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RESPONSE OF SUBTERRANEAN CLOVER TO DUAL INOCULATION WITH VESICULAR–ARBUSCULAR MYCORRHIZAL FUNGI AND A PLANT GROWTH-PROMOTING BACTERIUM, *PSEUDOMONAS PUTIDA*

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Summary—Plant growth and nodulation of subterranean clover (*Trifolium subterraneum* L.) were studied in nonsterile soil inoculated with a plant growth-promoting rhizobacterium (PGPR) isolate of *Pseudomonas putida* and indigenous vesicular–arbuscular mycorrhizal (VAM) fungi. Although inoculation with the PGPR or VAM fungi increased plant growth after 12 weeks, a significant increase in root dry weight, compared to uninoculated controls, was observed only when both the PGPR and VAM fungi were present. Shoot dry weight of plants inoculated with PGPR and VAM was significantly greater than with the PGPR alone, VAM fungi alone or uninoculated controls. Nodulation was enhanced significantly by either the PGPR or VAM fungi alone (× 1.50-fold increase over controls at 12 weeks), but was significantly greater (× 2.03-fold increase over controls) when both the PGPR and VAM fungi were present. Inoculation with the PGPR increased colonization by VAM fungi from 7 to 23% of the root system infected at 6 weeks, but colonization levels by VAM fungi were similar (ca. 50%) at 12 weeks. Populations of the PGPR increased similarly in the rhizosphere of both mycorrhizal and nonmycorrhizal plants. Concentrations of Fe, Cu, Al, Zn, Co and Ni were considerably greater in the shoots of plants inoculated with the PGPR and VAM fungi than in plants inoculated with the PGPR or VAM fungi alone.

INTRODUCTION

Specific strains of pseudomonads have been shown, when incorporated into soil, or inoculated onto roots or seed, to increase the growth and yield of some agricultural crops (Azcon-Aquilar and Barea, 1978; Burr *et al.*, 1978; Howie and Echandi, 1983; Kloepper *et al.*, 1980a; Suslow and Schroth, 1982). Such plant growth-promoting rhizobacteria are believed to function mainly by inhibiting populations of deleterious microorganisms, called subclinical pathogens or exopathogens, in the rhizosphere (Kloepper and Schroth 1981a, b; Suslow, 1982). When populations of deleterious microorganisms are reduced, the plant can achieve more of its growth potential (Kloepper and Schroth 1981a, b; Suslow, 1982; Woltz, 1978). Suslow (1982) discussed and reviewed the possible mechanisms involved.

Plants, including most agricultural plants, grown in nonsterile soil coexist with fungal root endophytes, forming mutualistic fungus–root associations called vesicular–arbuscular VA mycorrhizas. The fungal partners of the association are ubiquitous and the mycorrhizal condition is normal for most plant species. Once the fungus–root association is formed, the plant can benefit from an increased supply of phosphorus and other ions which the fungus can acquire from the soil beyond the zone of depletion at the root surface and can transport back to the root (Rhodes and Gerdemann, 1980). Other effects of VAM on plant growth and physiology are resistance to water stress and plant hormonal balance (Hayman, 1983) and resistance to plant pathogens (Dehne, 1982). The fungi are considered to be obligate symbionts, very well adapted to live with plant roots from which they obtain carbohydrates and in which they are protected from microbial antagonism in the soil (Hayman, 1978).

Because of the ubiquity of the mycorrhizal association in crop plants and their effects on plant growth, physiology and nutrition, their influence should be considered when testing the growth-promoting effects of beneficial bacterial inoculants such as the plant growth-promoting rhizobacteria. To achieve maximum benefit from beneficial microorganisms, the influence of bacterial inoculants on mycorrhiza development in nonsterile soil should also be studied for possible interactions, particularly antagonistic effects. No such studies on plant growth-promoting rhizobacteria and VA mycorrhizas have been reported. Therefore, we investigated the interactions between one known plant growth-promoting rhizobacterium, *Pseudomonas putida*, and indigenous VA mycorrhizal fungi and their effects on the growth

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and nodulation of subterranean clover (*Trifolium subterraneum* L.) in nonsterile soil with native soil *Rhizobium* inoculum.

MATERIALS AND METHODS

Soil and inoculum preparation

Nonsterile soil free from indigenous VA mycorrhizal fungi was prepared by mixing into approx. 10 l of pasteurized soil (65°C for 30 min using aerated steam) 2 l of a sieved garden soil suspension (200 g soil l^{-1} H_2O), containing *Rhizobium* and other indigenous bacteria. This amount of liquid suspension roughly brought the soil to field capacity where it was maintained for 2 weeks.

Spores of VA mycorrhizal fungi were isolated by wet-sieving and decanting (Gerdemann and Nicholson, 1963) soil from a 6-month-old sweet corn pot culture in garden soil containing an indigenous population of VA mycorrhizal fungi. The spores were surface disinfested by suspending them in an antibiotic solution containing 2% w/v chloramine T, 200 μg streptomycin ml^{-1} and a drop of Tween 20 for 10 min. They were then washed with 0.5 l of 0.5% sodium hypochlorite (Clorox) solution through a sterile Büchner apparatus, rinsed with 2 l sterile distilled (SD) H_2O and resuspended in SD H_2O. One-half of the soil was amended with the VAM spore suspension by hand-mixing to yield a concentration of 100 spores ml^{-1} soil. An aliquot of the final spore rinse water was mixed into the non-VAM soil to include any antibiotic or chlorox residues.

Pseudomonas putida isolate R20 marked with rifampicin antibiotic resistance (obtained from R. Osburn, University of California, Berkeley) can enhance growth of sugar beet (Osburn, unpublished) and decrease *Pythium* damping-off disease (Osburn *et al.*, 1983). We confirmed the growth-promoting potential of this isolate on clover and lettuce in mycorrhizal fungus-free, nonsterile soil, prepared as above. The bacterium was grown for 48 h on Kings B (KB) medium amended with 100 μg rifampicin ml^{-1}. The colonies were scraped into sterile 0.1 M $MgSO_4$ buffer solution to a final concentration of 10^8 cfu ml^{-1}. Aliquots of the bacterial suspension were mixed promptly by hand into one-half of the soil amended with VA mycorrhizal fungus spores or soil to which spores were not added (10 ml buffer suspension 50 ml^{-1} soil), the resulting concentration in each case being 2×10^7 cfu ml^{-1} soil. Soil not amended with the PGPR suspension received an identical amount of sterile 0.1 M $MgSO_4$ buffer solution.

Surface-disinfested (3% H_2O_2 for 30 min) subterranean clover seed (*Trifolium subterraneum* L. cv. Mt Barker) were germinated on moist sterile filter paper in glass Petri dishes. Fifty uniform 5-day-old seedlings were randomly assigned to one of the four treatments and transplanted into 50-ml tubes (Ray Leach Cone-Tainer Nursery, Canby, Oregon). Plants were grown for 6 and 12 weeks in the greenhouse (24°C day, 18°C night, 16 h photoperiod of 240 μE m^{-2} s^{-1} from high pressure sodium vapor lamps) and fertilized every 14 days with 20 ml of Long-Ashton nutrient solution (Hewitt, 1966) with 1/4 strength phosphorus (11 μg ml^{-1}).

Assay procedure

Twenty replicate plants per treatment were harvested at 6 weeks and 28 replicates at 12 weeks. To estimate PGPR populations in the rhizosphere soil, the tops were removed and 1 g (fresh wt) root samples were cut from the middle 1/3 of the root system with a sterile scalpel. The root samples were held with sterile forceps, tapped firmly against a hard surface to remove loosely adhering soil and placed in a sterile dilution bottle containing 100 ml 0.1% water agar dilutent. The bottle was shaken 25× by hand, the roots removed and serial dilutions prepared. Five replicate KB agar plates amended with 100 μg rifampicin ml^{-1} were inoculated with 0.5 ml of diluted rhizosphere soil suspension. The plates were held for 5 days at room temperature in the dark. Final dry wt of the rhizosphere soil samples was determined by pipetting 25 ml of the original suspension into tared weighing dishes, drying in a 70°C oven for 48 h and reweighing.

The population of PGPR in the rhizoplane (as distinct from those in the rhizosphere) was also assayed in the 12 weeks harvest by quickly determining the fresh wt of the root sample, macerating the roots in 100 ml of 0.1% water agar dilutent in a sterile Waring blender at high speed for 2 min and adding serial dilutions onto the rifampicin selective medium in the same manner as for the rhizosphere soil sample.

Root colonization by VAM fungi was assessed by clearing and staining an additional root sample (0.5 g fresh wt) by a modification of the technique of Phillips and Hayman (1970) and determining the proportion of 1 mm root sections containing mycorrhizal hyphae, vesicles, arbuscules or spores. Root nodules were counted on fresh roots and weights of tops and roots were taken after drying at 70°C for 48 h. Plant tissues were analysed with an ICP Spectrophotometer by the Plant Analysis Laboratory, Department of Horticulture, Oregon State University.

The data were analyzed with one-way analysis of variance, and the Least Significant Difference procedure was used to separate treatment means (Sokal and Rohlf, 1969).

RESULTS

Inoculation with PGPR, with or without VAM fungi, significantly stimulated shoot weight after 6 weeks. Plants grown with VAM fungi only had low levels of mycorrhizal fungus colonization and were not significantly larger than control plants after 6 weeks (Table 1). Although the PGPR or VAM fungi alone increased root and shoot weight after 12 weeks, a significant increase in root growth, as measured by tissue dry wt, compared to uninoculated controls, was observed only when both PGPR and VAM fungi were present. Shoot growth of plants inoculated with both PGPR and VAM fungi was significantly greater than any other treatment (Table 1).

VAM fungus infection in the roots was increased significantly from 7 to 23% by the presence of PGPR at 6 weeks (Table 1). At 12 weeks, about 50% of the root systems of all plants grown in VAM fungus-

Table 1. Dry weight, nodulation and mycorrhizal fungus colonization of subterranean clover inoculated with a plant growth-promoting bacterium (PGPR), vesicular–arbuscular mycorrhizal (VAM) fungi, or both

Treatment	Shoot dry wt (mg) 6 (weeks)	Shoot dry wt (mg) 12 (weeks)	Root dry wt (mg) 6 (weeks)	Root dry wt (mg) 12 (weeks)	No. nodules 6 (weeks)	No. nodules 12 (weeks)	% VAM 6 (weeks)	% VAM 12 (weeks)
Noninoculated control	39a[1]	240a	46a	200a	9.1a	32.6a	0	0
VAM	40ab	300ab	47a	230ab	12.7ab	49.3b	7a	50a
PGPR	62bc	360b	66a	250ab	20.5b	49.1b	0	0
PGPR + VAM	64c	460c	68a	280b	31.6c	66.2c	23b	44a
LSD	22	30	24	60	10.5	10.8		

[1]Column means followed by the same letter are not significantly different at $P = 0.05$.

amended soil, with or without PGPR, were mycorrhizal. Arbuscules, many vesicles and abundant external hyphae were observed. Plants grown in soil not amended with VAM fungus spores did not become mycorrhizal.

Nodulation was significantly enhanced by PGPR at 6 weeks and 12 weeks and by VAM fungi alone at 12 weeks. However, at both harvests, significantly more nodules had developed on the root systems of plants grown with both organisms compared to PGPR alone, VAM fungi alone or uninoculated controls (Table 1). The majority (>60%) of the nodules of plants treated with PGPR, VAM fungi or PGPR + VAM fungi appeared firm and pigmented at 12 weeks. Nodules of control plants were noticeably smaller and less pigmented.

The population of antibiotic-marked PGPR was easily monitored on the rifampicin-amended medium. No colonies grew at all from soil not inoculated with PGPR, indicating that spontaneous antibiotic resistance had not occurred in natural soil microorganisms, and were therefore not being included in the PGPR population counts. Populations of PGPR in the rhizosphere soil of both mycorrhizal and nonmycorrhizal plants increased during the 12 week experiment from 3×10^6 cfu g^{-1} dry rhizosphere soil to 4–5 $\times 10^6$ cfu. At 12 weeks, populations of PGPR on the rhizoplane of mycorrhizal and nonmycorrhizal plants grown in PGPR-amended soil were comparable, averaging 6×10^5 cfu g^{-1} dry root.

Concentrations ($\mu g\ g^{-1}$) of Fe, Al, Cu, Zn, Co and Ni were considerably greater at 6 weeks in the shoots of plants inoculated with both PGPR + VAM than in plants inoculated with either organism alone (Table 2). Concentration (% dry wt) of P was also higher in dual-inoculated plants. Concentrations of N, K, Ca, Mg, Mn, S and Mo in roots and shoots were similar in all treatments (Tables 2 and 3).

DISCUSSION

The rapid and sustained establishment of the *P. putida* isolate in the rhizosphere of inoculated plants resulted in significantly increased plant growth, in the presence or absence of VAM fungi. Although the indigenous VAM fungi used in this study did not significantly enhance growth when inoculated alone, it is noteworthy that the greatest growth and nodulation was achieved by plants grown with both organisms. Azcon-Aquilar and Barea (1978) noted similar results with whole-cell inocula of a *Pseudomonas* isolate, *Rhizobium meliloti* and a selected VAM fungus on alfalfa. In our study, root weight was increased significantly over the uninoculated controls only when both organisms were present and shoot weight of dual-inoculated plants was significantly greater at 12 weeks than those grown with PGPR or VAM fungi alone.

Possible explanations for the increased benefit to the plant by dual-inoculation with PGPR + VAM might include: (1) early VAM infection, and therefore early benefit by the mycorrhizal symbiosis, increased by the presence of PGPR; (2) enhanced VAM uptake of elements solubilized by PGPR; and (3) contributions by both organisms singly or in concert to nodule formation or activity. These mechanisms would not be necessarily mutually exclusive.

More rapid and extensive formation of VA mycorrhizae could result in more rapid development of hyphae and hence increased ion uptake and earlier

Table 2. Shoot tissue nutrient major and minor element concentration of subterranean clover (*Trifolium subterraneum* L.) inoculated with a plant growth-promoting bacterium (PGPR), vesicular–arbuscular mycorrhizal (VAM) fungi, or both[1]

Nutrient ($\mu g\ g^{-1}$)

Treatment	Fe 6	Fe 12	Cu 6	Cu 12	Al 6	Al 12	Zn 6	Zn 12	Co 6	Co 12	Ni 6	Ni 12	Mn 6	Mn 12	Mo 6	Mo 12
	(weeks)		(weeks)		(weeks)		(weeks)		(weeks)		(weeks)		(weeks)		(weeks)	
Noninoculated	633	716	11	14	449	506	24	27	0.27	0.33	0.17	2.53	161	120	2.2	1.9
VAM	800	573	13	16	578	401	24	27	0.23	0.27	0.18	2.36	160	115	1.8	1.5
PGPR	841	578	19	15	540	375	24	26	0.35	0.30	0.24	2.57	155	111	1.8	1.7
PGPR + VAM	1638	528	28	14	942	374	31	26	0.48	0.28	0.39	2.31	170	113	1.9	1.4

Nutrient (% dry wt)

Treatment	N 6	N 12	K 6	K 12	Ca 6	Ca 12	Mg 6	Mg 12	S 6	S 12	P 6	P 12
	(weeks)		(weeks)		(weeks)		(weeks)		(weeks)		(weeks)	
Noninoculated	2.61	3.18	2.16	2.58	2.06	2.11	0.55	0.55	0.25	0.26	0.10	0.14
VAM	2.42	2.99	2.22	2.40	2.05	2.14	0.53	0.50	0.27	0.25	0.12	0.15
PGPR	2.67	3.05	2.34	2.25	2.18	1.97	0.53	0.48	0.23	0.20	0.10	0.10
PGPR + VAM	2.46	3.30	2.29	2.16	1.39	2.09	0.52	0.46	0.23	0.23	0.14	0.13

[1]Combined treatment replicates were analyzed.

Table 3. Root tissue major and minor element concentration of subterranean clover (*Trifolium subterraneum* L.) inoculated with a plant growth-promoting bacterium (PGPR), vesicular–arbuscular mycorrhizal (VAM) fungi, or both[1]

	Nutrient ($\mu g\ g^{-1}$)															
	Fe		Cu		Al		Zn		Co		Ni		Mn		Mo	
	6	12	6	12	6	12	6	12	6	12	6	12	6	12	6	12
Treatment	(weeks)		(weeks)		(weeks)		(weeks)		(weeks)		(weeks)		(weeks)		(weeks)	
Noninoculated	5284	6832	31	34	5705	5231	36	32	1.97	1.98	7.5	8.4	170	178	6	3.0
VAM	6591	7217	18	42	6262	5910	30	35	2.16	2.11	9.9	8.8	177	186	5.1	2.7
PGPR	8614	5236	33	23	6654	4249	34	25	2.78	1.87	11.2	10.0	249	161	6	2.7
PGPR + VAM	8004	6787	36	25	7689	5914	35	28	2.26	1.91	12.4	8.7	240	169	4.5	2.3

	Nutrient (% dry wt)											
	N		K		Ca		Mg		S		P	
	6	12	6	12	6	12	6	12	6	12	6	12
Treatment	(weeks)		(weeks)		(weeks)		(weeks)		(weeks)		(weeks)	
Noninoculated	1.70	2.16	2.01	2.13	0.43	0.48	0.96	1.3	0.84	1.2	0.10	0.14
VAM	1.70	2.32	2.22	1.81	0.43	0.52	0.99	1.1	0.93	1.0	0.13	0.14
PGPR	1.84	2.30	1.89	1.68	0.42	0.47	0.97	1.2	0.83	0.9	0.12	0.11
PGPR + VAM	1.61	2.32	1.36	1.27	0.41	0.46	0.68	1.0	0.64	0.9	0.12	0.15

[1]Combined treatment replicates were analyzed.

induction of other physiological benefits from VAM. Early VA mycorrhizal colonization has been associated with improved nodule formation and function (Smith *et al.*, 1979). There is evidence that some pseudomonads can increase the plant susceptibility to VAM infection. Stimulation of VAM fungus colonization by a *Pseudomonas* sp. was noted by Mosse (1962) when a *Pseudomonas* contaminant of otherwise sterile root + VAM fungus spore culture induced changes necessary for fungal penetration of the roots. Low concentrations of a sterile pectolytic + cellulolytic enzyme preparation or sterile bacterial filtrates were also effective, suggesting that changes in cell wall plasticity was involved. The presence of microorganisms on roots modifies root exudation patterns and can thus affect root-infecting processes by fungi (Rovira, 1965). Azcon-Aquilar and Barea (1978) reported stimulation of VAM infection by a phosphate-solubilizing *Pseudomonas* strain and by a cell-free extract of the same. Azcon *et al.* (1978) also suggested the involvement of increased concentrations of plant growth regulators in the rhizosphere and demonstrated stimulation of VAM fungus colonization by a mixture of IAA, gibberellins and cytokinins. The *P. putida* isolate used in our study is an effective antagonist against *Pythium* spp (Osburn *et al.*, 1983) and may have influenced the rhizosphere microflora favorably for VAM fungus colonization. Although the presence of *Pseudomonas* spp or other bacteria is not essential for VAM establishment, as demonstrated by successful VAM fungus colonization in axenic root organ cultures (Mosse and Hepper, 1975), infection by VAM fungi in nature may be aided by the better infection conditions created at or near the root surface by these common rhizosphere bacteria. Once roots are colonized, continued spread of the colonization may be less influenced by rhizosphere microorganisms and more sustained by the established nutrient source and induced changes in root physiology. This may explain why VAM infection was not enhanced further by PGPR at the later harvest. It should be emphasized that pseudomonads in the rhizosphere are a diverse group of bacteria and their interactions with the plant and with mycorrhizal fungi will differ between strains. For example, Bowen and Theodorous (1979) observed a fluorescent pseudomonad markedly suppressing the development of several ectomycorrhizal fungal species in *Pinus* roots.

It is of interest that dual-inoculated plants had higher shoot tissue concentration of Fe, Al, Cu, Zn, Co and Ni compared to those inoculated with VAM fungi or PGPR alone. As mycorrhizal and non-mycorrhizal PGPR-inoculated plants were the same size at 6 weeks, this indicates uptake of these elements by the VAM fungi. Fe, Al, Zn, Co and Ni, among other elements, can be solubilized by 2-ketogluconic acid (Duff *et al.*, 1963), a chelating agent produced through glucose metabolism by many pseudomonads (Richards, 1974) including *P. putida* (Grimes and Mount, 1984). A bacterial chelating action combined with fungal uptake may explain the higher concentration of these elements observed in plants colonized by PGPR and VAM fungi. Phosphates are also solubilized by ketogluconic acid and held in a form available to plants (Richards, 1974). P concentrations in dual-inoculated plants were slightly higher than those inoculated with PGPR or VAM fungi alone, but since the treatment replicates were combined, these data could not be statistically analysed. PGPR have been shown to produce a strong siderophore (Kloepper *et al.*, 1980b) which is considered to play a role in the antagonistic abilities of these bacteria. To our knowledge, it is not known whether the Fe chelated on the siderophore, called ferric pseudobactin (Kloepper *et al.*, 1980b), is available to plants.

Our results confirm other studies in which *Rhizobium* nodulation was enhanced by *Pseudomonas* spp (Krasilnikov and Korenyako, 1944; Azcon-Aquilar and Barea, 1978; Grimes and Mount, 1984). That nodulation of actinorrhizal plants by *Frankia* spp was greatly enhanced in gnotobiotic culture by bacterial isolates inoculated singly or together was observed by Knowlton *et al.* (1980). They proposed that the "helper" organisms, including *Pseudomonas cepacia* strains, facilitate a close association between the symbiont and host by causing massive root hair deformation which served to trap the *Frankia* filaments. They pointed out the close analogy of their study with the observations of Mosse (1962) in which colonization by a VAM fungus was also greatly facilitated by a "helper" organism in gnotobiotic

culture. The mechanisms by which inoculation with some *Pseudomonas* strains is thought to increase plant susceptibility to VAM fungus colonization, e.g. changes in cell wall plasticity and root exudation patterns, growth regulator concentration in the rhizosphere, nutrient availability and uptake, and perhaps alteration of the rhizosphere microflora, are also possible explanations for the enhanced *Rhizobium* nodulation by PGPR observed in our study, since these mechanisms have been implicated in the *Rhizobium* nodulation process (Gibson and Jordan, 1983; Nutman, 1965).

Enhanced *Rhizobium* nodulation and N_2-fixation in mycorrhizal plants is well recognized and is attributed to increased P nutrition, although other as yet undetermined secondary effects may also be involved (Asimi *et al.*, 1980; Barea and Azcon-Aquilar, 1983; Crush, 1974; Daft, 1978; Smith *et al.*, 1979). In our study, inoculation with PGPR or VAM fungi enhanced nodulation, but nodulation was increased significantly again when both organisms were present. This may be an additive effect of both microorganisms operating singly or in a concerted action, e.g. bacterial chelation of P, Fe and Co, all essential nutrients for nodule formation and function (Gibson and Jordan, 1983), coupled with enhanced uptake by the fungal hyphae. The isolation of compounds from *Phaseolus vulgaris* roots which cause agglutination of *P. putida* cells (Anderson, 1983; Grimes and Mount, 1984) suggests a close binding of precipitated cells to the root surface, where bacterial metabolic activity would have the greatest potential to affect the plant.

The enhanced mineral uptake by plants inoculated with VAM fungi and PGPR, however, suggests a close association of the bacterium with the VAM fungal hyphae. Abundant fungal mycelium was observed in the mycorrhizal plants and it is now recognized that VAM fungus colonization spreads throughout the root by the growth of hyphae along the root surface from established infection points (Hayman, 1978). Because of the dynamic nature of microbial colonization and activity in the rhizosphere, the elucidation of cause and effect will have to take into account both indirect effects of each organism on the plant as well as direct microbial interactions. The ubiquity of VAM fungi, *Pseudomonas* spp and *Rhizobium* spp in the rhizosphere of legumes suggests consideration of an adaptation towards mutual enhancement, although not total dependency.

Our results indicate that some of the growth-promoting effects attributed to inoculation with PGPR may be due in part to additive effects with VAM fungi. Among some practical implications to be explored in this context is the encouragement of effective VAM development by cultural practices, or the inoculation with efficient VAM fungus strains, to ensure the efficacy of beneficial microorganisms. Although PGPR are known to be antagonistic towards some soil fungi and can affect species composition in the rhizosphere (Suslow, 1982; Osburn *et al.*, 1983), our study indicates that at least some of the VAM fungi were not inhibited.

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