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A rhizobacterium modifies plant and soil responses to the mycorrhizal fungus *Glomus mosseae*

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Abstract

The effects of a rhizobacterium (*Bacillus* sp. strain BH-II) on plant (*Pisum sativum* L.) parameters (biomass, root/shoot ratio, seed/plant ratio), on root colonization by a vesicular–arbuscular mycorrhizal (VAM) fungus (*Glomus mosseae*) and on the formation of water-stable soil aggregates were investigated in two soils in a greenhouse pot study. The soils were a grey silt-loam of high P and a yellow clay-loam of low P content. The rhizobacterium had no significant effect on plant biomass production in either soil in the absence of the VAM fungus, but decreased plant growth by more than 30% in its presence. It enhanced the root/shoot and seed/plant ratios in plants of both VAM and non-VAM treatments. Without the VAM fungus, both soils disaggregated. This slaking of water-stable aggregates (over 0.5 mm) was significantly less severe when the rhizobacterium was present. With the VAM fungus, aggregation increased up to 27% during the experiment, but the rhizobacterium did not affect this process significantly. It is concluded that rhizo-organisms acting in concert may have both positive and negative effects on the plant and soil components of an agrosystem.

1. Introduction

Vesicular–arbuscular mycorrhizal (VAM) fungi affect the development of their host plants (Bethlenfalvay, 1992) and host soils (Miller and Jastrow, 1994) as well as the structure and functioning of natural and agricultural plant communities (Barea and Jeffries, 1995). Because of their beneficial effects on seed yield and soil structure (Bethlenfalvay and Barea, 1994), VAM fungi are of particular interest to alternative agricultural practices that emphasize both plant productivity and soil quality. As colonists of root, rhizosphere and bulk soil, the mycelia of VAM fungi are in intimate contact with the cells of the root cortex and with those of the soil microbiota. This close association with soil microbes may stimulate as well as inhibit mycorrhiza formation (see Linderman, 1992) and can selectively influence the development of VAM fungi (Azcón et al., 1990). Likewise, some VAM fungi may increase while others may reduce total bacterial populations in the plant–soil system, or affect the proliferation of specific groups of bacteria (see Christensen and Jakobsen, 1993). Combined VAM-fungus and soil-microbe effects on plants are known, as in phosphate solubilization (Azcón-Aguilar et al., 1986a), N nutrition (Barea et al., 1992) and growth regulation (see Barea, 1986). In other cases, plant responses were ascribed to plant-growth promoting (PGPR; Burr and Caesar, 1984) or deleterious (DRB; Suslow and Schroth, 1982) rhizobacteria without considering mediation or

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modification of microbe-initiated plant responses by VAM fungi (Paulitz and Linderman, 1991). Recognition of the importance of microbial ecology in the agrosystem (Doran and Linn, 1994) and an awareness of the involvement of VAM fungi in the processes of soil aggregation (Tisdall and Oades, 1982) are now extending VAM research from plant science to soil science (Tisdall, 1994; Miller and Jastrow, 1994), but the combined effects of VAM fungi and rhizobacteria on plant and soil have not been reported. The purpose of the present experiment was to include soil and plant responses to a rhizobacterium in the study of plant–VAM fungus relationships, by determining the effects of a rhizobacterium on plant development and VAM colonization, and by relating these symbiotic functions to changes in the status of water-stable soil aggregates (WSA).

2. Materials and methods

2.1. Experimental design

Plants were grown in two different soils with (+VAM) or without (−VAM) a VAM fungus. The soils of each of these four treatments were either inoculated with a rhizobacterium (+BAC) or not (−BAC). The experimental units (potted plants, one plant per pot) of the resulting eight treatments of this $2 \times 2 \times 2$ factorial design were arranged in a completely random manner. There were five replications per treatment. Results were evaluated by analysis of variance and two-tailed *t*-test. Actual probability values are presented instead of the arbitrary 5% level to give a more precise reading of the probability and to permit an individual interpretation of its significance (Nelson, 1989). We may interpret differences as 'significant' up to a probability value of $P = 0.099$. The data reported here were part of a larger experiment.

2.2. Biological materials, soils and growth conditions

Pea (*Pisum sativum* L. cv. 'Lincoln') plants were (1) inoculated with the VAM fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe and a rhizobacterium *Bacillus* sp., strain BH-II (+V+B), (2) with *G. mosseae* alone (+V−B), (3) with the *Bacillus* alone (−V+B), or (4) were left uninoculated as controls

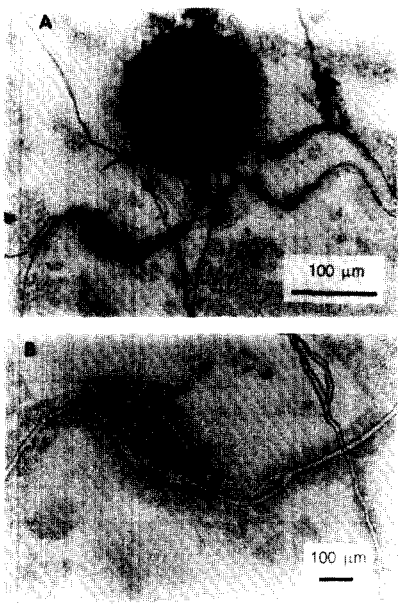

Fig. 1. Spore (A) and enlarged hyphae (B) of the VAM fungus *Glomus mosseae* surrounded by *Bacillus* sp. strain BH-II on water agar, with the fungus as the only carbon source for BH-II.

(−V−B). Seeds were pre-germinated for 3 days, and the seedlings were selected for uniformity. A saturating amount (at least 1000 spores and sporocarps and 500 heavily colonized root fragments) of the VAM inoculum (*G. mosseae*, Rothamsted isolate, obtained from B. Mosse and cultured on *Sorghum halepense* at the Estación Experimental del Zaidín) was used. The rhizobacterium was isolated from hyphae of surface-sterilized (Mosse, 1962) spores of *G. mosseae* that grew on water agar (pH 7.0, 25°C) following 15 days of incubation (Azcón-Aguilar et al., 1986b), and was cultured on LB medium ($g\,l^{-1}$: tryptone, 10; yeast extract, 10; NaCl, 5; agar, 15; Sambrock et al., 1989). The rhizobacterium had not been detected on other species of VAM fungi tested under similar conditions, but was previously observed to grow preferentially on plants colonized by *G. mosseae* (G. Andrade, unpublished data, 1993), and to form sheaths of dense mucigel around hyphae of *G. mosseae* on water agar (Fig. 1). Tests to identify this organism were performed by the American Type Culture Collection (ATCC), Rockville, MD. Results derived from the Automated Bacterial Identification System (Biolog) and from fatty acid methyl ester gas chromatography (FAME) indi-

cated closest commonality (46.8%) with *Bacillus megaterium*. However, further efforts by ATCC using an extensive array of morphological, biochemical and physiological tests revealed closest (though weak) resemblance to *Bacillus simplex*. Thus, awaiting DNA hybridization, the organism remains unidentified as to species at this time. The bacterial inoculum was cultured in liquid LB medium (28°C, 48 h). The culture was then centrifuged at $6000 \times g$ for 10 min. The bacterial pellet was washed twice with distilled water and resuspended in sterile distilled water. Suspensions (20 ml, 10^9 colony-forming units (CFU) ml^{-1}) were added at planting to the stems of the seedlings (+BAC plants) at the soil surface and to the remaining soil surface, and mixed into the soil to a depth of 2–3 cm. The VAM inoculum was mixed evenly into the entire soil volume of the +VAM plants prior to planting. The two soils utilized were: Soil 1, a gray silt-loam (43% sand, 48% silt, 9% clay) of pH 7.6, organic matter (OM) content of 11.4 g kg^{-1}, and P content ($NaHCO_3$-extractable) of 44.1 mg kg^{-1}; Soil 2, a yellow clay-loam (42% sand, 40% silt, 18% clay) of pH 7.0, OM content of 3.2 g kg^{-1}, and 20.6 mg kg^{-1} of $NaHCO_3$-extractable P. The soils were air-dried, crushed, sieved (2 mm) and mixed with coarse sand (over 2 mm). The mix (soil:sand, 1:1, w:w) was sterilized by autoclaving for 1 h on 3 consecutive days prior to planting. Plants were watered from below (4 mM $Ca(NO_3)_2$ solution) twice weekly, and daily during the last 3 weeks. The initial watering was from above to facilitate distribution and movement of the bacteria (Huysman and Verstraete, 1993). Sievings (30 μm sieve pores) of the VAM inoculum free of VAM propagules were added to all soils. Plants were deliberately not inoculated with *Rhizobium* to maximize VAM and rhizobacterium interactions. The high N content of the nutrient solution kept the roots nodule-free. Plants were grown in pot cultures from January to March (1993) in a greenhouse in Granada, Spain. Temperatures were generally controlled (15/32°C, night/day), but upon occasional control failure varied more widely (4/41°C). Plants were harvested after 8 weeks of growth.

2.3. Assays

The growth medium (soil and coarse-sand mix) was permitted to dry to a point before harvest where it could be shaken off the roots and crumbled. It was then air-dried, crushed, and sieved in order to (1) separate the coarse sand (over 2 mm) and (2) to divide the remaining soil into two size fractions (over 0.5 mm and less than 0.5 mm). The roots were washed, and the dry weights of roots, shoot and seeds were each determined. Root colonization by the VAM fungus was determined by clearing in 5% KOH (90°C, 30 min), washing thoroughly with water, acidifying in 0.01 N HCl (60 min), and staining with trypan blue (0.05%) in lactic acid/glycerol (90°C, 15 min). Percentage colonization was estimated by the grid-line intersect method (Ambler and Young, 1977). Formation or disaggregation (slaking) of water-stable soil aggregates (WSA) was determined according to Kemper and Rosenau (1986), with modifications described earlier (Thomas et al., 1993; Bethlenfalvay and Barea, 1994). The WSA status of the soils was determined before and after the experiment. Samples (30 g) of the soil fraction containing large (over 0.5 mm) particles were placed on a sieve (0.5 mm pore size) to determine WSA content by washing (16 vertical strokes min^{-1} for 3 min through a 10 cm water column) in water. The materials that did not pass the sieve were dried (110°C), weighed, and dispersed in dilute NaCl solution to separate soil from sand particles (over 0.5 mm). The sand fraction was dried, weighed, and the mass was subtracted from the mass of the total sand-containing soil. The difference was evaluated as WSA. Two measures of water stability were used. One was calculated as the percentage by weight of WSA (size over 0.5 mm) of total soil. The other was expressed as the change in soil WSA content during the course of the experiment. Proliferation of the inoculated rhizobacterium during the experiment was estimated using the soil fraction containing small (less than 0.5 mm) particles, where populations were expected to be smaller and more uniform than in the large aggregates or rhizosphere soil. The dry-sieved soils of the individual replications were combined and thoroughly mixed. Slurries made from 1 g samples of the combined replicate soils were serially diluted through 10^{-5}, and plated on LB medium (pH 7.0, 28°C, 48 h). CFU were counted on dilutions 10^{-2} through 10^{-5}. The numbers of total CFU at dilutions 10^{-3} and 10^{-4} were calculated from regression curves of the four dilutions vs. their actual CFU counts. Colonies counted at dilutions 10^{-3} and 10^{-4} were classified as gram-positive or negative rods. The

proportionate number of gram-positive rods were reported per gram of soil. The results are based on duplicate samplings, each of which was plated in triplicate.

3. Results

3.1. Plant effects

Inoculation with the rhizobacterium (*Bacillus* sp.) did not significantly (for *P*-values see tables) affect dry mass in −VAM plants in either Soil 1 or Soil 2 (Table 1). In +VAM plants, however, the presence of the rhizobacterium was associated with highly significant decreases in plant growth (Table 1) which amounted to −33.5% for Soil 1 and −31.3% for Soil 2 (Table 2, +BAC vs. −BAC). Responses to VAM colonization in −BAC plants were positive and significant in both soils (Table 1). In +BAC plants, the VAM responses were significant (Table 1), but negative in Soil 1 (−24.6%) and positive in Soil 2 (25.2%, Table 2). The small VAM response of −BAC plants in the high-P Soil 1 and the large VAM response in low-P Soil 2 reflects the well-known mycorrhizal P effect (Jakobsen, 1986). In the +BAC plants, these VAM responses were smaller in Soil 2, and negative in Soil 1. The impact of the experimental factors on plant response is shown by the highly significant main effects of the factors (Table 3). The significant Soil × VAM interaction (Table 3, Fig. 2(A)) shows a difference in both the direction and magnitude of plant response to VAM (VAM effect) in the two soils. Thus, the two factors were not independent: the VAM fungus was more effective in enhancing plant growth in Soil 2 than in Soil 1, and the rhizobacterium modified only the extent but not the direction of this interaction. The significant VAM × BAC interaction was also complex (Fig. 2(B)). In both soils, the BAC response was smaller in the presence of VAM than without VAM; showing differences in both magnitude and direction in Soil 1, and in magnitude only in Soil 2. These effects indicate that the BAC and VAM factors not only acted on plant growth in concert, but that their action was also modified by the soils in which the plants grew. The lack of significant Soil × BAC interaction (Table 3) indicates similar and independent plant responses to BAC in both soils. While the rhizobacterium did not have significant effects on −VAM plant dry mass and decreased that of +VAM plants (Table 1), it significantly increased the root/shoot ratios in plants of all treatment combinations (Table 4). This effect was more pronounced in +VAM plants than in −VAM plants (Table 2), indicating a localized, VAM-depend-

Table 1
Plant, soil and soil microbe parameters in two different soils. Soils were inoculated with the vesicular–arbuscular mycorrhizal (VAM) fungus *Glomus mosseae* and the rhizobacterium *Bacillus* sp. (BAC), or with one of the organisms, or with neithger. The significance of differences (*P* by *t*-test) is shown horizontally for the +VAM vs. −VAM comparison and vertically for the +BAC vs. −BAC comparison. Numbers are means of five replications

Parameter		Soil 1			Soil 2		
		+BAC	−BAC	*P*	+BAC	−BAC	*P*
Plant dry mass (g)	+VAM	3.4	5.2	<0.001	3.6	5.2	0.003
	−VAM	4.6	4.6	0.950	2.9	3.1	0.802
	P	<0.001	0.018		0.056	<0.001	
WSA (% change)	+VAM	+24.5	+16.8	0.230	+27.1	+22.1	0.287
	−VAM	−36.5	−53.5	0.037	−15.4	−25.8	0.041
	P	<0.001	<0.001		<0.001	<0.001	
Vam colonization (%)	+VAM	16.0	18.8	0.095	49.8	64.6	<0.001
Rhizobacterium[a]	+VAM	7.6	5.7		18.2	16.5	
	−VAM	11.3	7.2		25.5	18.1	

[a]CFU (g soil)$^{-1} \times 10^4$.

Table 2
Changes in plant parameters as a result of microbial inoculation. Soils were inoculated with the vesicular–arbuscular mycorrhizal (VAM) fungues *Glomus mosseae* and the rhizobacterium *Bacillus* sp. (BAC), or with one of these organisms, or with neither. Values are presented as percentages of change between inoculated (+) and uninoculated (−) treatments

Parameter	Soil 1				Soil 2			
	+VAM vs. −VAM		BAC vs. −BAC		+VAM vs. −VAM		+BAC vs. −BAC	
Plant dry mass	+BAC	−24.6	+VAM	−33.5	+BAC	+25.2	+VAM	−31.3
	−BAC	+11.7	−VAM	−1.5	−BAC	+69.2	−VAM	−7.1
Root/shoot ratio	+BAC	+37.2	+VAM	+75.1	+BAC	+9.8	+VAM	+61.8
	−BAC	−10.2	−VAM	+14.7	−BAC	−17.8	−VAM	+21.1
Seed/plant ratio	+BAC	+5.5	+VAM	+7.4	+BAC	+3.5	+VAM	+4.2
	−BAC	+3.1	−VAM	+5.0	−BAC	+5.7	−VAM	+6.4

Table 3
Analysis of variance of plant, soil (water-stable aggregate, WSA) and fungal (VAM) parameters

ANOVA factors	Parameters				
	Plant dry mass	Root/shoot ratio	Seed/plant ratio	WSA % change	VAM colonization
Soil (S)	<0.001	<0.001	0.079	0.001	<0.001
VAM	<0.001	0.037	0.003	<0.001	
BAC	<0.001	<0.001	<0.001	0.014	0.036
Soil×VAM	<0.001	0.005	0.848	0.012	
Soil×BAC	0.901	0.271	0.763	0.547	0.137
VAM×BAC	<0.001	<0.001	0.936	0.343	
Soil×VAM×BAC	0.065	0.0591211	0.404	0.769	

Table 4
Root/shoot and seed/plant ratios of plants inoculated with the vesicular–arbuscular mycorrhizal (VAM) fungus *Glomus mosseae* and the rhizobacterium *Bacillus* sp. (BAC), or with one or the organisms, or with neither

Parameter		Soil 1			Soil 2		
		+VAM	−VAM	*P*	+VAM	−VAM	*P*
Root/shoot ratio	+BAC	0.310	0.226	0.002	0.246	0.224	0.060
	−BAC	0.177	0.197	0.013	0.152	0.185	0.001
	P	<0.001	0.042		<0.001	0.002	
Seed/plant ratio	+BAC	0.462	0.438	0.074	0.467	0.451	0.098
	−BAC	0.430	0.417	0.115	0.448	0.424	0.079
	P	0.049	0.002		0.059	0.030	

ent effect on plant development rather than general growth promotion or inhibition ascribed to PGPR or DRB organisms. Conversely, VAM effects on the root/shoot ratio were positive in +BAC plants, and negative in −BAC plants (+VAM vs. −VAM, Table 2), indicating synergistic effects of the two organisms on root growth. Analysis of variance of the root/shoot ratio paralleled the main effect and interaction evaluations

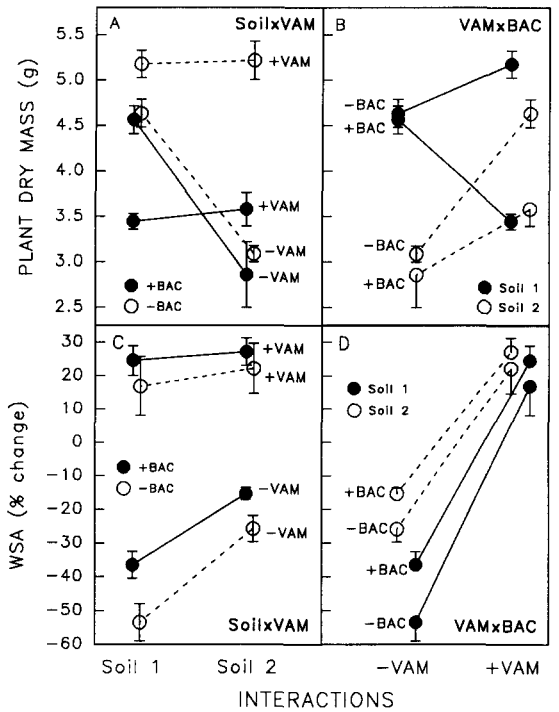

Fig. 2. Interactions of the experimental factors Soil and VAM (Soil × VAM) and of VAM and BAC (VAM × BAC) are illustrated for the response variables plant dry mass and water-stable aggregate (WSA). Interactions for Soil × VAM are shown for both +BAC (●, solid line) and −BAC (○, dashed line). Interactions for VAM × BAC are shown for Soil 1 (●, solid line) and for Soil 2 (○, dashed line). Bars through datapoints are the standard error of the mean. Lines connecting points do not denote functional progression, they merely indicate which points are being compared.

of plant dry mass (Table 3). Small, but significant increases were also observed in the seed/plant ratios of the +BAC plants compared with those of the −BAC plants (Tables 2 and 4). Likewise, the seed/plant ratios were larger in the +VAM than in the −VAM plants in both soils. The +VAM+BAC plants had the largest seed/plant ratios, and the −VAM−BAC plants the smallest ratios (Table 4), indicating a synergistic, localized effect (as with the root shoot ratio) of the two organisms on seed yield.

3.2. Soil effects

Soil aggregation changed significantly during the course of the experiment as a result of inoculation with the two rhizo-organisms. In +VAM soils, new formation of WSA was observed, while the −VAM soils disaggregated (Table 1). The rhizobacterium tended to increase WSA in the +VAM soils, but the trend was not significant ($P > 0.2$). In the −VAM soils, however, disaggregation was significantly smaller in the +BAC than in the −BAC soils. All three factors had significant main effects on WSA (Table 3), but only the Soil × VAM interaction was significant (Table 3, Fig. 2(C)). Unlike the Soil × VAM interaction in plant dry mass, there was only a difference in the magnitude of the soil responses to the VAM factor: the Soil 2 vs. Soil 1 response was larger for −VAM than for +VAM, regardless of the presence of BAC. A comparison of the effects reveals VAM-dependent aggradative or degradative properties of the two soils. The lack of significant VAM × BAC interaction for WSA is illustrated by the essentially parallel lines that connect each of the two response of WSA to BAC at different levels of VAM (Fig. 2(D)). Since BAC effects at each level of VAM (or VAM effects at each level of BAC) were the same, these factors influenced WSA status independently.

3.3. Microsymbiont effects

Root colonization by the VAM fungus was smaller in +BAC than in −BAC soils (Table 1), with a significant main effect of both the Soil and BAC factors on VAM development (Table 3). We ascribe the soil effect to on VAM colonization differences in soil P content, and the BAC effect to an antagonism between the two organisms. The count of gram-positive bacteria (CFU) was higher in the +BAC treatments, indicating survival and proliferation of the inoculated organism over the background of other organisms that originated from the application of sievings free of VAM propagules and from other nonsterile sources (watering) during the experiment. In both soils, counts of the rhizobacterium were lower in the +BAC than in the −BAC treatments, indicating that the antagonism between the two organisms was mutual.

4. Discussion

Many groups of rhizobacteria (Paulitz and Linderman, 1991) have been reported to promote plant growth or the development of plant organs in interac-

tion with VAM fungi (Azcón, 1989; Dhillon, 1992; Staley et al., 1992). These bacteria also affect VAM fungi (Meredith and Anderson, 1992) and may enhance or inhibit their growth (Garbaye, 1991), but when an antagonistic rhizobacterium becomes dominant in the rhizosphere, mycorrhiza formation and plant growth (Azcón-Aguilar and Barea, 1985) can be severely reduced. Depending on plant response, these rhizobacteria are classified as PGPR or DBR. However, when the stability of the plant–soil system (agrosystem, see Bethlenfalvay and Schüepp, 1994) rather than only plant growth is of interest, rhizobacterium effects on both plant and soil must be considered. The salient finding of our experiment was the divergence in the combined effects of our two rhizo-organisms (*G. mosseae* and *Bacillus* sp.) on plant and soil. When used together, the organisms inhibited plant growth but produced the best level of aggregation in both soils (Table 1). Thus, a rhizobacterium, such as our *Bacillus* sp., may have no effect on −VAM plants and act as a DRB in +VAM plants, but at the same time function as a 'soil-aggregation-promoting rhizobacterium' (SAPR), especially if not antagonized by a VAM fungus (Table 1). We are not aware of other reports of juxtaposed plant and soil effects produced by combinations of rhizo-organisms, even though the literature of the influence of microorganisms on plant development or soil formation separately is voluminous (Bethlenfalvay and Schüepp, 1994; Pankhurst et al., 1994). Is the rhizobacterium we used here a PGPR or a DRB, or perhaps a SAPR? Most likely it is all of these, and understanding its ecology and developing it (and others) as a product may be one of our greatest challenges (Mahaffee and Kloepper, 1994). A present finding that has a bearing on this was the improvement (PGPR) of certain aspects of plant development (root vs. shoot and seed vs. plant, Table 4) in the +BAC vs. −BAC treatments, while at the same time reducing total plant growth (DRB). The positive root and seed effects in the +BAC plants are noteworthy, for these are the real measures, rather that 'growth' in general, of the use-value of a soil organism in soil stability and yield production, the central aspects of sustainable agriculture. The data also confirmed the findings of others (Azcón-Aguilar and Barea, 1985) that antagonistic VAM–rhizobacterium interactions can affect plant growth, and that this effect may be mediated by decreases in mycorrhiza formation (Table 1). However, the data extended the effects of VAM–rhizobacterium interactions to the soil, as demonstrated by the large and significant decrease in the slaking of WSA in the −VAM soils, accompanied by only a small and insignificant improvement in aggregate formation in the +VAM soils (+BAC vs. −BAC, Table 1). It is expected that mutualistic interactions between VAM fungi and rhizobacteria could result in important improvements in soil structure.

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