ELSEVIER

Plant Science 159 (2000) 57–63

PLANT SCIENCE

www.elsevier.com/locate/plantsci

Growth promoting effect of two *Sinorhizobium meliloti* strains (a wild type and its genetically modified derivative) on a non-legume plant species in specific interaction with two arbuscular mycorrhizal fungi

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Received 15 February 2000; received in revised form 2 May 2000; accepted 15 June 2000

Abstract

In the present study, we have investigated whether the ubiquitous rhizosphere soil organism *Sinorhizobium meliloti* has a plant growth promoting (PGP) effect on non-leguminous plant species. Such PGP activity was investigated for both a wild type strain and its genetically modified (GM) derivative, which had an enhanced biofertilizer capability. The PGP effect of these rhizobial strains was tested in interaction with two arbuscular-mycorrhizal (AM) fungi: *G. mosseae* or *G. intraradices* on lettuce (*Lactuca sativa L.)* plants. Both rhizobial strains were efficient in increasing lettuce biomass and also induced modifications on root morphology, particularly in mycorrhizal plants; thus these strains behave as plant growth promoting rhizobacteria. In non-mycorrhizal plants, both strains exhibited a similar growth promoting effect on lettuce. However, both rhizobial strains differed in mycorrhizal plants with regard to (i) biomass production, (ii) the length of axis and lateral roots, and (iii) the number of lateral roots formed; effects which were, in turn, affected by the AM fungus involved. Microbial treatments were more effective on root growth and morphology at earlier developmental stages (20 days of plant growth) but, in a later stage (after 40 days), the microbial effects were more relevant at increasing plant biomass. The interaction between the GM rhizobial strain and *G. mosseae* produced the highest growth promoting effect (476% over control), in spite of the fact that *G. intraradices* showed a quicker and higher colonization ability than *G. mosseae*. Microbial interactions inducing PGP effects did not benefit AM colonization nor the succinate dehydrogenase activity in the AM fungal mycelium. Irrespective of the underlying mechanisms, which are being now investigated, the interactions between rhizobial strains, as free-living saprophs, and AM fungi are noteworthy, and depend on the microbial genotype involved.

Keywords: Sinorhizobium; Arbuscular mycorrhizal fungi; Specific interactions; Non-legume plant; PGPR

1. Introduction

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium and Azorhizobium* are known for their capacity to fix atmospheric nitrogen in a symbiotic relationship with the root of leguminous plants. However, Rhizobiaceae have also the ability to form non-specific associative interactions with roots of other plants without forming nodules [1]. It has been suggested that rhizobial strains may be able to produce plant growth regulators, and some of them have been considered as a plant growth promoting rhizobacteria (PGPR) [2]. This term was first coined in 1978 by Kloepper and Schroth [3] for beneficial bacteria colonizing niches closed to plant roots. Several genera of bacteria have been recognized as having PGPR

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0168-9452/00/$ - see front matter
PII: S0168-9452(00)00321-6

activity [4–6]. Plant growth promoting rhizobacteria represent a diverse group of rhizosphere colonizing bacteria and diazotrophic microorganisms [7]. Several mechanisms have been proposed to account for plant growth stimulation including the involvement of a range of metabolites that stimulate plant growth either directly or indirectly [8,9].

In young plants, the main reported effect of beneficial inoculation was an improvement of root development, which affects root length and branching. Microbial metabolites such as auxins, cytokinins and gibberellins are know to stimulate root development, and they are produced by both rhizosphere bacteria and arbuscular mycorrhizal (AM) fungi [10–13]. However, whether these microbial phytoactive metabolites are able to stimulate root growth in situ is still under discussion.

Among the microorganisms inhabiting the rhizosphere and contributing to soil fertility, AM fungi must be considered [14]. Combined inoculations of rhizobial strains with AM fungi produced growth stimulating effects that surpassed those of individual inoculations in legumes. However, it has been shown that plant responses depend on

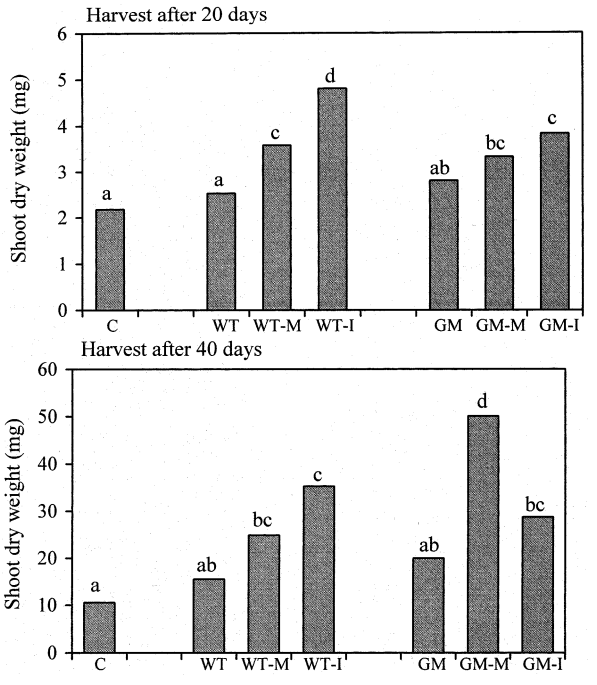

Fig. 1. Effect of *S. meliloti* strains (the wild type ⟨WT⟩ and its genetically modified ⟨GM⟩ derivate) and mycorrhizal fungus (*G. mossae* ⟨M⟩ or *G. intraradices* ⟨I⟩) on the shoot dry biomass of lettuce plants at two harvest times (20 and 40 days after sowing). Bars representing mean values (five replicates), not sharing a letter, differ significantly at $P < 0.05$ by Duncan's test.

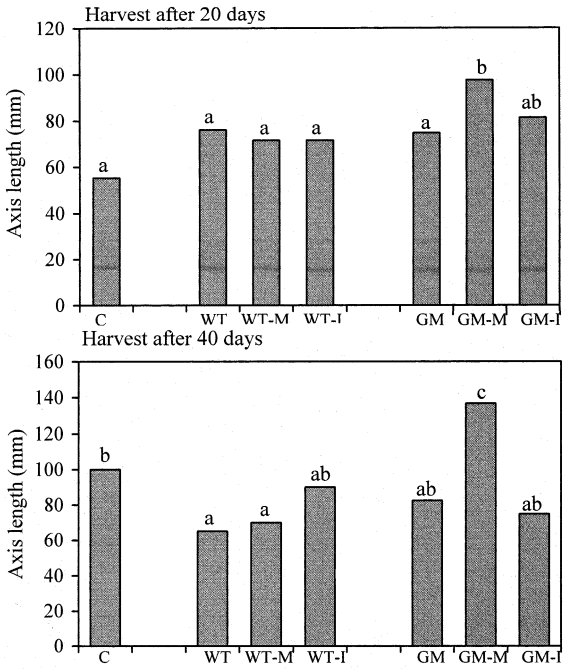

Fig. 2. Effect of *S. meliloti* strains (the wild type ⟨WT⟩ and its genetically modified ⟨GM⟩ derivate) and mycorrhizal fungus (*G. mossae* ⟨M⟩ or *G. intraradices* ⟨I⟩) on the axis root length of lettuce plants at two harvest times (20 and 40 days after sowing). Bars representing mean values (five replicates), not sharing a letter, differ significantly at $P < 0.05$ by Duncan's test.

the particular combinations of rhizobial strains and AM fungal isolates because physiological and biochemical processes are successfully shared in the triple association [15]. Selected combinations of *Sinorhizobium* strains and specific AM fungi enhanced the positive effects achieved by each microbial group improving the acquisition of nutrients [15]. Particular and specific interactions between these endophytes need to be compatibilized at a physiological level [16]. In general, for an effective growth stimulation, a close interaction between efficient microorganisms and host plants is a prerequisite for the utilization by the partners of plant assimilates or microbial metabolites, respectively [4].

There is no information concerning the saprophite plant growth promoting effect of *Sinorhizobium meliloti* species. Thus, the objective of this study was to evaluate the activity of both a wild *S. meliloti* and its genetically derivative strain on non-leguminous plants. These rhizobial strains were found to improve processes related to mycorrhiza formation and plant responses by *G. mosseae* on *Medicago sativa* [17]. In the present study, the

PGPR activity of the wild-type (WT) *S. meliloti* and its genetically modified (GM) derivative was evaluated in a soil microcosm system at two growth periods (after 20 and 40 days of sowing), on lettuce plants either mycorrhizal or not. Shoot biomass, root development and the impact of the *Sinorhizobium* strains on AM colonization by two AM fungi, and the levels of succinate dehydrogenase activity (SDH) of the AM fungal development in the root cortex were evaluated.

2. Materials and methods

2.1. Experimental design

The experiment used a randomized complete-block design. The factors were: two AM isolates, each one combined with each one of two *Sinorhizobium* strains (WT and GM), and controls without AM inoculum. Ten replicates per treatment were used for a total of 70 pots to allow for two harvests.

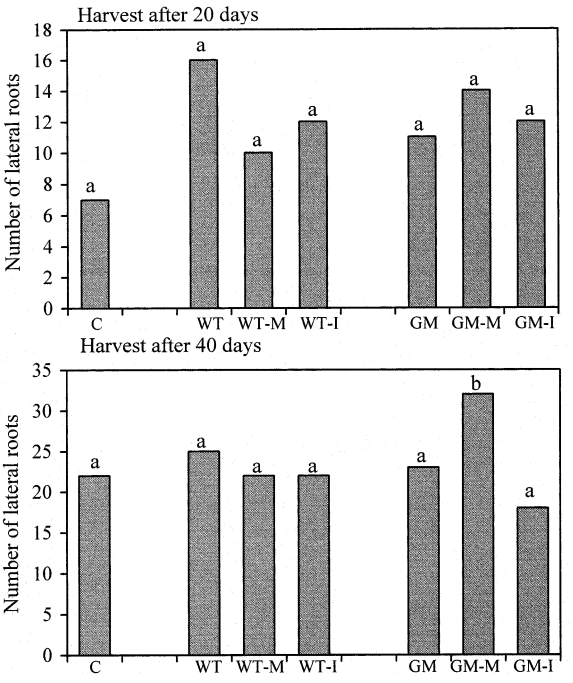

Fig. 3. Effect of *S. meliloti* strains (the wild type ⟨WT⟩ and its genetically modified ⟨GM⟩ derivate) and mycorrhizal fungus (*G. mossae* ⟨M⟩ or *G. intraradices* ⟨I⟩) on the number of lateral roots of lettuce plants at two harvest times (20 and 40 days after sowing). Bars representing mean values (five replicates), not sharing a letter, differ significantly at $P < 0.05$ by Duncan's test.

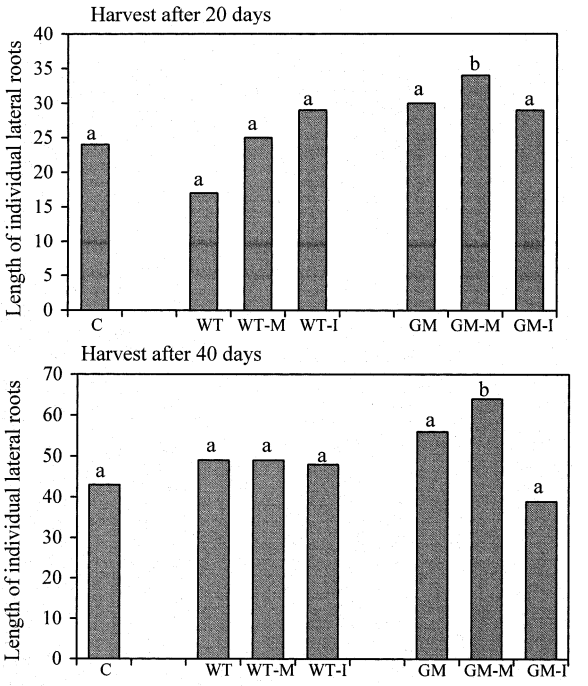

Fig. 4. Effect of *S. meliloti* strains (the wild type ⟨WT⟩ and its genetically modified ⟨GM⟩ derivate) and mycorrhizal fungus (*G. mossae* ⟨M⟩ or *G. intraradices* ⟨I⟩) on the length of individual lateral roots of lettuce plants at two harvest times (20 and 40 days after sowing). Bars representing mean values (five replicates), not sharing a letter, differ significantly at $P < 0.05$ by Duncan's test.

2.2. Soil and biological materials

The experimental soil was collected from a field area in the Estación Experimental del Zaidín (Granada) [18], sieved (2 mm), diluted with quartz sand (1/1, v/v) and autoclaved (100°C for 1 h on each of three consecutive days). The soil/sand mixture had pH 8.1, 1.8% (w/w) organic matter, and the following nutrient concentrations (mg kg^{-1}): N, 2.5; P ($NaHCO_3^-$ extractable P), 6.24; K, 132. It consisted of 35.8% sand, 46.3% silt and 20.5% clay. Experiment pots were filled with 300 g sterilized soil/sand (1/1 v/v) mixture.

Mycorrhizal inoculum from each endophyte was multiplied in an open pot culture of *Lactuca sativa*, and consisted of soil, spore, hyphae and AM root fragments. The AM fungal species, belonging to the collection of Estación Experimental del Zaidín [18], were *G. mosseae* (Nicol. and Gerd.) Gerd and Trappe or *G. intraradices* (Schenck and Smith). Five grams of each inoculum, having similar characteristics (an average of 30 spores g^{-1} and root fragments with 75% of colonized roots length), were applied below the seeds of the test

plant, *L. sativa* L. cv. Romana (as described later). Non-mycorrhizal treatments received the same amount of autoclaved inoculum.

The *S. meliloti* strains tested were the WT GR4, and its GM derivate GR4 (pCK3), developed by Sanjuán and Olivares [19] to improve the nodulation competitiveness of the wild-type strains.

The rhizobial strains were grown on Ty medium [20]. The inoculum of both the WT and the GM strains was applied at a rate of 1 ml per seedling (10^8 cfu ml^{-1}).

2.3. Test plant and growth conditions

Seeds of lettuce (*L. sativa* L. cv. Romana) were sterilized with H_2O_2 per 30 min before sowing. Plants were grown in the greenhouse for 20 and 40 days under controlled environmental conditions of a 16 h/8 h light/dark cycle, a 25°C/15°C day/night temperature, 50% relative humidity and with a photosynthetic photon flux density of 500–750 mmol m^{-2} s^{-1}. Water was supplied after daily weighing to maintain the water-holding capacity of the test soil/sand mixture near 100% throughout the experiment.

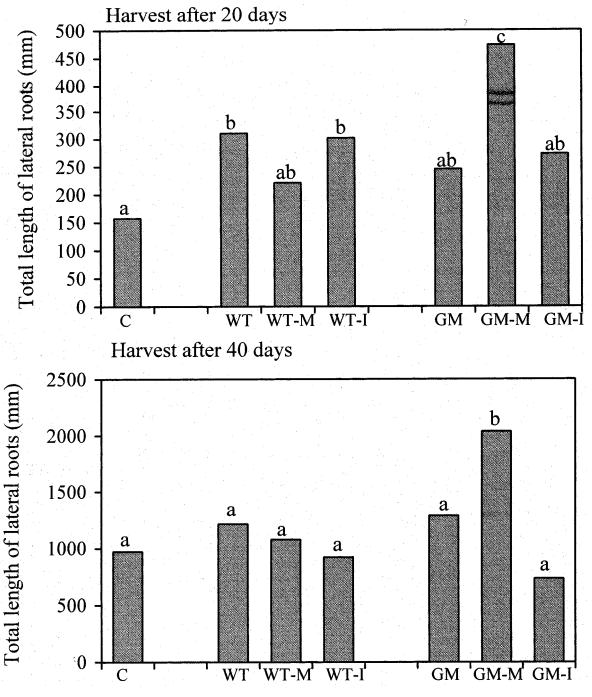

Fig. 5. Effect of *S. meliloti* strains (the wild type ⟨WT⟩ and its genetically modified ⟨GM⟩ derivate) and mycorrhizal fungus (*G. mossae* ⟨M⟩ or *G. intraradices* ⟨I⟩) on the total lateral root length of lettuce plants at two harvest times (20 and 40 days after sowing). Bars representing mean values (five replicates), not sharing a letter, differ significantly at $P < 0.05$ by Duncan's test.

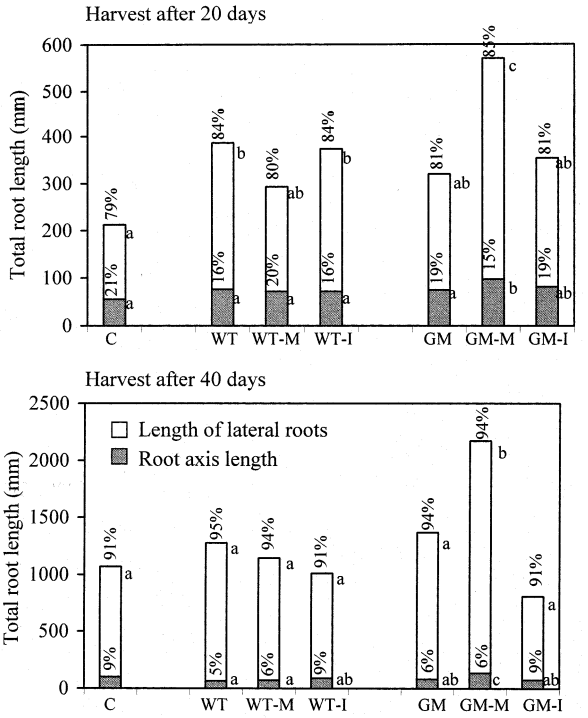

Fig. 6. Effect of *S. meliloti* strains (the wild type ⟨WT⟩ and its genetically modified ⟨GM⟩ derivate) and mycorrhizal fungus (*G. mossae* ⟨M⟩ or *G. intraradices* ⟨I⟩) on the total root growth (axis plus lateral). Numbers inside the bars represent the proportion percentage between axis and lateral roots of lettuce plants at two harvest times (20 and 40 days after sowing). Bars representing mean values (five replicates), not sharing a letter, differ significantly at $P < 0.05$ by Duncan's test.

2.4. Determination of growth and symbiotic parameters

At harvest (20 or 40 days after planting), the root system was separated from the shoot, and the dry weight of shoot was recorded after drying at 70°C. Image analysis methods were applied to study the effect of biological treatments on root development. Roots were separated into axes and laterals, and the root length of each order measured [21]. Mycorrhizal colonization was microscopically assessed using the gridline intersect method [22], after staining [23]. To evaluate the physiological state of the AM fungi in the root system, the living proportion of the AM development showing activity (SDH) was measured [24].

Data for each experimental variable from the five replicates were analysed statistically using analysis of variance and Duncan's test.

3. Results

Dual inoculation of rhizobial strains and AM fungi increase shoot biomass. An early effect of the WT rhizobial strain was evidenced after 20 days of plant growth, in *G. intraradices* mycorrhizal plants with a 220% increase over the control. However, the most efficient treatment at final harvest was the GM strain co-inoculated with *G. mosseae*, which increased shoot growth by 476% at this time (Fig. 1).

Fig. 2 shows that the axis root length was only increased in plants co-inoculated with *G. mosseae* together with the GM rhizobial strain. The number of lateral roots (Fig. 3) was not significantly increased by microbial inoculants at the first harvest, but it was at the second (40 days) by co-inoculation with *G. mosseae* and the GM rhizobial strain. The length of individual roots was also stimulated (at both harvest) by the same microbial combinations (Fig. 4). As Fig. 5 shows, all biological treatments increased lateral root length (significantly in most cases) at the first harvest, but this effect was more evident in plants co-inoculated with GM rhizobial strain and *G. mosseae*. Both rhizobial treatments increased lateral root length in non-mycorrhizal plants. However, such an effect was increased by 300% (20 days) and by 276% (40 days) under dual GM–*G. mosseae* inoculation. The length of lateral roots colonized by *G. mosseae* was significantly affected by rhizobial strain inoculation.

The total root length (axis plus lateral roots) and the relative proportion between axis and lateral roots is represented in Fig. 6. Biological treatments tend to increase the proportion of laterals.

The length of AM-colonized root (AM%) was higher in the case of *G. intraradices*-colonized plants (Fig. 7). *G. mosseae* resulted in a less infective endophyte but the most effective, at any growth stage.

Similarly, the amount of living mycelium, i.e. the proportion of AM colonization showing SDH activity, was higher in *G. intraradices*-colonized plants. For *G. mosseae*-colonized roots, the amount of living colonization increased along the time but the proportion non-vital/vital colonization decreased with time. In fact, at the first harvest, all the fungal mycelium developed by *G. mosseae* in the root cortex showed SDH activity and, in the last determination (40 days), the amount of SDH-active mycelium decreased to a 30% relative to total AM colonization.

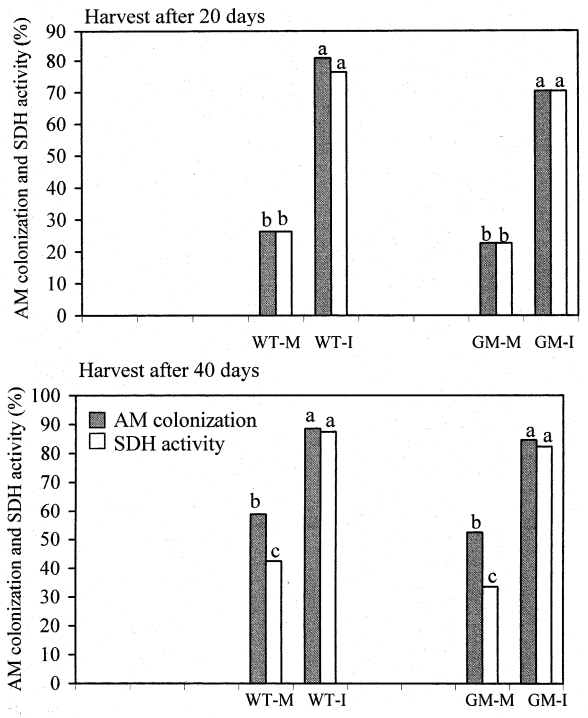

Fig. 7. Effect of *S. meliloti* strains (the wild type ⟨WT⟩ and its genetically modified ⟨GM⟩ derivate) on mycorrhizal colonization and its succinate dehydrogenase (SDH) activity in lettuce roots at two harvest times (20 and 40 days after sowing). Bars representing mean values (five replicates), not sharing a letter, differ significantly at $P < 0.05$ by Duncan's test.

4. Discussion

Inoculation of *S. meliloti* strains, as free-living rhizosphere microorganisms, increase shoot and root biomass of a non-legume mycorrhizal plant species. The positive benefits from rhizobial inoculation, either the WT strain or its GM derivative, may be attributed to several mechanisms such as the secretion of plant growth hormones, known to enhance plant growth by acting on plant metabolic processes [9]. Phytoactive substances can cause morphological and physiological changes in the root system and, according to results presented in this paper, the lateral root development was improved by the GM rhizobial strain early after inoculation. The growth-promoting effect of *G. mosseae* co-inoculated with

the GM strain on the development of lateral roots seems to be a direct microbial interactive mechnism because both the GM and the WT strains produced similar effects on shoot biomass. At the final harvest, GM strain associated with *G. mosseae* resulted in the most efficient treatment, increasing lateral roots (by 276%), principal root (by 37%) and shoot biomass (by 476%). These growth effects may not be attributed to the highest *G. mosseae* colonizing ability, as Tobar et al. [17] observed a direct interaction of rhizobial and AM fungus independently of root colonization. Both rhizobial strains were more effective in mycorrhizal plants. The effectivity of these dual inoculations demonstrates direct and specific interactions between particular microorganisms (bacterium and AM fungus).

The specific compatibilities *Glomus* sp. and *S. meliloti* strains have been previously tested in the legume *M. sativa* using six wild types of *S. meliloti* strains in co-inoculation with three AM fungi [15]. The interactions involved in the tripartite symbioses on the mutualistic processes have been discussed [16,18]. Bianciotto et al. [25] also found that the degree of attachment between rhizobia to spores and/or hyphae of AM fungi depended upon the strain. The effect of other *Sinorhizobium* sp., like *S. leguminosarum*, as growth-promoting bacteria in non-legume plant was previously tested [2,26].

The here-in described effect of rhizobial strains on root development has been shown for other microorganisms that influence root growth and branching, which is fundamental for root function [27–29]. The fact that the GM strain is more effective to improve lateral root development than the WT strain in mycorrhizal plants was also found by Barea et al. [21] using a legume plant. In fact, the PGPR ability of *S. meliloti* strains can be envisaged as an additional mechanism to the N-fixing activity involved in the nodulated *Medicago* sp. plant responses. It therefore seems that free-living rhizobia behave as other PGPRs like *Azospillum* [30] or *Pseudomonas* inoculants [31].

In conclusion, this new aspect of rhizosphere ecology/biology emphasizes the ecological and practical importance of rhizobial isolates as free-living microorganisms for legume and non-legume plants, and the positive interaction developed with other members of soil microbiota as the AM fungi.

Acknowledgements

Two of the authors (C.G. and C.A.) are grateful to AECI, Spain, for financial support during their stay at the EEZ-CSIC. This research was supported by the European Union, TMR Network ERB FMR XCT 960039.

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