacterial cells and by the dialysed cell extract, compared with the treatment with the mycorrhizal fungus alone. The same trend was also observed for mycelium associated with the root surface: the active mycelium was significantly stimulated by living bacterial cells and by dialysed cell extract, whereas the increase in total length of aseptate hyphae was significant only for the dialysed cell extract. As shown in Table 3, plant shoot dry weight and leaf area were only significantly different from uninoculated controls in treatments inoculated simultaneously with the mycorrhizal fungus and living bacterial cells. The shoot-root ratio was not affected by any of the experimental treatments.

Experiment 2

In vitro observation showed that the proliferation of AMF extraradical mycelium from the root segments was inhibited by dialysed bacterial culture liquid (Table 4) when compared with the control treatment. None of the bacterial culture fractions significantly stimulated proliferation of hyphal growth. Higher amounts of coarse aseptate hyphae were observed in treatments with dialysed cell extract. The hyphal lengths recorded in this treatment were significantly higher than treatments with the cell extract. The percentage contamination by other fungi was significantly lower in the presence of living bacterial cells, and was significantly higher when the bacterial culture liquid (S) was used as the incubation medium than in the treatment without the bacterial fraction.

Discussion

Our results show a stimulatory effect of living bacterial cells of *P. putida* on the development of mycorrhizal infection and extraradical mycelium of *G. fistulosum*. A similar effect of pseudomonads has previously been observed for early stages of root colonisation by mycorrhizal fungi (Meyer and Linderman 1986b). Stimulation of hyphal growth was also observed when surface-sterilised spores of *G. mosseae* were supplemented by rhizospheric bacteria culture as well as by the cell-free supernatant of the culture (Azcón 1987).

There are several possible mechanisms for the stimulatory effects. Bacteria may produce some biologically active molecules that directly or indirectly (*via* plant physiology) affect the mycorrhizal fungus. Fluorescent pseudomonads (like *P. putida*) produce numerous metabolites, including the plant growth regulators auxins, gibberellins and ethylene, biotin, nicotinic acid and

pantothenic acid, which affect the growth of plants and microorganisms in soil (Hussain and Vancura 1970). The production of physiologically active concentrations of indole-3-acetic acid and some other auxin molecules has been reported for *P. putida* (Prikryl et al. 1985). All these products are small molecules with molecular weights lower than 10 000 and can pass through dialysis membranes. The stimulation of mycelial growth by the dialysed cell extract in our experiments excludes the possibility that only small molecules caused the effect. The effect of *P. putida* on plant biomass accumulation can be attributed to antibiotic activity by the bacterium. The suppression of contamination by other fungi on root segments in vitro showed that the bacteria are able to inhibit the growth of saprophytic micromycetes which grow in the rhizosphere and inhibit plant growth in hydroponic culture. Compared with the dialysed cell extract, the crude extract significantly inhibited the development of intraradical infection and extraradical mycelium of *G. fistulosum* in experiment 1 (Tables 1, 2) but inhibited the growth of extraradical mycelium in vitro in experiment 2 (Table 4). This suggests the presence of some low molecular weight suppressive compounds. Most bacterial extracellular products are polysaccharides continuously produced in the soil either as root exudates or during the decomposition of organic compounds (Wagner and Tang 1976). If added to soil, they can induce changes in the structure of soil microbial populations (Andreyuk and Iutinskaya 1989). There are few reports of the production of extracellular polysaccharides by fluorescent pseudomonads (Eagon 1956) but these bacteria rapidly utilise polysaccharide compounds (Vancura et al. 1979), which correlates with their extensive proliferation in the rhizosphere of various plants (Vancura 1980). This may cause qualitative and quantitative changes in the population of saprophytic microbes as well as mycorrhizal fungi. In our experiment, the dialysed macromolecular products of the bacterial culture caused no positive growth response of AMF extraradical mycelium or root infection, so that extracellular polysaccharides or other macromolecules are probably not responsible for this effect.

The mechanism of plant growth stimulation by fluorescent pseudomonads may involve competition with deleterious microbes for available iron (Kloepper et al. 1980). The iron-chelating capacity of fluorescent pseudomonads increases with soil water content (Mamoun and Olivier 1990), and such conditions are common in hydroponic culture, which is saturated continuously with nutrient solutions. This is probably not the basis of the stimulatory effects in our experiment, because control plants were not damaged by any pathogen, and the culture was constantly supplied with nutrient solution containing Fe. Unlike crude cytoplasm, the dialysed cytoplasm produced a stimulatory effect on the mycorrhizal fungus. This suggests the presence of some macromolecular compounds with molecular weights higher than 10 000, or their degradation products, which must act at very low concentrations, because the organic molecules applied per plant at the start of the experiment corresponded to a c-equivalent of only 302 μg glucose.

The addition of very small amounts of organic matter to a nonsterile substratum initially poor in organic carbon, may cause remarkable changes in microbial populations. These microorganisms can further influence the behaviour of propagules of mycorrhizal fungi due to the production of bioactive compounds. The validity of this explanation and the nature of the possible compounds involved will be the subject of future research.

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