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Arbuscular Mycorrhizal Symbiosis Changes the Colonization Pattern of *Acacia tortilis* spp. *Raddiana* Rhizosphere by Two Strains of Rhizobia

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ABSTRACT

The aim of the study was to assess the effect of the mycorrhizosphere of *A. tortillis* spp. *raddiana* mycorrhized with *Glomus intraradices* on the root nodulation by *Sinorhizobium terangae* (ORS 1009) and/or *Mesorhizobium plurifarium* (ORS 1096) in two different culture substrates (sandy soil and sand). The endomycorrhizal fungus only stimulated plant growth in the sandy soil. Moreover, arbuscular mycorrhizal infection enhanced the nodulation process in both culture substrates. Beside the stimulatory effects of the mycorrhizosphere on both rhizobia development, fungal symbiosis induces two different dynamics of each bacterial strains in the sand-grown plants. These results suggest specific relationships could occur during the development of the tripartite symbiosis, at physiological and molecular level. From a practical point of view, the role of arbuscular mycorrhizas in improving nodulation and N_2 fixation is universally recognized. The fungal symbiosis could modify the development of bacterial inoculants along the root systems. This effect is of particular interest in the controlled inoculation of selected rhizobia.

Introduction

Mycorrhizal plants usually transfer more assimilates to the roots than nonmycorrhizal plants [16]. This effect results from the carbon needs of the mycorrhizal fungus and from the higher respiration rate of the mycorrhizal roots than of the nonmycorrhizal roots [18, 27]. As root exudates provide most of the low molecular weight carbon compounds that are easily used by microorganisms in the soil, mycorrhizal symbiosis exerts a significant effect on the bacterial community composition in the rhizosphere. This fungal effect is commonly named the "mycorrhizosphere effect" [28]. Around the mycorrhized roots, the microbial communities differ greatly from those of the uninfected roots and of the surrounding soil [20, 21, 25] and specific relationships occur between mycorrhizal fungi and mycorrhizosphere microbiota. Mycorrhizal infection can stimulate some bacterial groups or, in contrast, suppress others. For instance, the population densities of fluorescent pseudomonads are often decreased by mycorrhizal infection [2, 17, 29].

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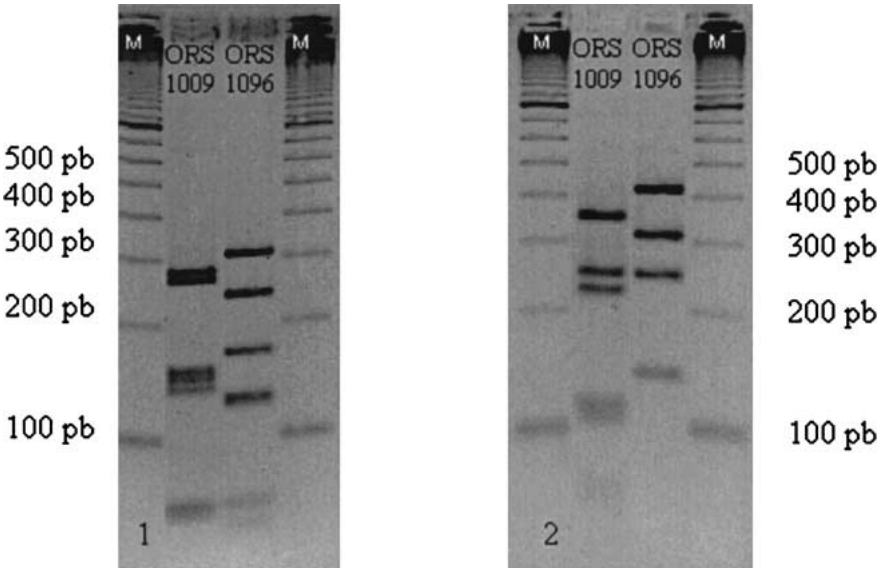

Fig. 1. Gel electrophoresis of PCR-amplified 16S-23S rDNA fragments of *S. terangae* (ORS 1009) and *M. plurifarium* (ORS 1096), digested with two restriction enzymes (1, *Msp*I. 2, *Hae*III. M, molecular weight markers).

Many N_2-fixing trees and shrubs are especially dependent on mycorrhizae to absorb nutrients required for plant growth and efficient N_2 fixation [11, 19]. A positive global effect of mycorrhizas on the size of the rhizobial populations has been demonstrated [6, 23, 30]. For example, inoculation of *Acacia tortillis* spp. *raddiana* with the arbuscular mycorrhizal fungus *Glomus mosseae* increased nodule weights by 10- to 12-fold [11]. However, the influence of the mycorrhizal symbiosis on the competitive interactions between inoculated rhizobial strains is largely unknown.

The aim of the present study was to assess the effect of the mycorrhizosphere of *A. tortillis* spp. *raddiana* with mycorrhizal fungus *Glomus intraradices* on root nodulation by *Sinorhizobium terangae* (ORS 1009) and/or *Meso rhizobium plurifarium* (ORS 1096) in two different culture substrates.

Materials and Methods

Plant

Seeds of *Acacia tortillis* spp. *raddiana* (provenance Bel Air, Dakar, Senegal) were surface sterilised in sulfuric acid (36 N) for 60 min. The acid solution was then decanted and the seeds rinsed and imbibed for 6 h in four changes of sterile distilled water. Seeds were then transferred aseptically in Petri dishes filled with 4% (w:v) water agar. The plates were incubated for 2 days at 28°C. The germinating seeds were used when radicules were 1–2 cm long.

Fungal Inocula and Rhizobia

The arbuscular mycorrhizal fungus *Glomus intraradices* Schenk & Smith (DAOM 181602, Ottawa Agricultural Herbarium) was propagated on leek (*Allium porrum* L.) for 12 weeks under greenhouse conditions on Terragreen substrate. This calcined clay (particule size average 5 mm), Oil-Dri US-special Ty/IIIR (Oil-Dry Company, Chicago, USA) is an attapulgite from Georgia [32]. The leek plants were then uprooted; the roots were gently washed and cut into pieces 0.5 cm long (around 250 vesicules cm^{-1}). Nonmycorrhizal leek roots, prepared as above, were used for the control treatment without endomycorrhizal inoculation.

The isolates of *Sinorhizobium terangae* (ORS 1009) and *Mesorhizobium plurifarium* (ORS 1096) were isolated from nodules collected in Senegal under *Acacia laeta* and *A. raddiana*, respectively [12, 13]. They were cultured in glass flasks containing liquid yeast extract–mannitol medium [37] at 37°C for 2 days on an orbital shaker. The bacterial suspensions were approximately 10^9 cfu mL^{-1}.

Preparation of Growth Substrates

Two culture soils were used. Soil was collected from a 17-year-old plantation of *A. holosericea* in an experimental station localised at Sangalkam (50 km east of Dakar, Senegal). The soil was crushed, passed through a 2-mm sieve, and autoclaved for 40 min at 120°C. After autoclaving, the physicochemical characteristics of the soil were as follows: pH (H_2O) 5.3; clay (%) 3.6; fine silt (%) 0.0; coarse silt (%) 0.8; fine sand (%) 55.5; coarse sand (%) 39.4; carbon (%) 0.17; nitrogen (%) 0.02; C/N 8.5; total P (ppm) 39; and Olsen P (ppm) 4.8.

A contrasting substrate was a sand collected from dunes near Dakar. Before autoclaving (40 min, 120°C), the sand was washed with tap water to eliminate salt traces.

These particular substrates were used to test the influence of soil nutrient availability on rhizobial and mycorrhizal colonization by comparing a soil with another substrate, the sand, that was very deficient in P and N content.

The germinated seeds were individually grown in 0.5-L pots filled with the autoclaved soil or sand. One hole (1 cm by 5 cm) was made in each pot and filled with 1 g fresh mycorrhizal leek

Table 1. Influence of *G. intraradices* on the plant growth, *A. raddiana* nodulation by *S. terangae* (ORS 1009) and/or *M. plurifarium* (ORS 1096), microbial biomass, and soil nitrogen content in the disinfected sandy soil after 4 months culture

Treatments	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)	Number of nodules per plant	Microbial biomass (μg C g^{-1} of soil)	NH_4^+ (μg g^{-1} of soil)	NO_3^- (μg g^{-1} of soil)
Control	117 a*	180 a	0.7 a	18.7 a	2.0 b	9.0 b
ORS 1009	195 a	161 a	0.7 a	25.3 b	1.8 ab	7.0 ab
G. intraradices	701 b	321 b	7.3 b	20.3 ab	1.7 ab	0.9 a
G. intraradices + ORS 1009	786 b	375 b	11.3 b	24.3 b	1.3 a	2.3 a
Control	117 a	180 a	0.7 a	18.7 a	2.0 b	9.0 b
ORS 1096	108 a	195 b	0.0 a	18.0 a	1.8 ab	17.2 c
G. intraradices	701 b	321 b	11.3 b	20.3 a	1.7 ab	0.9 a
G. intraradices + ORS 1096	774 b	617 c	23.7 b	16.7 a	1.3 a	2.9 a
Control	117 a	180 a	0.7 a	18.7 a	2.0 b	9.0 b
ORS 1009 + ORS 1096	239 a	226 ab	2.0 a	20.3 a	1.9 a	8.9 b
G. intraradices	701 b	321 b	11.3 b	20.3 a	1.7 a	0.9 a
Gi + ORS 1009/ORS 1096	824 b	453 b	11.7 b	12.7 a	1.5 a	2.1 a

*For each rhizobial inoculation, data in the same column followed by the same letter are not significantly different according to the one-way analysis of variance.

root (not mycorrhizal for the control treatments). The holes were then covered with the same autoclaved soil. After 1 week culture, seedlings were inoculated with 5 mL of the ORS 1009 or ORS 1096 suspensions (10^9 bacterial cells) or 5 mL of the culture medium without bacteria for the control treatments. The same volume of bacterial inoculum was added in the treatments combining ORS 1009 and ORS 1096. The planted pots were kept in a greenhouse (25°C day, 20°C night, 10-h photoperiod) and were watered regularly with nonsterilized water (120°C, 20 min). The pots were arranged in a randomized complete block design.

Quantitative Evaluation

The plants were harvested after 4 months and the shoots and roots were separated. Shoot dry matter was determined after drying at 80°C for 1 week. Root systems were gently washed. Root nodules were counted, surface disinfected with calcium hypochlorite (33 g L^{-1}) for 5 min, then with 96° ethanol for 5 min, and rinsed with sterile distilled water. They were cryopreserved at −80°C in glycerol 20%. For the determination of the mycorrhizal infection, root systems were cleared and stained according to the method of Phillips and Hayman [31]. Roots were cut into 1 cm pieces, mixed, and placed on slides for microscopic observations [10]. About 100 root pieces were observed per plant under a microscope (magnification × 400). The endomycorrhizal rates were expressed as (number of mycorrhizal root pieces/number of nonmycorrhizal root pieces) × 100). Then root dry weight (60°C, 1 week) was determined for each plant. The soil of each pot, 10 replicates per treatment, was mixed and 20 g of moist soil were sampled to determine the microbial biomass using the fumigation extraction method [1]. The NH_4^+ and NO_3^- contents were determined according to the method described by Bremner [9].

For evaluation of rhizobial infection, nodules were collected in each treatment with ORS 1009 and/or ORS 1096 and crushed in 150 μL sterile distilled water with a plastic pestle. Then 150 μL of CTAB/PVPP buffer (0.2 M Tris-HCl, pH 8; 0.04 M EDTA pH 8; 2.8 M NaCl; 4% w:v CTAB; 2% w:v PVPP) was added to the crushed nodule suspension. The mixture was incubated at 65 °C for 60 min and centrifuged for 10 min at 11,000 *g* to remove cell fragments. Supernatant was mixed with 150 μL of phenol–chloroform–isoamyl alcohol (25:24:1; v:v:v) and centrifuged at 13,000 *g* for 15 min. DNA was purified from phenol by adding 150 μL of chloroform–isoamyl alcohol (24:1; v:v) followed by a centrifugation at 13,000 *g* for 15 min. DNA from the aqueous phase was precipitated overnight at −20°C by adding sodium acetate–absolute ethanol (1:25; v:v). The solution was centrifuged at 13,000 *g*. The DNA pellet was washed with ethanol 70% and the suspension was centrifuged at 13,000 *g* for 15 min, vacuum dried, and desorbed into 25 μL of ultrapure water. DNA samples were stored at −20°C for further analysis.

The primers MBAS3 (5′-TGCGGCTGGATCACCTCCTT-3′) and MBAL2 (5′-GTGGGTTCCCCATTCGG-3′) were used to amplify the 16S-23S rDNA spacer region. The amplification was made in a total volume of 25 uL and performed with lyophilized beads (Ready to Go PCR beads, Pharmacia Biotech) containing 1.5 U of *Taq* polymerase, 10 mM of Tris-HCl at pH 9 and ambient temperature, 50 mM of KCl, 1.5 mM of $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTP), 1 μM of each primer, and 4 μL of pure total DNA extract (10–50 ng μL^{-1}). Amplification reactions were performed in a Gene-Amp PCR System 2400 automatic thermocycler (PerkinElmer Applied Biosystems, California). The program was as follows: initial denaturation 5 min at 95°C, 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 55°C) and extension (1 min at 72°C), and the last extension (7 min at 72°C). PCR-amplified DNAs in 3-μL aliquots were visualized by horizontal electrophoresis on 1% (w:v) agarose gel (type II, Sigma, La Verpilliere, France). The gels were stained for 30 min with ethidium bromide (1 mg L^{-1}) and integrated with image analysis software BIOCAPT (Vilbert Lourmat, France) under a 260-nm UV source.

PCR products (7 μL) were digested in a total volume of 20 μL at 37°C for 2 h using the endonucleases HaeIII and MspI (Gibco

Table 2. Influence of *G. intraradices* on the plant growth, *A. raddiana* nodulation by *S. terangae* (ORS 1009) and/or *M. plurifarium* (ORS 1096), microbial biomass, and soil nitrogen content in the disinfected sand after 4 months culture

Treatments	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)	Number of nodules per plant	Microbial biomass (μg C g^{-1} of soil)	NH_4^+ (μg g^{-1} of soil)	NO_3^- (μg g^{-1} of soil)
Control	106 a*	147 a	3.0 a	1.3 a	1.7 a	2.5 a
ORS 1009	122 a	148 a	8.7 b	5.0 ab	1.6 a	3.0 a
G. intraradices	113 a	155 a	11.0 b	7.3 ab	1.2 a	0.07 a
G. intraradices + ORS 1009	343 b	223 a	12.0 b	13.0 b	1.4 a	0.5 a
Control	106 a	147 a	3.0 a	1.3 a	1.7 b	2.5 a
ORS 1096	136 a	154 a	13.7 b	4.3 ab	1.4 ab	2.9 a
G. intraradices	113 a	155 a	11.0 b	7.3 b	1.2 a	0.07 a
G. intraradices + ORS 1096	196 a	125 a	13.7 b	4.7 ab	1.4 ab	1.7 a
Control	106 a	147 a	3.0 a	1.3 a	1.7 a	2.5 a
ORS 1009 + ORS 1096	137 ab	167 a	4.7 a	8.7 b	1.6 a	1.2a
G. intraradices	113 a	155 a	11.0 a	7.3 b	1.2 a	0.07 a
Gi + ORS 1009/ORS 1096	195 b	222 a	11.0 a	11.0 b	1.4 a	1.6 a

*For each rhizobial inoculation, data in the same column followed by the same letter are not significantly different according to the one way analysis of variance.

BRL, Cergy Pontoise, France) as described by the manufacturer. Restricted DNA was analyzed by horizontal electrophoresis in a 2.5% (w:v) Metaphor gel (FMC, Rockland, MD; USA). After 3 h of running at 80 volts, the gels were stained with ethidium bromide (1 mg L^{-1}) and photographed under UV light.

Statistical Analysis

Data were subjected to a one-way analysis of variance and means were compared with the Student's t test ($P < 0.05$). Fungal percentage colonization was transformed by arcsin ($\sqrt{x}$) before statistical analysis. The distributions of both rhizobial strains along the root systems of mycorrhized and not mycorrhized plants were compared with 2×2 contingency tables and chi-square test (ψ^2 test) and Yates correction for small numbers.

Results

Mycorrhizal colonization of *A. raddiana* seedlings ranged from 38.3% to 40.7%. No significant differences were recorded between the two cultural substrates.

Nodulation

Neither rhizobial strains stimulated the growth of *A. raddiana* seedlings grown on either substrates (soil or sand) (Tables 1 and 2).

Fungal inoculation alone significantly increased shoot biomass on soil (Table 1) but not on sand (Table 2). On soil, the shoot growth response to dual inoculation, *G. intraradices* + ORS 1009 and/or ORS 1096 was not significantly different than the response to *G. intraradices* inoculation alone (Table 1). In contrast, on sand the dual inoculations (*G. intraradices* + ORS 1009 or *G. intraradices* added with both rhizobial strains) significantly increased shoot growth of *A. raddiana* seedlings (Table 2). Root biomass was not significantly affected by microbial inoculants on sand (Table 2). In contrast, fungal inoculation with or without ORS 1009 and/or ORS 1096 significantly stimulated root growth on soil. Moreover, a significant difference in root growth was found between the treatments *G. intraradices* + ORS 1096 and *G. intraradices* alone, when grown on soil (Table 1).

Although the soil and dune sand were autoclaved and the seed surfaces disinfected, the presence of nodules on control plant roots indicates that plants were contaminated with indigenous rhizobia (Tables 1 and 2). One explanation for this contamination was that the irrigation water may have contained N_2-fixing bacteria. *G. intraradices* singly inoculated significantly increased this contamination in both culture substrates as judged by nodule numbers per plant (Tables 1 and 2). Neither rhizobial strain singly inoculated infected *A. raddiana* root systems in the soil experiment (Table 1). However, rhizobial colonization occurred when both rhizobia were inoculated in soil or sand (Tables 1 and 2), and when singly inoculated in dune sand substrate (Table 2). The arbuscular mycorrhizal fungus significantly increased the root infection of both rhizobial strains inoculated together or singly in all the treatments in soil (Table 1), but not in sand (Table 2).

Table 3. Identification of rhizobial strains from nodules collected in each rhizobial treatment in soil and sand by restriction analysis of PCR-amplified 16S-23S rDNA spacer region

Treatments	Number of isolates[a]	
	ORS 1009	ORS 1096
Soil		
ORS 1009	0	0
G. *intraradices* + ORS 1009	100 (9)[a]	0
ORS 1096	0	0
G. intraradices + ORS 1096	0	100 (11)
ORS 1009 + ORS 1096	20 (1)	80 (4)
Gi + ORS 1009/ORS 1096	48 (25)	52 (27)
Sand		
ORS 1009	100 (11)	0
G. intraradices + ORS 1009	100 (6)	0
ORS 1096	0	100 (7)
G. intraradices + ORS 1096	0	100 (5)
ORS 1009 + ORS 1096	0	100 (32)
Gi + ORS 1009 /ORS 1096	13 (3)	87 (20)

[a] Percentage (and number of analyzed nodules) of each rhizobial strain from each treatment.

Restriction Analysis

Restriction analysis was performed with 77 nodules from plants grown in soil and 84 nodules from plants grown in sand. Nodules collected from the roots of plants not inoculated with ORS 1009 or ORS 1096 yielded PCR/RFLP patterns different from those of ORS 1009 and 1096 (data not shown). Presumably, these represented the contaminating rhizobia. Nodules from ORS 1009 or ORS 1096 treatments with or without *G. intraradices* exhibited the same patterns as the inoculated rhizobial strains in both experiments (Table 3). In the soil-grown plants, when both rhizobial strains (but not the fungus) were inoculated, the ORS 1009 strain was detected in 20% of the treated nodules compared to 80% for ORS 1096 (Table 3) (Fig. 1). When *G. intraradices* was inoculated, both rhizobial strains were equally represented (47% for ORS 1009 and 51% for ORS 1096). However, this mycorrhizal effect was not significantly different according to the ψ^2 test ($\psi^2 = 1.45$, $P = 0.25$). In sand-grown plants, only the ORS 1096 strain was recorded from nodules collected in the dual rhizobial inoculation (Table 3). In contrast, when *G. intraradices* was present, the ORS 1009 strain was recorded from 13% of the total number of nodules compared to 87% for ORS 1096 (Table 3). The distributions of root nodule rhizobia with and without *G. intraradices* were significantly different according to the ψ^2 test ($\psi^2 = 4.4$, $P = 0.04$).

In the soil-grown plants, microbial biomass was significantly higher in the ORS 1009 + *G. intraradices* than in the control (Table 1). NO_3^- content was significantly lower in the dual inoculation treatment (rhizobia + *G. intraradices*) than in the control. The same effect was recorded in ORS 1009 + *G. intraradices* or ORS 1096 + *G. intraradices* treatments with NH_4^+ content (Table 1). In the sand-grown plants, no significant differences between the treatments were found for NO_3^- and NH_4^+ (Table 2). Microbial biomass was significantly higher in the dual inoculation and ORS 1009 + *G. intraradices* treatments than in the control (Table 2). In soil-grown plants, shoot biomass was positively correlated with the number of nodules per plant and negatively to the soil nitrogen content (NH_4^+ and NO_3^-) (Table 4). The number of root nodules was also negatively correlated with the soil nitrogen content. In the sand-grown plants, shoot biomass was only correlated with the root biomass and the microbial biomass. No significant correlations were recorded between the number of root nodules per plant and the other parameters (Table 4).

Discussion

Not surprisingly, *Glomus intraradices* dramatically stimulated plant growth. Shoot biomass of *A. raddiana* seedlings was stimulated nearly 6-fold on a sandy soil commonly found in West Africa. This beneficial effect of the arbuscular mycorrhizal symbiosis on *A. raddiana* was previously described with *G. mosseae* [11] and with *G. aggregatum* [14]. However, a positive effect of mycorrhizal inoculation was not observed in plants grown in sand. It is well known that soil chemical and physical properties influence mycorrhizal establishment [15, 24, 33]. Most likely, the sand lacked some critical material necessary for growth of the mycorrhizal fungus.

Co-inoculation of the fungus + rhizobia in soil did not significantly influence plant growth compared to the *G. intraradices* treatment, but the fungus did enhance the nodulation process. With sandy soil, neither rhizobial strain infected root systems when they were inoculated without *G. intraradices*. On the other hand, ORS 1009 and ORS 1096 nodules were detected by restriction fragment analysis when the fungus was also present. Our results agree with those of others, that arbuscular mycorrhizal infection generally helps nodule formation and function

Table 4. Correlation matrix between the plant growth parameters, root nodulation, soil nitrogen content, and microbial biomass with each cultural substrate (soil and sand)

	SB	RB	NP	MB	NHC	NOC
Soil						
Shoot biomass (SB)	1.000	0.79**	0.70**	NS	−0.61**	−0.67**
Root biomass (RB)		1.000	0.65**	NS	−0.40*	−0.42*
Number of nodules per plant (NP)			1.000	NS	−0.62**	−0.42*
Microbial biomass (MB)				1.000	NS	NS
NH_4^+ content (NHC)					1.000	NS
NO_3^- content (NOC)						1.000
Sand						
Shoot biomass (SB)	1.000	0.56**	NS	0.75**	NS	NS
Root biomass (RB)		1.000	NS	0.61**	NS	NS
Number of nodules per plant (NP)			1.000	NS	NS	NS
Microbial biomass (MB)				1.000	NS	NS
NH_4^+ content (NHC)					1.000	NS
NO_3^- content (NOC)						1.000

*Significant at $P < 0.05$; **Significant at $P < 0.01$; NS, not significant.

under stress conditions (drought, salinity, low-nutrient soil, etc.) [3]. Mechanisms by which mycorrhizae facilitate nodule formation include increased root growth, which favors rhizobia colonization and infection. In addition, root exudation is modified both qualitatively and quantitatively by the presence of an active arbuscular mycorrhizal symbiosis [26, 38]. Extraradical mycelium of mycorrhizal fungi influence the chemical composition, pH of the soil and release substances into the soil [4, 39]. In particular, many fungi produce trehalose [35]. This carbohydrate has been identified in mycorrhizas such as arbuscular endomycorrhizas [7, 34]. As both rhizobial strains used in this experiment can metabolize this compound (data not shown), release of trehalose could stimulate the rhizosphere colonization of ORS 1009 and ORS 1096. These changes could increase rhizobial success in the mycorrhizosphere. Beside the stimulatory effects of the mycorrhizosphere on both rhizobia development, *G. intraradices* infection induces two different dynamics of each bacterial strains in sand-grown plants. ORS 1096 seems to be more competitive than ORS 1009 in sand. Mycorrhizal infection increases the competitiveness of ORS 1009. This mycorrhizosphere effect could be also attributed to a generalized stimulation of host nutrition [15]. However more specific relationships could occur during the development of the tripartite symbiosis, at physiological and molecular level [8, 36]. From a practical point of view, the role of arbuscular mycorrhizas in improving nodulation and N_2 fixation is universally recognized and is a fact of great relevance to any sustainable approach [22]. Moreover, fungal symbiosis could modify the development of bacterial inoculants along the root systems. This effect is of particular interest in the controlled inoculation of selected rhizobia.

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