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Saprophytic Actinomycetes Promote Nodulation in *Medicago sativa-Sinorhizobium meliloti* Symbiosis in the Presence of High N

Mariana Solans · Gernot Vobis · Luis Gabriel Wall

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Abstract Saprophytic rhizoactinomycetes isolated from the root nodule surface of the nitrogen-fixing actinorhizal plant *Discaria trinervis*, *Streptomyces* MM40, *Actinoplanes* ME3, and *Micromonospora* MM18, previously shown to stimulate nodulation in *Frankia-Discaria trinervis* symbiosis, were assayed as co-inoculants with *Sinorhizobium meliloti* 2011 on *Medicago sativa*. When plants were fertilized with a low level of N (0.07 mM), the inoculation of the actinomycetes alone did not show any effect on plant growth. Meanwhile, when actinomycetes were co-inoculated with *S. meliloti*, nodulation and plant growth were significantly stimulated compared to plants inoculated with only *S. meliloti*. The analysis of nodulation kinetics of simultaneously or delayed co-inoculations suggests that the effect of the actinomycetes operates in early infection and nodule development counteracting the autoregulation of nodulation by the plant. Because the actinomycete effect was found in the symbiotic nitrogen-fixing state of the plant, we investigated the effects of the actinomycetes, in single inoculation or co-inoculation with *S. meliloti*, on plants grown under a high level of N (7 mM) that was inhibitory for nodulation by *S. meliloti*. The inoculation of the actinomycetes alone did not show any effect on plant growth although high N was available. Unexpectedly, the co-inoculation of actinomycetes with *S. meliloti* on plants grown with high N (7 mM) significantly stimulates nodulation, clearly counteracting the inhibition of nodulation by high N. These results corroborate that the interaction of rhizoactinomycetes would interfere with the autoregulation of nodulation in alfalfa mediated by high N, opening new research lines of potential agronomical applications.

Keywords Rhizoactinomycetes · Autoregulation · Nitrogen fixation · PGPR

M. Solans · G. Vobis
Departmento de Botánica, Centro Regional Universitario Bariloche, Universidad Nacional del Comahue, Quintral 1250, 8400 S. C. de Bariloche, Río Negro, Argentina

M. Solans
e-mail: msolans@crub.uncoma.edu.ar

L. G. Wall (✉)
Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, B1876BXD Bernal, Buenos Aires, Argentina
e-mail: lgwall@unq.edu.ar

Introduction

Saprophytic actinomycetes are known to be common rhizoplane- and rhizosphere-colonizing bacteria (Frioni 1999; Solans and Vobis 2003), which have a high capacity to synthesize an array of biodegradative enzymes, antibiotics, phythormone, and antifungal metabolites (Goodfellow and Cross 1974; Takana and Omura 1990; Tokala and others 2002; Gregor and others 2003). The bacterial community of the rhizosphere varies between plant species and over time. Different zones of the same root can support distinct bacterial communities, suggesting qualitative and quantitative differences in root exudation. In addition, soil type has a key role in determining the specific dominant bacteria colonizing the rhizosphere (Marschner and others 2001). The rhizosphere supports diverse groups of bacteria that can interact with the plant in different ways, being deleterious or beneficial to plant growth. Bacteria that may have negative effects on plant growth without infecting root tissues may operate by the production of phytotoxins and phytohormones, competition for nutrients, and the inhibition of mycorrhizal fungi (Glick 1995; Morgan and

others 2005). The bacteria that provide benefit to plants without establishing a mutualism symbiosis with the plant are usually referred to as plant-growth-promoting rhizobacteria or PGPR (Kloepper and others 1989).

Plant-growth-promoting rhizobacteria operate by a wide variety of mechanisms, including N_2 fixation, enhanced solubilization of P, and phytohormone production (Glick 1995; Vessey 2003; Barea and others 2005). A number of various nonsymbiotic bacteria may be considered to be PGPR, such as *Pseudomonas, Azospirillum*, and *Bacillus,* which are associative root colonizers (Kloepper and others 1989). It is common to see the effects of PGPR via synergism or the enhancement of the beneficial effects of a third-party rhizospheric microorganism. In these cases, the PGPR aiding the other host-symbiont relationship is often referred to as "helper" bacteria (Knowlton and others 1980; Vessey 2003; Banerjee and others 2005).

A number of environmental factors such as pH, nitrogen availability, calcium, and the presence of different rhizospheric microorganisms have been shown to influence the N_2-fixing symbioses between legumes and rhizobia. It has long been known that high availability of N has inhibitory effects on nodulation and nitrogen fixation (Streeter 1988). However, studies in hydroponic sand and growth-pouch cultures have shown that in pea (*Pisum sativum*), low, static concentrations of ammonium ($\leq$0.5 mM) actually result in stimulation in whole-plant nodulation (Gudden and Vessey 1997; Fei and Vessey 2003). It is known that the forms and concentrations of external nitrogen influence the levels of endogenous hormones, particularly cytokinins and indole acetic acid (Mercier and Kerbauy 1991; Fei and Vessey 2004). Consequently, changes in the levels of auxins and cytokinins have been implicated in the normal infection and nodulation process of various legumes (Ferguson and Mathesius 2003).

Agricultural practices using free-living plant-growth-promoting rhizobacteria have become a significant component of modern agriculture in many countries (Bashan and Holguin 1997). Traditional studies on the effect of PGPR on legumes with rhizobacteria such as *Azospirillum*, *Pseudomonas*, and *Bacillus* have been very descriptive (Kloepper and others 1989; Liste 1993; Höflich and others 1994; Cattelan and others 1999; Guo and others 2002), but there are few investigations of the promoting effect on legume-rhizobia symbiosis with actinomycetes (Tokala and others 2002; Gregor and others 2003).

Recent studies on a nonlegume nitrogen-fixing symbiosis, the actinorhizal *Discaria trinervis-Frankia,* show a "helper" effect of rhizoactinomycetes on this symbiosis (Solans 2007). The helper effect of rhizoactinomycetes *Streptomyces* MM40, *Actinoplanes* ME3, and *Micromonospora* MM18 was expressed as a promotion of plant growth of *Discaria trinervis* in symbiosis with *Frankia*; meanwhile, no plant-growth-promoting effect was found when the rhizoactinomycetes were applied alone to the plant (Solans 2007). This observation prompted us to study the rhizoactinomycete effect on a different N_2-fixing symbiosis as a legume-rhizobia system.

The aim of this research was to evaluate the effect of saprophytic actinomycetes on the growth and nodulation of *M. sativa* when co-inoculated with *S. meliloti* under different experimental conditions, including high N availability in plant nutrient solution.

Materials and Methods

Bacterial Growth

Sinorhizobium meliloti 2011 (Dr. Lagares IBBM-Universidad Nacional de La Plata, Argentina) was cultured in liquid yeast extract maltose medium with continuous agitation at room temperature (20–22°C) during the day and static at 28°C overnight, for 72 h. Cells were harvested by centrifugation at 5000 rpm for 15 min and washed twice with the same volume of 1/10 Evans solution (Huss-Danell 1978) with 0.07 mM N as NH_4NO_3. Resuspended cells were stored at 6°C in the same solution to be used for inoculation. The plants were inoculated with 0.1 ml of the rhizobia suspension at an optical density of 0.7 at 600 nm, containing approximately 10^6 bacteria per plant.

Rhizoactinomycetic strains *Streptomyces* sp. (BCRU-MM40), *Actinoplanes* sp. (BCRU-ME3), and *Micromonospora* sp. (BCRU-MM18) were originally isolated from the rhizosphere and rhizoplane of the actinorhizal plant *Discaria trinervis* (Solans and Vobis 2003). The inocula of rhizoactinomycetes were prepared from nonwashed homogenized culture, including mycelia and supernatant of each strain and grown in liquid Emerson YpSs medium (Vobis 1992) in Erlenmeyer flasks with continuous agitation at room temperature (20–22°C) during daytime and static at 28°C overnight for 8 days. Plants were inoculated with 1 ml of each homogenized culture of rhizoactinomycetes containing 5×10^6 cfu/ml.

Plant Growth

Alfalfa (*Medicago sativa*) seeds (Salamida, S.C. de Bariloche) were surface sterilized by washing for 2 min in 96% ethanol, followed by 15 min in 0.2% $HgCl_2$ in 0.5 v/v HCl, and then washed five times with sterile distilled water. For germination, the surface-sterilized seeds were sown in Petri dishes containing vermiculite-sand, moistened with nutrient 1/10 Evans solution (Huss-Danell 1978) with 0.07 mM N as NH_4NO_3, and cultured for 5 days. The germination and plant growth took place in a growth

chamber with 16-h photoperiod (photosynthetic photon flux density = 318 μmol m^{-2} s^{-1}) at 20–26°C and 35–60% relative humidity. After germination, single plants were transferred aseptically to glass tubes (2.5 × 20 cm) containing vermiculite-sand as substrate and fertilized with 1/10 Evans solution (Huss-Danell 1978) with 0.07 mM N as NH_4NO_3 until inoculation.

In addition, growth in the pouch system was carried out with two plants transferred to each pouch (Mega International, Minneapolis, MN, USA) and fertilized with Evans solution containing 0.07 mM N as NH_4NO_3 until inoculation. After 15 days from transference, the plants were inoculated with the rhizobia suspension and rhizoactinomycetes strains.

Experimental Designs

To study the effect of rhizoactinomycetes strains on growth and nodulation of *M. sativa*, the plants were inoculated with each strain alone or co-inoculated with *S. meliloti* 2011 and cultivated either in glass tubes or the growth-pouch system. The last growth system was chosen to follow the rate of nodulation throughout the experiment. In both cases, the plants were watered after inoculation with 1/10 Evans solution with 0.07 mM N. The following different treatments were assayed: noninoculated control plants (C); single inoculations with the symbiotic rhizobial strain *S. meliloti* 2011 (R), *Streptomyces* MM40 (S), *Actinoplanes* ME3 (A), or *Micromonospora* MM18 (M); double inoculations (co-inoculations) with the rhizobia strain *S. meliloti* 2011(R) and each of the rhizoactinomycetes strains (RS, RA, RM); and a triple inoculation with *S. meliloti* 2011, *Streptomyces* MM40, and *Actinoplanes* ME3 (RSA).

To investigate if the effect of rhizoactinomycetes operates before or after the interaction of *S. meliloti* with the plant, the double inoculation on plant growth pouches was delayed 14 days between microorganisms. The plants were inoculated with *S. meliloti* 2011 first and then, 14 days later, inoculated with each saprophytic actinomycete, or they were first inoculated with each rhizoactinomycete strain and then with *S. meliloti* 2011. Thus, the different treatments were as follows: control plants with single inoculation with the rhizobial symbiotic strain *S. meliloti* (R); double inoculation with *S. meliloti 2011* first and then with *Streptomyces* MM40 or with *Streptomyces* MM40 first and then with *S. meliloti* 2011 (RS/SR); *S. meliloti* 2011 first and then *Actinoplanes* ME3 or *Actinoplanes* ME3 first and then *S. meliloti* 2011 (RA/AR); *S. meliloti* 2011 first and then *Micromonospora* MM18 or *Micromonospora* MM18 first and then *S. meliloti* 2011 (RM/MR); *S. meliloti* 2011 first and then *Streptomyces* MM40 together with *Actinoplanes* ME3 or *Streptomyces* MM40 together with *Actinoplanes* ME3 first and then *S. meliloti* 2011 (RSA/ASR). In all cases plants were watered before and after inoculation with 1/10 Evans solution containing 0.07 mM N.

To test the effect of N concentration on alfalfa growth and/or nodulation in the presence of rhizoactinomycetes, the plants were fertilized with either low N concentration (0.07 mM) or high N concentration (7 mM) and inoculated simultaneously with rhizobial strain and rhizoactinomycete strains as described above.

Nodulation and Plant Growth Parameters: Statistical Analysis

For plants grown in pouches, nodulation was recorded weekly. After 7–10 weeks postinoculation, plants were harvested and the following plant growth parameters were measured: shoot length, shoot dry weight, root length, root dry weight, number of nodules per plant, and nodule dry weight. Nitrogen fixation activity of nodulated plants was estimated by acetylene reduction assay (ARA) as described elsewhere (Ferrari and Wall 2008). Data were analyzed with one-way analysis of variance (ANOVA) and two-way ANOVA. *Post-hoc* comparisons of means (Tukey test) were performed when statistically significant results at $p \leq 0.05$ were found. Treatments were run with ten replicates for glass tube experiments and with eight replicates for growth pouch systems in all experiments, except for the delayed co-inoculations where five replicates were used.

Results

Effect of Co-inoculations of Rhizoactinomycetes and *S. meliloti* on Plant Growth and Nodulation of *M. sativa*.

In the first experiment, the plants were cultivated in glass tubes and fertilized with 0.07 mM N, which allowed full nodulation by *S. meliloti* 2011. Plants inoculated with only rhizoactinomycetes strains showed a similar growth rate to that of noninoculated control plants. Thus, there were neither beneficial nor deleterious effects compared with controls. However, when plants were co-inoculated with each of the rhizoactinomycetes strains together with *S. meliloti* 2011, both nodulation and plant growth were significantly stimulated compared with single inoculation with *S. meliloti* (Table 1). This stimulation was observed in total plant dry weight and especially in the biomass of nodules (Table 1); co-inoculation with *Sinorhizobium* + *Actinoplanes* (RA) and *Sinorhizobium* + *Micromonospora* (RM) produced increments of about 42% in dry weight of nodules compared with plants inoculated with only *S. meliloti* (R) (Table 1).

To analyze the course of nodulation in the presence or absence of the co-inoculated rhizoactinomycetes, we

Table 1 Effect of rhizoactinomycetes on the growth and nodulation of alfalfa plants growing in glass tubes

Treatments	Number of nodules	Shoot DW (mg)	Root DW (mg)	Nodule DW (mg)
C	0.0	11.4 ± 0.8	23.6 ± 2.1	0.0
S	0.0	13.3 ± 1.2	34.5 ± 2.1	0.0
A	0.0	11.3 ± 0.9	20.2 ± 1.8	0.0
M	0.0	14.9 ± 1.2	30.5 ± 2.2	0.0
R	16.2 ± 1.9	57.6 ± 6.7	84.3 ± 8.6	1.8 ± 0.2
RS	17.1 ± 1.9	89.2 ± 5.1	187.2 ± 13.1	2.5 ± 0.2
RA	19.4 ± 2.0	107.9 ± 9.8	214.3 ± 12.1	3.0 ± 0.3
RM	18.4 ± 2.9	137.8 ± 16.9	190.2 ± 21.2	3.2 ± 0.2

Inoculum source: C, noninoculated control plants, S, *Streptomyces* MM40, A, *Actinoplanes* ME3, M, *Micromonospora* MM18, R, *Sinorhizobium meliloti* 2011, RS, *S. meliloti* 2011 + *Streptomyces* MM40, RA, *S. meliloti* 2011 + *Actinoplanes* ME3, RM, *S. meliloti* 2011 + *Micromonospora* MM18, DW, dry weight

Plants were watered with 1/10 Evans solution with 0.07 mM N as NH_4NO_3. Values are mean ± SE, $n = 10$

repeated the experiments growing the alfalfa plants in pouches. With this method it was possible to follow nodulation appearance during the experiment.

The kinetics of nodulation show a clear difference between treatments during the first week after inoculation. The nodulation rate was clearly higher for co-inoculated plants compared with single inoculations with *S. meliloti* (Fig. 1). After 1-2 weeks after inoculation, the nodulation rate was similar between treatments. Thus, the difference at the nodulation level at the end of the experiment seemed to be a consequence of the differences caused by the rhizoactinomycetes at the very early steps of infection and/or nodule development (Fig. 1). The co-inoculated plants with rhizoactinomycetes and with rhizobia treatments RS, RA, RM, and RSA showed higher shoot growth and a greater

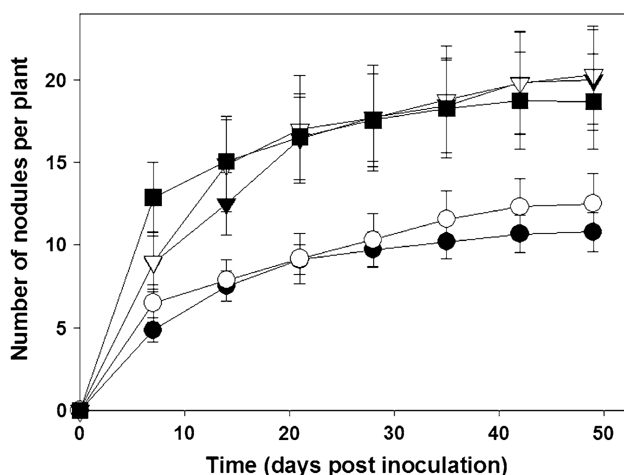

Fig. 1 Nodulation kinetics of alfalfa with different inoculum treatments: R, *Sinorhizobium meliloti* 2011(●); RS, *S. meliloti* 2011 + *Streptomyces* MM40 (○); RA, *S. meliloti* 2011 + *Actinoplanes* ME3 (▼); RM, *S. meliloti* 2011 + *Micromonospora* MM18 (▽); RSA, *S. meliloti* 2011 + *Streptomyces* MM40 + *Actinoplanes* ME3 (■). Plants were inoculated and grown as indicated in Materials and Methods. Values represent mean ± SE, $n = 7$

number of nodules per plant than plants inoculated with only *S. meliloti* (Fig. 2). Although the differences were not significant, probably because of low *n* of plants, the tendency was in the same direction as previously observed in the glass tube assay (Table 1) and on *Discaria trinervis* nodulation (Solans 2007). Plants co-inoculated with rhizobia and *Streptomyces* (RS) had a significantly higher nodule dry weight than plants with single inoculation with rhizobia ($p \leq 0.05$) (Fig. 2). The plants inoculated with only rhizoactinomycetes did not show that there was any effect on the growth of alfalfa plants and they grew similar to noninoculated control plants (Fig. 2).

At the end of the experiments, nitrogenase activity was estimated as acetylene reduction activity (ARA). Specific activity was similar between plants co-inoculated with rhizoactinomycetes or single inoculated with *S. meliloti* 2011. There was no statistical difference in ARA values between treatments, ranging from 0.0186 to 0.0531 µmol ethylene h^{-1} g^{-1} nodule.

Effects of a 14-Day Delay Between the Inoculations of Both Microorganisms

To investigate whether rhizoactinomycetes promotion has an effect before or after the interaction of *S. meliloti* with the plant, the plants were inoculated at different times with *S. meliloti* and the rhizoactinomycetes with a delay of 14 days between the first and the second inoculation; inoculation was either first with the *S. meliloti* and then the rhizoactinomycete or vice versa. It is worth noting that independent of the order of the inoculation, the addition of rhizoactinomycetes produced a positive effect on nodulation by *S. meliloti*. In both cases, the addition of actinomycetes sustained the initial nodulation rate for a longer time than the single inoculation with only *S. meliloti* (Fig. 3). The effect was clearer when the actinomycetes were inoculated 14 days before (Fig. 3b) than after *S. meliloti* (Fig. 3a).

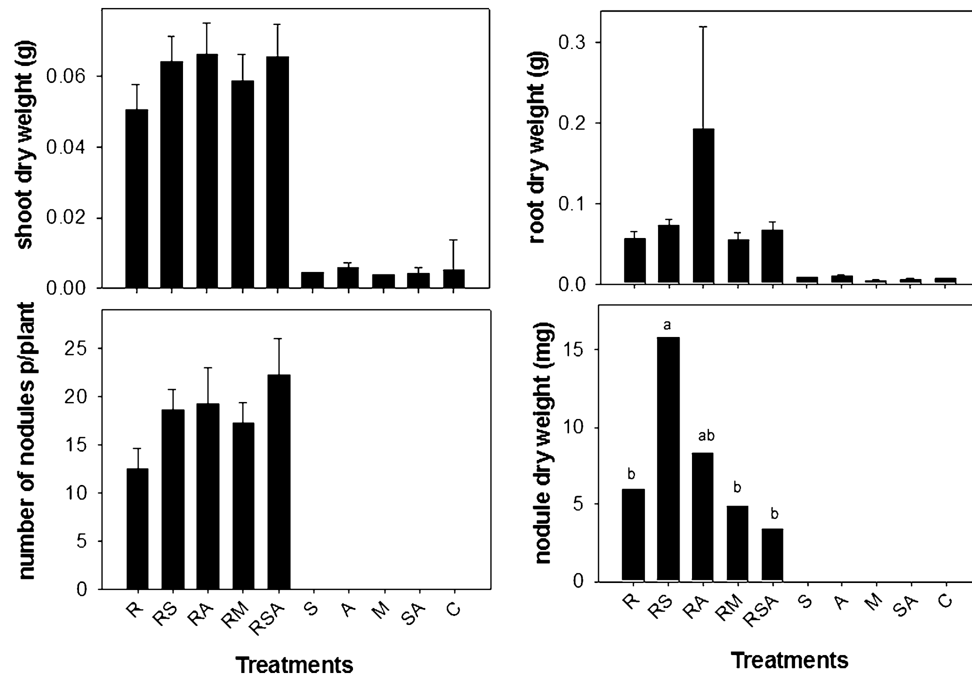

Fig. 2 Effect of rhizoactinomycetes on the growth and nodulation of alfalfa plants inoculated and cultivated in pouches. Inoculum source: R, *S. meliloti* 2011; RS, *S. meliloti* 2011 + *Streptomyces* MM40; RA, *S. meliloti* 2011 + *Actinoplanes* ME3; RM, *S. meliloti* 2011 + *Micromonospora* MM18; RSA, *S. meliloti* 2011 + *Streptomyces* MM40 +*Actinoplanes* ME3; S, *Streptomyces* MM40; A, *Actinoplanes* ME3; M, *Micromonospora* MM18; SA, *Streptomyces* MM40 + *Actinoplanes* ME3; C, noninoculated control plants. Plants were inoculated and grown as indicated in Materials and Methods. Nine weeks after inoculation plants were harvested, nodules were counted, and biomass was estimated as dry weight. Values represent mean ± SE, $n = 8$. Different letters denote significant differences at $p < 0.05$

At the end of the experiment, 70 days after the first inoculation, the number of nodules per plant remained constant in all treatments and was significantly higher in plants co-inoculated with *S. meliloti* and *Micromonospora* (RM) and with *S. meliloti* and *Streptomyces* together with *Actinoplanes* (RSA), with 15.4 and 17.2 nodules plant^{-1}, respectively, independent of the order of the double inoculation (Fig. 4). Single inoculated plants with *S. meliloti* had 8.8 nodules plant^{-1}. The plants inoculated with RM showed a significantly high nodulation (number and biomass) with 15 nodules and 7 mg compared to inoculation with RA (13 nodules and 4 mg) and R (9 nodules and 5 mg) ($p \leq 0.05$) (Fig. 4).

Co-Inoculation of Rhizoactinomycetes with *S. meliloti* in the Presence of High N Concentration

As expected, plants fertilized with 0.07 mM N and inoculated with *S. meliloti* normally developed root nodules, but the development of nodules was inhibited by a N concentration as high as 7 mM (Fig. 5, first two bars). However, the plants co-inoculated with *S. meliloti* and each of the rhizoactinomycetes strains developed root nodules at N concentration of 7 mM (Fig. 5, compare gray bars with gray control R), although the number of nodules (means/plant) was lower than that at low N concentration (Fig. 5, compare gray bar with paired black bar for each treatment). At low N, the single inoculated plants with *S. meliloti* developed 10.8 ± 1.2 nodules per plant (mean ± SE), whereas co-inoculated plants with rhizoactinomycetes developed between 12.5 ± 1.8 nodules (RS) and 20 ± 3.1 nodules (RA) (Fig. 5, black bars). However, the plants fertilized with high N concentration and inoculated with *S. meliloti* (R) did not form root nodules, whereas those plants co-inoculated presented between 8.4 ± 1.9 nodules (RS) and 15 ± 1.8 nodules (RM) (Fig. 5, gray bars).

The kinetics of nodulation at high N (Fig. 6) showed the same pattern as at low N (Fig. 1), with a very early effect of rhizoactinomycetes already operating during the first week. The average initial nodulation rate is clearly higher and sustained for the co-inoculation treatments compared to single inoculation by *S. meliloti*. Under low N concentration, the plants co-inoculated showed an initial rate of nodulation between 5.2 and 12.8 nodules plant^{-1} week^{-1} (Fig. 1), whereas at high N concentration the nodulation rate was between 5.8 and 10.5 nodules plant^{-1} week^{-1} (Fig. 6). After 1 week, the nodulation rate was similar between treatments probably because autoregulation was already operating on the plants in a similar way for all treatments.

There was no effect with respect to plant growth from single inoculation by each of the rhizoactinomycetes alone at any of the different N concentration growth conditions.

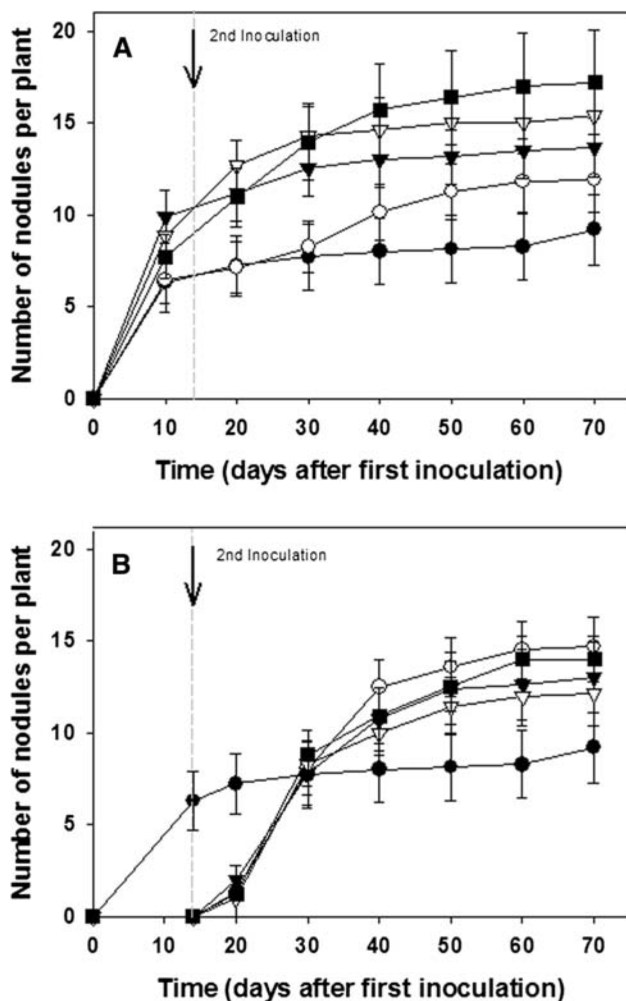

Fig. 3 Nodulation kinetics of alfalfa when both inoculants were added at different times. **a** *S. meliloti* 2011 was inoculated first, and 14 days later (*gray line* and *arrow*) the rhizoactinomycetes were added to the plant. **b** The rhizoactinomycetes were inoculated first, and 14 days later (*gray line* and *arrow*) *S. meliloti* 2011 was added to the plants. Plants inoculated with only *S. meliloti* 2011 at day zero were used as the same control to compare nodulation rate in the co-inoculated plants. Co-inoculation treatments: R, *Sinorhizobium meliloti* 2011(●); RS, *S. meliloti* 2011 + *Streptomyces* MM40 (○); RA, *S. meliloti* 2011 + *Actinoplanes* ME3 (▼); RM, *S. meliloti* 2011 + *Micromonospora* MM18 (▽); RSA, *S. meliloti* 2011 + *Streptomyces* MM40 +*Actinoplanes* ME3 (■). Values represents mean ± SE, $n = 8$

Of course, high N was better than low N for plant growth measured as dry weight biomass. Thus, there was no plant growth promotion effect by rhizoactinomycetes even if high N was available for the plant.

Discussion

The results presented here show that at least three different actinomycetes of different genera, *Streptomyces* MM40, *Micromonospora* MM18, and *Actinoplanes* ME3, can stimulate nodulation by *Sinorhizobium meliloti* 2011 on alfalfa when inoculated on plant roots either before, at the same time, or a few days after the inoculation of *S. meliloti* (Table 1, Figs. 1 and 3). Those actinomycetes also stimulate plant growth when co-inoculated with *S. meliloti*, but this plant growth effect seems to be a consequence of the stimulation of root nodulation because the actinomycetes alone were not able to stimulate plant growth (Figs. 2 and 4). The fact that the actinomycetes alone were not able to induce any plant growth change, even when high N was available in the nutrient solution, indicates that these microorganisms do not fit the definition of plant-growth-promoting rhizobacteria (PGPR) (Kloepper and others 1989), although they are still beneficial to plant growth in a more complex interaction involving the true plant symbiotic partner.

The beneficial interaction of actinomycete-*S. meliloti*-alfalfa studied here seems not to be specific for the plant, for the microsymbiont, or for the legume-rhizobia symbioses per se. The positive effect of stimulating nodulation and plant growth in alfalfa-*S. meliloti* symbiosis was completely equivalent to what had already been found and described for the actinorhizal symbiosis of *D. trinervis*-*Frankia* BCU110501 (Solans 2007). In that study, co-inoculation of the same actinomycetes together with *Frankia* BCU110501 on *Discaria trinervis* roots stimulated nodulation and plant growth of the plant (Solans 2007). Moreover, the actinomycetes *Streptomyces* MM40, *Micromonospora* MM18, and *Actinoplanes* ME3 have been isolated from the nodule surface and rhizosphere of native *Discaria trinervis* growing in northwest Patagonia, Argentina, where there is no alfalfa or *Medicago* plants (Solans and Vobis 2003).

The analysis of the kinetics of nodulation in the cases of co-inoculation (Figs. 1, 3 and 6) suggests that the effect of the actinomycete operates at the beginning of the infection and nodulation of the plant roots, sustaining the initial nodulation rate for a longer time than when only *Sinorhizobium meliloti* is inoculated. Because alfalfa is infected via root hair (Rhijn and Vanderleyden 1995) and *D. trinervis* is infected via intercellular invasion (Valverde and Wall 1999), the apparently common effect of actinomycete promoting nodulation in both symbioses should operate via a common mechanism in both symbioses. Considering that actinomycete promotion of nodulation is expressed in the presence of high N concentration (Figs. 5 and 6), otherwise counteracting the inhibition of nodulation by N, and considering that autoregulation of nodulation (Caetano-Anollés and Bauer 1988, 1991) and inhibition of nodulation by N (Streeter 1988) are somehow connected, as suggested by hypernodulating legume mutants (Krusell and others 2002), we think that the actinomycetes effect on promotion of nodulation operates at the autoregulation level of the plant nodulation mechanism.

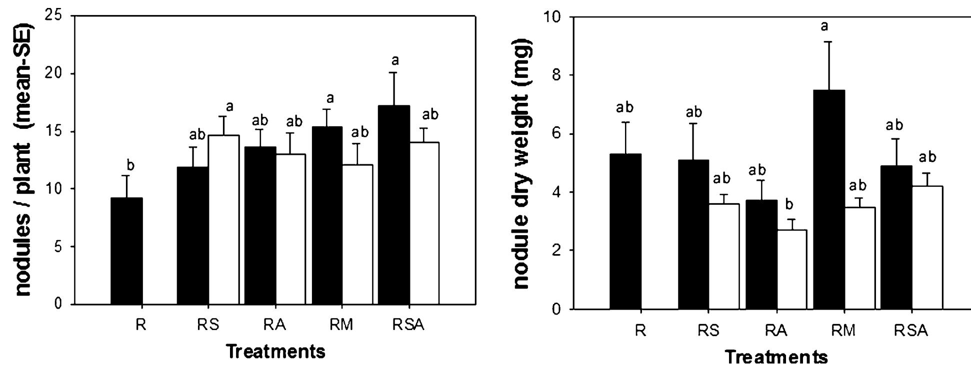

Fig. 4 Alfalfa nodulation level after delayed co-inoculation. The plants of the experiment referred to in Fig. 3 were harvested 7 weeks after inoculation and the final number of nodules per plant and nodule biomass were measured. *Black bars* denote the treatment when *S. meliloti* 2011 was inoculated first, 14 days before the actinomycetes were added. *White bars* denote the opposite treatment when the rizoactinomycetes were inoculated first and 14 days later *S. meliloti* 2011 was added. Treatments: R, *S. meliloti* 2011*;* RS, *S. meliloti* 2011 + *Streptomyces* MM40; RA, *S. meliloti* 2011 + *Actinoplanes* ME3; RM, *S. meliloti* 2011 + *Micromonospora* MM18; RSA, *S. meliloti* 2011 + *Streptomyces* MM40 + *Actinoplanes* ME3. Values are mean ± SE, $n = 8$. Different letters denote significant differences at $p < 0.05$

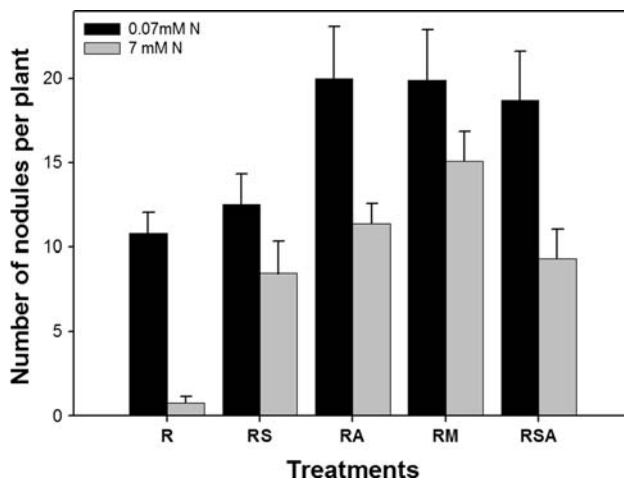

Fig. 5 Effect of rhizoactinomycetes co-inoculation on alfalfa nodulation under different nitrogen concentrations. Before and after the inoculation, plants were watered with nutrient solution containing either 0.07 mM N (*black bars*) or 7 mM N (*gray bars*) as ammonium nitrate. Seven weeks after the first inoculation, plants were harvested and nodules were counted. Treatments: R, *S. meliloti* 2011*;* RS, *S. meliloti* 2011 + *Streptomyces* MM40; RA, *S. meliloti* 2011 + *Actinoplanes* ME3; RM, *S. meliloti* 2011 + *Micromonospora* MM18; RSA, *S. meliloti* 2011 + *Streptomyces* MM40 + *Actinoplanes* ME3. Values are mean ± SE, $n = 8$

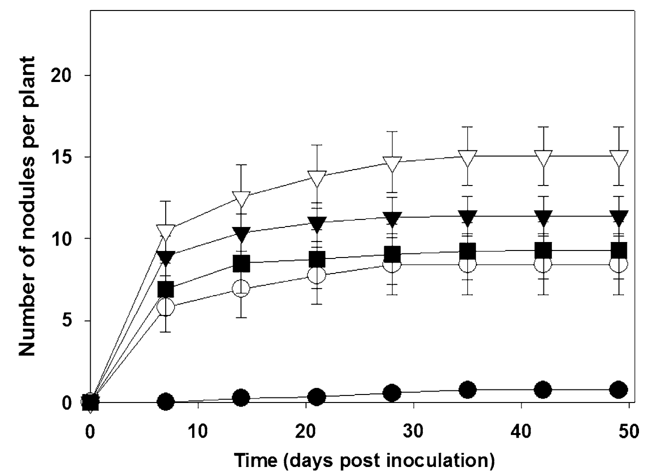

Fig. 6 Nodulation kinetics of alfalfa growing with 7 mM N as NH_4NO_3 and co-inoculated with rhizoactinomycetes. Inoculum treatments: R, *S. meliloti* 2011(●); RS, *S. meliloti* 2011 + *Streptomyces* MM40 (○); RA, *S. meliloti* 2011 + *Actinoplanes* ME3 (▼); RM, *S. meliloti* 2011 + *Micromonospora* MM18 (▽); RSA, *S. meliloti* 2011 + *Streptomyces* MM40 + *Actinoplanes* ME3 (■). Plants were inoculated and grown as indicated in Materials and Methods. Values represent means ± SE, $n = 7$

It has been shown that bacterial phytohormones are involved in the interaction between plant and free-living PGPR (Liste 1993; Höflich and others 1994; Glick 1995), because IAA is one of the most important phytohormones in terms of triggering the biochemical signal that leads to plant growth improvement (Glick and others 1999; Ribaudo and others 2006). Cytokinins and auxins may act synergistically to initiate cell division and nodule primordial formation, mediating plant responses to rhizobia, integrating the pathway signaling mediated by nod factors (Mulder and others 2005). Even more, a pathway for nodulation that is independent of nod factors has recently been described and the signals involved seem to be phytohormone related (Giraud and others 2007). Different studies have shown that ethylene mediates plant autoregulation of nodulation in legumes such as alfalfa (Peters and Crist-Estes 1989) and in actinorhizal plants such as *Discaria trinervis* (Valverde and Wall 2005).

The three rhizoactinomycetes used in this study, *Streptomyces* MM40, *Actinoplanes* ME3, and *Micromonospora* MM18, produce and release IAA, GA_3, and zeatine in defined media. It was possible to identify and quantify

these phytohormones in bacterial culture supernatant using gas chromatography-mass spectrometry with selective ion monitoring (GC-MS-SIM) for IAA and GA_3 and using HPLC-UV for zeatine (M. Solans and others, unpublished). Nevertheless, we cannot rule out yet if hormone production and/or another kind of compound is related to the stimulation of nodulation described here.

In conclusion, the positive interaction between rhizoactinomycetes and the symbiosis by *M. sativa* and *S. meliloti*, stimulating nodulation even at high available N for the plant, open new lines of research to investigate the phenomena related to the autoregulation of nodulation. The plant growth promotion caused by the co-inoculation of rhizoactinomycetes also reveals new lines of research on a topic of potential agronomical application.

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