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SELECTIVE INTERACTION BETWEEN FREE-LIVING RHIZOSPHERE BACTERIA AND VESICULAR–ARBUSCULAR MYCORRHIZAL FUNGI

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Summary—Plant growth and nutrient uptake of tomato (*Lycopersicum esculentum*) were studied in a sand–vermiculite medium inoculated with two rhizosphere bacteria (A or P) and three vesicular–arbuscular mycorrhizal (VAM) fungi (*G. mosseae*, *G. fasciculatum* or *G.* sp; E_3 type). A selective interaction between free-living rhizosphere microorganisms and VAM fungi was tested. The effect of *Glomus* species on plant growth and nutrient content was related to the bacterial associated groups. Generally, bacterial inoculation increased the growth of mycorrhizal plants. Inoculation with A did not affect responses to *G. mosseae* treatment. Mycorrhizal plants with *G. fasciculatum* showed increased growth with A or P inoculation. This effect was significantly greater with dual A plus P inoculation. In the case of *G.* sp. (E_3 type) P treatment did not increase plant growth. Germination, hyphal growth and vegetative spores production of *G. mosseae* spores, cultivated *in vitro* under axenic conditions, were increased in the presence of P or A bacterial inoculum. P was more effective than A. The effects of A and P were not correlated with the degree of mycorrhizal colonization quantified at the end of the experiment.

INTRODUCTION

Little is known about the interrelationships between vesicular–arbuscular (VA) endomycorrhizal fungi and ubiquitous soil-inhabiting microorganisms. Vesicular–arbuscular mycorrhizal (VAM) and rhizosphere soil organisms interactions have mainly been observed on selected non-pathogenic microorganisms involved in nutrient transformations (Azcón and Barea, 1975; Bagyaraj and Menge, 1978; Brown and Carr, 1979; Subba Rao *et al.*, 1985). The objective of studying these dual VAM fungal–bacterial interactions has focused upon obtaining an additive effect on plant growth by microbial inoculants acting singly or in a concerted form.

VAM fungi and rhizosphere microorganisms can also influence their mutual development which might result in a synergistic interaction. It is known that introduced saprophytic bacteria can stimulate the growth of spores of *G. mosseae* (Azcón, 1987) and the percentage of root length colonized by the mycorrhizal fungus (Azcón-Aguilar and Barea, 1978; Barea *et al.*, 1983; Brown and Carr, 1979). Also, the bacterial growth in the presence of VA mycorrhizas was increased (Ames *et al.*, 1984; Barea *et al.*, 1975; Raj *et al.*, 1981). The host plant might also influence microbial interactions within the rhizosphere since plant physiology and root metabolism are affected by the introduction of such organisms.

Any of these singly or combined facts have relevance to VAM fungal and bacterial interactions. In unpublished studies particular effective VA mycorrhizal fungal–bacterial associations have been observed, depending on the selected microorganisms. It has been shown that VA endophytes vary enormously in their symbiotic effectiveness depending on the associated microbial population. Results are presented on the effect of two soil bacteria in association with three endomycorrhizal *Glomus* species on tomato plants. Tomato growth and nutrient content was measured to see the effect of each bacterial–*Glomus* strain combination. The study was conducted in a sand–vermiculite medium. A second experiment was carried out *in vitro* in axenic conditions and in the absence of the host-plant to test microbial interactions amongst themselves. In this assay we determined the effect of the two bacterial cultures on the germination, hyphal growth and production of new spores by *Glomus mosseae* spores.

MATERIALS AND METHODS

Tomato (*Lycopersicum esculentum*) plants were grown in sand–vermiculite (1:1 v/v) medium which had been washed and sterilized by autoclaving and distributed in 300 ml capacity pots. At sowing, plants were inoculated with one of three endomycorrhizal *Glomus* species: *Glomus mosseae* (Nicol and Gerd.) Gerd. and Trappe; *Glomus fasciculatum* (Taxter sensu Gerd.) Gerd. and Trappe or *Glomus* sp. (unidentified form of *Glomus*, type E_3). VAM endophytes came from the stock culture collection. The three *Glomus* species were cultured on *Medicago sativa* that were grown under greenhouse controlled conditions for 6 months and afterwards stored for several months in polyethylene bags at 5°C. Mycorrhizal inocula were placed into the planting hole (3 g per pot). Soil containing spores, mycelium and infected root fragments were used as inocula. The three mycorrhizal fungi were assayed as single inocula and with each free-living rhizosphere bacterium separately or in dual bacteria combination. Pots were supplied with filtered washings of a natural soil to provide the natural microflora. In this way a similar microflora was established in all the treatments.

The saprophytic microorganisms assayed were *Azotobacter vinelandii* (A) strain ATCC 12837 selected on the basis of its ability to produce aminoacids and vitamins (González-López *et al.*, 1983). This organism was grown in nitrogen-free medium (Wilson and Knight, 1952) in 250 ml flasks. The other bacteria (P) was a strain belonging to the *Enterobacteriaceae* family isolated from local soil, pH = 7.4 (water). It was grown in soil extract enriched nutrient broth (8 g l^{-1}). Both bacteria were grown in shake culture at 28°C for 14 days in 250 ml flasks containing 50 ml of medium. Two millilitres of bacterial cultures (10^8 colony forming units ml^{-1}) per pot, 1 ml per seed, were added to the corresponding treatments at sowing.

Plants were grown for 40 days under greenhouse conditions with temperatures ranging from 19 to 25°C and 16–8 h light–dark photoperiod. The relative humidity was 70–90%. Plants were fertilized twice weekly (50 ml pot-week) using the nutrient solution described by Hepper and O'Shea (1984). The pH was adjusted to 6.8–7.0. There were five replicates per treatment and two plants per pot.

Once plants were harvested the weight of shoots and roots were recorded and shoot tissue analysed for their N, P and K content. Part of the root systems were assessed for VAM infection by the stain method of Phillips and Hayman (1970). The percentage of total root length infected was calculated from the data obtained by the gridline intersect plate technique (Giovannetti and Mosse, 1980).

In the second experiment a single mycorrhizal species (*G. mosseae*) was used. This study was carried out in Petri dishes under axenic conditions in order to observe directly any bacterial–VA fungus interactions. *G. mosseae* sporocarps were obtained from the stock culture that had been utilized as inoculum in the first part of the experiment. Uniform and undamaged spores were selected from excised sporocarps. Spores were surface sterilized with a mixture of chloramine T (2%), streptomycin (0.03%) and a drop of Tween-80 (Mosse, 1962) for 20 min and washed several times in sterile deionized water prior to transferring them to water–agar (10 g l^{-1} of Difco Bacto agar) at pH 7. Spores were individually placed in Petri dishes at approx. 2 cm around a sterilized central disc (Whatman No. 1 filter paper) of 1 cm dia; 1 ml of the corresponding bacterial culture was added to the discs. Discs in control plates were amended with sterile culture medium used to grow the bacterium assayed in this experiment. There were 10 replicate plates per treatment with 6–8 single spores per replicate. Plates were sealed with parafilm and held in the dark at 25°C.

Sequential determination of germination, formation of new spores and mycelial growth were made after 4, 9, 13, 17 and 25 days of spore incubation. A spore was considered germinated when the germ-tube was clearly visible (2–3 mm at 40×). Further elongation was described as hyphal growth. To evaluate mycelial growth and development three categories: namely a = weak, b = moderate and good and c = strong and very strong were established according to the area covered by hyphal extension. The standard error was calculated for the mean number of germinated spores in each Petri dish.

Table 1. Shoot dry weight (mg) of *Lycopersicum* plants inoculated with VAM fungi and the free-living rhizosphere bacteria A and P

Mycorrhiza treatment	Bacteria treatment			
	None	P	A	A + P
G. mosseae	800f	1060dc	850fdc	1010fdc
G. fasciculatum	990dc	1500b	1400b	1870a
G. sp. (E_3 type)	850fd	940d	1280c	1120dc

Values followed by the same letter are not significantly different ($P \leq 0.05$).

RESULTS

The two A and P microorganisms assayed, in single or dual inoculation normally increased growth of mycorrhizal tomato plants (Table 1). Nevertheless, bacterial treatments had different effects according to the associated *Glomus* species. A was ineffective with *G. mosseae* and P with *G.* sp. but both were effective with *G. fasciculatum*. A plus P addition reached higher yield than single A or P applications in plants associated with *G. fasciculatum*. Conversely, *G.* sp. (E_3 type) mycorrhizal plants response to P or A plus P were not significantly different (Table 1). The root dry weight was also modified under different bacterial and mycorrhizal treatments (Table 2). A, P or A plus P inoculum did not increase weight of root in the *G. mosseae* treatment. Those bacteria, when inoculated together, showed a high efficiency in stimulating root weight in plants with *G. fasciculatum* and *G.* sp. Bacterial treatments did not change the R/S ratio in plants with *G. mosseae* and *G. fasciculatum* (Table 2). Nutrient data are presented both on the basis of concentration (percentage of dry matter, Table 3) and as total accumulation (the product of dry matter and concentration, Table 4). Because of differences in vegetative production between treatments, a dilution effect can be observed associated with growth (percentage of dry matter, Table 3).

The N, P and K plant content was affected by microbial treatments (Table 4). Plants infected with *G. mosseae* had an increased N, P and K content

Table 2. Root dry weight (mg) and R/S ratio of *Lycopersicum* plants inoculated with VAM fungi and the free-living rhizosphere bacteria A and P

Mycorrhiza treatment	Bacteria treatment							
	None		P		A		A + P	
	R	R/S	R	R/S	R	R/S	R	R/S
G. mosseae	325a	0.40x	394ab	0.37x	382ab	0.45xy	435abc	0.43xy
G. fasciculatum	471c	0.47xy	684d	0.45xy	579d	0.41x	984f	0.52y
G. sp. (E_3 type)	425b	0.50y	578d	0.61z	520cd	0.41x	654d	0.58z

Values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 3. Shoot N, P, K concentration (%) in *Lycopersicum* plants inoculated with VAM fungi and the free-living rhizosphere bacteria A and P

Mycorrhiza treatment	Nutrients (%)	Bacteria treatment None	P	A	A + P
G. mosseae	N	3.28ab	3.92bc	4.16bc	3.85b
	P	0.125z	0.123z	0.129z	0.129z
	K	4.38h	4.48h	4.60ht	4.50h
G. fasciculatum	N	3.38ab	3.08a	3.23ab	2.48a
	P	0.121z	0.093x	0.100xy	0.107xy
	K	3.88f	3.66f	4.24gh	4.38gh
G. sp. (E_3 type)	N	4.55c	3.85b	2.89a	3.25ab
	P	0.118y	0.106xy	0.086x	0.107xy
	K	4.18g	4.39h	3.91fg	4.67t

Values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 4. Shoot N, P, K, content (mg) in *Lycopersicum* plants inoculated with VAM fungi and the free-living rhizosphere bacteria A and P

Mycorrhiza treatment	Nutrients	Bacteria treatment None	P	A	P + A
G. mosseae	N	26.3a	41.6cd	35.4bc	38.9c
	P	1.0x	1.3yz	1.1x	1.3y
	K	35.0g	47.5g	39.1fg	45.5g
G. fasciculatum	N	33.5bc	46.2d	45.2d	46.4d
	P	1.2xy	1.4z	1.4z	2.05w
	K	38.5fg	55.0h	59.4h	82.0j
G. sp. (E_3 type)	N	38.7c	36.2c	37.0c	36.4c
	P	1.0x	1.0x	1.1x	1.2xy
	K	35.5f	41.3fg	50.0gh	52.3h

Values followed by the same letter are not significantly different ($P \leq 0.05$).

when they were associated with P or A plus P bacterial cultures. The two rhizobacteria, singly and especially in combination, enhanced the N, P and K content in the *G. fasciculatum* treatment. No effect of bacterial inoculation was found on N and P absorption by *G.* sp. mycorrhizal plants. A and A plus P increased K in this treatment.

The degree (%) mycorrhizal infection produced by the three *Glomus* species assayed (Table 5) was similar and bacterial inoculation slightly modified this response. In treatments with only A inoculation the percentage of root infection was slightly increased. Combined A plus P inoculum decreased such a response in *G. fasciculatum* and *G.* sp. plants. Bacterial treatments enhanced the root length infected in nearly all mycorrhizal treatments (Table 5).

Table 6 shows the effect of A and P on germination of *G. mosseae* spores *in vitro*. Spore germination was not changed by bacterial treatments (A or P) at 4 and

Table 6. Effect of the free-living rhizosphere bacteria (A and P) on germination spores of *G. mosseae*

Treatments	Percentage germination (times–days)[a] 4	9	13	17	25	30
C	3 ± 3	20 ± 13	42 ± 12	50 ± 15	68 ± 9	68 ± 9
A	16 ± 15	21 ± 16	58 ± 17	92 ± 13	95 ± 8	95 ± 8
P	5 ± 7	17 ± 11	73 ± 15	87 ± 15	90 ± 10	90 ± 10

[a]Days after *G. mosseae* spores were inoculated into the Petri dish on water–agar medium. SEMs are given ($P = 0.05$) on the mean of number of spores per Petri dish.

9 days. In the subsequent determinations some effect can be observed in the presence of these bacteria.

A significant increase in hyphal growth and secondary vesicles formation occurred in A or P treatments (Table 7). The first new vegetative vesicles were observed after 9 days of incubation of spores in presence of P inoculum. In the treatment without bacterial inoculation, secondary vesicles were observed at 25 days. P inoculum produced the highest stimulation of growth of *G. mosseae* spores considered as hyphal elongation and new secondary vesicles formed.

DISCUSSION

The results show that the effect of *Glomus* species on plant growth and nutrition is related to the associated bacterial groups. The data indicate a *Glomus* species–bacteria strains interaction in which certain specific effects can be observed. A notable result is the selective effect of A, P and A plus P on each *Glomus* species assayed (Tables 1, 2, 4 and 5). The rhizosphere bacteria assayed did not decrease plant growth and nutrient content in any *Glomus* treatment. A positive effect of bacterial–fungal inocula was evidenced in some cases. P-*Glomus* sp. and A-*G. mosseae* inoculation did not significantly increase the effect of each endophyte. Mycorrhizas and rhizosphere microorganisms can influence the mutual development of each other. The associated A, P or A plus P microorganisms contributed to the mycorrhizal effects on plant growth.

Results of plant growth stimulation produced by specific endophyte-bacteria inoculation did not arise from a direct effect on the percentage of infection measured at the end of the experiment. None of the most effective microbial combinations here used produced an increase on percentage of root colonization (Tables 1 and 5). Normally plant growth increases, by dual VAM fungus–bacteria inoculation, were related with increased VAM infection (Azcón-Aguilar and

Table 5. VAM root infection (% and root length infected total) in *Lycopersicum* roots inoculated with VAM fungi and the free-living rhizosphere bacteria A and P

Mycorrhiza treatment	Bacteria treatment None %	None Total	P %	P Total	A %	A Total	P + A %	P + A Total
G. mosseae	12a	390.0x	17ab	669.0y	19b	725.8y	17b	739.5y
G. fasciculatum	13a	612.3y	14ab	957.6z	17b	984.3z	7c	688.8y
G. sp. (E_3 type)	16ab	680.0y	12a	693.6y	17b	884.0yz	11ac	719.4y

Values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 7. Effect of the free-living rhizosphere bacteria (A and P) on hyphal growth (H) and secondary vesicle (E) arising from spores of *G. mosseae*

	Hyphal development[a] (days)											
	4		9		13		17		25		30	
Treatments	H	E	H	E	H	E	H	E	H	E	H	E
C	a	—	a	—	a	—	a	—	a	0,1	a	0.1
A	a	—	a	—	b	1 ± 0.8	b	2.1 ± 0.8	b	2 ± 0.4	b	2 ± 0.4
P	a	—	a	0.8 ± 0.4	c	2.3 ± 1.5	c	4 ± 2	c	6 ± 3	c	6 ± 3

[a]The three categories established, i.e. a, b and c, indicate dimensions of mycelial development: a, slight: the largest dimension was <5 mm; b, moderate: between 5–10 mm; c, extensive: 10 mm.

Barea, 1978; Subba Rao *et al.*, 1985). Data about infection levels on root are not related to the ability of endophyte to stimulate plant growth.

Changes in numbers of inoculated bacteria were not measured in this experiment. Results of Meyer and Linderman (1986b) show that VAM affects specific groups of bacteria. Azcón *et al.* (1976) observed a different evolution in numbers of bacteria affected by mycorrhizal treatment. The most effective bacterial inoculum on plant growth were: P in *G. mosseae* treatments; A plus P in *G. fasciculatum* treatments and A in *G.* sp. treatments (Table 1). But no significant differences were found in N and K contents in *G. mosseae*-P and N content in the A plus P-*G. fasciculatum* and in *G. mosseae* and A and P in *G. fasciculatum*. From this it would seem that phosphorus is the main nutrient implicated in the plant growth effect. A more effective mycorrhizal phosphorus uptake might be the relevant factor. It is possible that, as the establishment of the infection is preceded by same fungus growth, such as propagule germination or pre-infective hyphal elongation, VAM can be stimulated by bacteria before it comes into contact with root cells (Azcón, 1987). This could result in a more rapid and extensive formation of the mycorrhiza with earlier plant benefits (Abbott and Robson, 1978, 1981). The positive effect can also be a stimulation on elongation, distribution or surviving of external post-infective mycelium. Any of these possibilities can increase VAM fungus activity. These effects can be motivated by a direct bacterial action on VAM fungus or through the host plant (Rovira, 1965). The bacteria might affect plant growth by the plant hormones which they synthesise. These growth substances of the auxin, gibberellin and cytokinin types also can be involved in the microbial interaction. Plant response to these bacteria can result in activities which favour VAM establishment or influences (Gibson and Jordan, 1983). Since plants can be modified in root morphology, as cell wall plasticity and physiology, as qualitative and quantitative root exudation (Stzelczyk and Pokojska-Burdziej, 1984), these changes affect extraradical fungal development. Bacterial production of auxins (Azcón and Barea, 1978; Barea *et al.*, 1976; Meyer and Linderman, 1986a) can result in translocation of soluble sugars to the root which can enhance the metabolic activity of the fungus in the root. The value of certain bacteria may be ascribed to the production of active metabolites (Lynch, 1976) such as vitamins and small amounts of some inorganic acids (González-López *et al.*, 1983), able to stimulate the biosynthetic capacity of germinated spores (Hepper and Jakobsen, 1983). Microbial–plant interactions are complex and interrelated. Whatever the mechanism involved might be the major findings from my study is the selective effect of VAM and rhizospheric bacteria on their mutual development and the effect on plant growth. This selective influence suggests that mechanisms involved in VAM stimulation are specific for each *Glomus* species.

A characteristic of the three *Glomus* species, which may distinguish them from each other, is their different abilities to form external hyphae in soil with respect to the length of root infected. The fact has been tested among species of VA fungi by Abbott and Robson (1985). According to Graham *et al.* (1982) different isolates of *G. fasciculatum* seem to differ in their capacity to form external hyphae in soil. Although they all produced similar degree of infection, an increased proliferation of extraradical mycelium, could explain the bacterial effect.

Results of studies done *in vitro* (Tables 6 and 7) show that A and P were able to stimulate *G. mosseae* germination, hyphal growth and the formation of secondary vesicles under axenic conditions. This agrees with previous reports by Azcón (1987), Azcón-Aguilar *et al.* (1986) and Mayo *et al.* (1986). Here P was more effective than A in enhancing better independent growth of *G. mosseae* spores. Using *G. mosseae* inoculum P also was more effective on plant growth (Table 1) than A. But this effect was not evidenced in the percentage of root infection (Table 5) at the harvest. If mycorrhizal colonization was affected at any time during the experiment it was not detected. Inoculation with the bacterium *Pseudomonas* increased colonization by VAM fungi at 6 weeks of plant growth, but colonization levels were similar at 12 weeks (Meyer and Linderman, 1986a,b). These results suggest that bacterial stimulation can cause an early infection (Azcón-Aguilar and Barea, 1985) which according to Abbott and Robson (1978, 1981) was always correlated with the extent of growth increase.

Mycorrhizal mycelium might act as typical rhizosphere organism and showed microbial competence or synergism before the establishement of the infection. This fact could explain why dual A plus P bacterial inoculation in *G.* sp. mycorrhizal plants was less effective than single A inoculation (Table 1). Microbial competition can avoid early VAM colonization. Fungus is out of nutritional competition only when root cells are penetrated. In the present experimental conditions competition for min-

eral nutrients can be excluded, since nutrients for plant growth were sequentially added to the sand–vermiculite medium. In both, *G. fasciculatum* and *G.* sp., the colonization level (%) decreased in the presence of A plus P bacteria. As because of the results of plant growth, a microbial incompatibility is improbable (Table 1). I suppose, that in these treatments the further mycorrhizal colonization by phosphorus content in the shoot (Table 5) was affected negatively (Azcón *et al.*, 1978; Graham *et al.*, 1981). Soil inhabiting microorganisms exert marked stimulatory influences on the root area when sharing a natural habitat with VAM fungi. The study of these microbial groups must be considered in conjunction. This is necessary for a better knowledge and understanding of such interactions in order to utilize VAM fungi successfully.

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