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Symbiosis

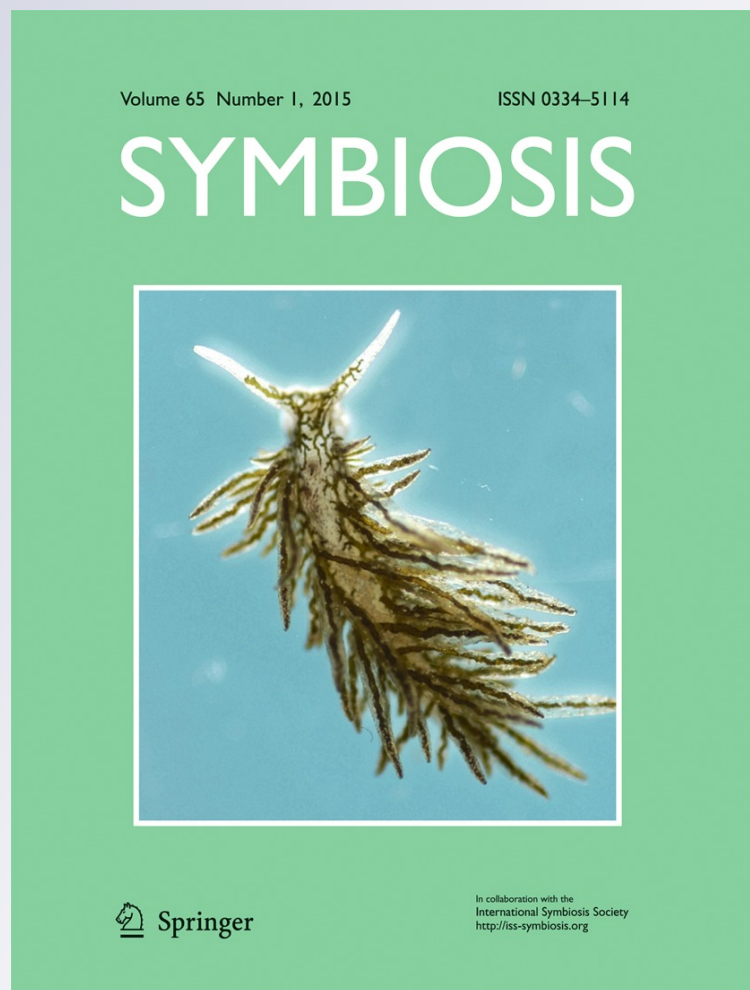
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Effect of actinobacteria on *Lotus tenuis* – *Mesorhizobium loti* symbiosis: preliminary study

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Abstract The effect of the actinobacteria on the legume *Lotus tenuis*, a model forage plant of the Flooding Pampa region in Argentina was to promote plant growth and root nodulation. *L. tenuis* seedlings were inoculated either with the symbiotic N₂-fixing strain *Mesorhizobium loti*, or with saprophytic strain of *Streptomyces* MM40, *Actinoplanes* ME3, or *Micromonospora* MM18, or co-inoculated with a combination of one, two or three of them, together with the rhizobium. The plants were grown in pouches and fertilized with three nitrogen levels (low, medium and high). *L. tenuis* plants co-inoculated with the symbiotic *M. loti* and actinobacteria showed an increase in plant biomass and nodulation. Plants fertilized with high N levels (inhibitory for nodulation by *M. loti*) were nodulated if the *M. loti* strain was added simultaneously with actinobacteria. These preliminary results show that actinobacteria have potential agronomic application which should be further explored.

Keywords Actinobacteria · *Lotus tenuis* · Plant growth · Nodulation · N- fertilization

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1 Introduction

Legumes have the ability to establish mutualistic symbiotic relationships with soil bacteria, collectively known as rhizobia, allowing the legume to be independent of nitrogen supply in the soil. In these associations, a unique structure, the nodule, develops on the root of the plant after the diazotroph and its host have recognized each other. The type of nodule depends on the host plant (Hirsch 1992). Among the legumes, Alfalfa (*Medicago sativa*) is important as a forage species in Argentina as well as worldwide. This because of the quality and palatability of forage, and the ability to grow in a wide range of climatic and soil conditions. The forage genus *Lotus* is used for experimental studies but also as an alternative to the traditionally cultivated fodder plants in Argentina. Local studies on *Lotus* sp. especially in the Salado River basin in the province of Buenos Aires have involved mainly *Lotus tenuis* Waldst et Kit (Estrella et al. 2009; Escaray et al. 2012). This species is widely cultivated by livestock producers in the Flooding-sensitive Pampa region (Cahuepé 2004; Coria et al. 2005) due to its nutritional value, high productivity, natural resistance and adaptation to soils in this area, which are characterized by waterlogging and alkalinity (Vignolio and Fernández 2006; Paz et al. 2012). Part of the success of *L. tenuis* in this region is related to its ability to form symbiotic associations with nitrogen-fixing bacteria, *Mesorhizobium loti* (Sannazaro et al. 2011) and associations with plant growth-promoting rhizobacteria (PGPR) like *Azospirillum* sp. Both microorganisms stimulate plant growth but through different physiological mechanisms. Biological nitrogen fixation is the most important but the production or metabolism of phytohormones (mainly auxins, cytokinins and gibberellins; Cassán et al. 2003) plays a smaller role. However, there is little information available on saprotrophic actinobacteria, or their interaction with *L. tenuis* plants.

Within the soil microbiota, actinobacteria are commonly known to colonize the rhizoplane and rhizosphere and to be involved in diverse physiological processes. They are also considered as important producers of bioactive secondary metabolites and extracellular enzymes (Bérdy 2005). Previous studies on the actinorhizal symbiosis between *Ochetophila trinervis* (Kellermann et al. 2005), also known as *Discaria trinervis* (Hook et Arn.) Reiche, and *Frankia* revealed promotion and nodulation in co-inoculation assays with saprophytic actinobacteria strains (Solans 2007). In addition, alfalfa seedlings inoculated with actinobacteria also showed a significant increase in plant and nodule biomass (Solans et al. 2009).

The aim of the present study was to see whether promotion of plant growth and nodulation could be induced in the model legume, *L. tenuis* following inoculation with a combination of actinobacteria and nitrogen fixing *Mesorhizobium*.

2 Materials and methods

2.1 Plant material and culture conditions

Seeds of *L. tenuis* cv. Esmeralda provided by INTECH were scarified and surface sterilized with concentrated sulfuric acid for 2 min. Seeds were thoroughly washed with sterile water and distributed on the surface of 1 % water agar plates for 7 days at 25 °C. Germination and plant growth took place in a growth chamber with a photoperiod of 16 h (photosynthetic photon flux density 318 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 26 °C temperature and 35 % relative humidity. Seedlings were aseptically transferred to pouches (Mega International Minneapolis, MN, USA) and fertilized with modified Evans 1/10 full strength solution (Huss-Danell 1978). Fifteen days old seedlings were inoculated and fertilized with modified Evans nutrient solution with different N levels using a previously published protocol (Solans et al. 2009).

2.2 Bacterial strains: cultivation and inoculum preparation

We used the rhizobium strain *Mesorhizobium loti* NZP2213 provided by INTECH. It was grown in liquid TY medium (tryptone 5 g L^{-1} ; yeast extract 3 g L^{-1} ; CaCl_2 0.5 g L^{-1}) in a shaker at 28.5 °C for 72 h. The inoculum concentration were standardized spectrophotometrically (OD_{600} values) to ensure reproducibility of the test conditions and 1 ml of non-washed bacterial suspension per plant was used for inoculation.

Three actinobacterial strains, *Streptomyces* sp. MM40, *Actinoplanes* sp. ME3 and *Micromonospora* sp. MM18, previously isolated (Solans and Vobis 2003) and characterized by their potential capability as promoters of plant nodulation (Solans and Vobis 2013) were cultured as described previously (Solans et al. 2009). 16S rRNA Gene sequencing data of the strains are available in GenBank under the following

accession numbers: FJ771041, FJ771040 and FJ771042, respectively.

2.3 Experimental design

To study the effect of actinobacterial strains on growth and nodulation of *L. tenuis*, the plants were inoculated with each strain alone or co-inoculated with *M. loti* NZP 2213 and cultivated in a growth-pouch system. After inoculation, the plants were watered with modified Evans 1/10 full strength solution with 0.07 mM N as NH_4NO_3 .

In addition, we analyzed the effect of the N concentration on *L. tenuis* growth and nodulation in the presence of actinobacteria. The plants were fertilized at three levels of N (ammonium nitrate): low N (0.07 mM), medium N (0.7 mM) or high N level (7 mM) in the growth-pouch system, and inoculated simultaneously with rhizobial and actinobacterial strains as described by Solans et al. (2009).

Various treatments were performed: a) single inoculation with the rhizobial strain *M. loti* (R); b) single inoculation with rhizospheric actinobacterial strains: *Streptomyces* sp. (S), *Actinoplanes* sp. (A) and *Micromonospora* sp. (M); c) co-inoculations with rhizobia and actinobacteria in double and triple combinations: rhizobia+*Streptomyces* (RS), rhizobia+*Actinoplanes* (RA), rhizobia+*Micromonospora* (RM), rhizobia+*Streptomyces*+*Actinoplanes*+*Micromonospora* (RSAM), rhizobia+*Streptomyces*+*Actinoplanes* (RSA); d) uninoculated control plants. At the end of the experiment, the following parameters were determined eight weeks post-inoculation as previously described by Solans (2008): shoot and root length, number of root nodules, dry weight of shoot, roots and nodules and nodulation kinetics. Treatments were run with eight replicates.

2.4 Data analysis

Data were analyzed using analysis of variance: one-way ANOVA, repeated measures ANOVA and two-way ANOVA with two fixed factors: type of treatments and type of N fertilization. Post-hoc comparison of means (Tukey's HSD test) were performed when statistically significant results were found. All results were considered statistically significant when $p < 0.05$.

3 Results

The root nodulation kinetics of *Lotus tenuis* seedlings co-inoculated with actinobacteria and fertilized with 7 mM N could be observed at the beginning of the second week post-inoculation. Co-inoculated plants had a higher number of nodules as compared with plants inoculated only with *Mesorhizobium loti* (R). During the nodulation experiments,

actinobacteria modified the rate of nodulation, as can be seen by the change in the slope of the curve at different stages of the experiment. The nodulation rate was higher for plants co-inoculated with actinobacteria than for plants inoculated only with *M. loti* (data not shown).

Besides this change on nodulation kinetics in *L. tenuis* plants caused by actinobacteria, at the end of the experiment a positive effect was found in the growth parameters of these plants, especially in the number of nodules ($F=4.12$; $p=0.000001$) and dry mass ($F=5.41$; $p=0.00078$) (Fig. 1). The greatest differences between plants inoculated with R and RSAM were observed in the dry weight of shoots and roots, presenting almost three times more biomass for RSAM than for R plants ($p=0.0008$) (Fig. 1a and b). Increased nodulation was observed in plants co-inoculated with rhizobium+*Actinoplanes* (RA) ($p=0.0031$), followed by triple co-inoculation RSAM ($p=0.0037$) and double inoculation RSA

($p=0.014$), with 31, 27 and 25 average numbers of nodules per plant (Fig. 1c). Also, the nodule dry mass was higher in co-inoculated plants than in R plants ($p<0.05$) (Fig. 1d). Plants inoculated only with actinobacteria showed growth similar to that of uninoculated control plants ($p>0.05$) (data not shown).

The N fertilization applied resulted in changes to almost all the variables measured: shoot length ($F=6.97$; $p=0.0012$); number of nodules ($F=31.08$; $p=0.00001$); shoot biomass ($F=11.11$; $p=0.000029$); root biomass ($F=4.47$; $p=0.0127$) and nodule biomass ($F=27.14$; $p=0.00002$). Plants fertilized with a high N levels had reduced values in all growth parameters, as well as in the number of nodules. This applied particularly to plants inoculated with rhizobia only. All plants inoculated with rhizobia and co-inoculated with actinobacteria were nodulated independent of the N level in the nutrient solution. Plants co-inoculated plants with actinobacteria

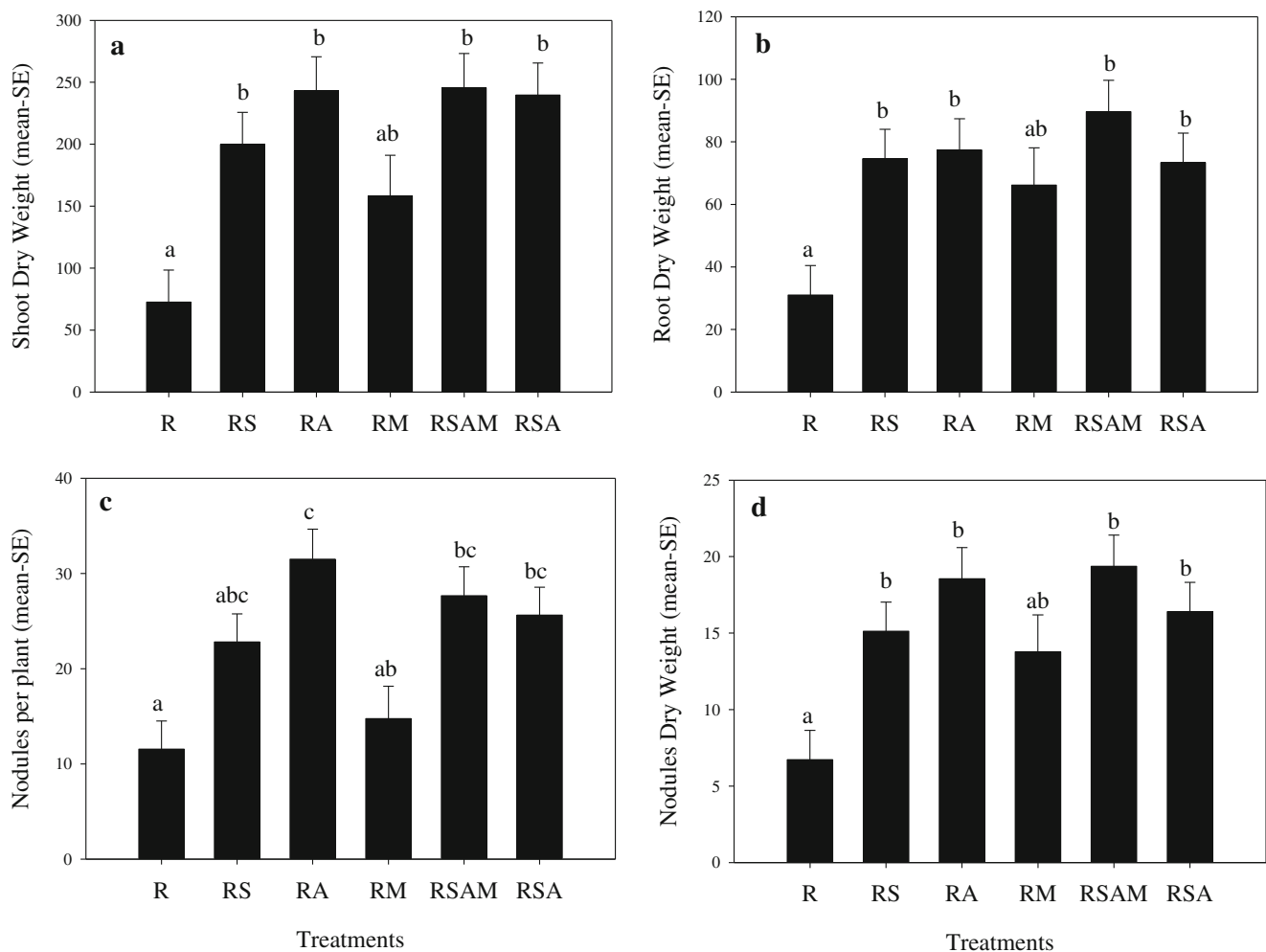


Fig. 1 Number of nodules and dry mass of *Lotus tenuis* plants inoculated with *M. loti* NZP2213 and actinobacteria, grown in pouches system. R: rhizobia (*Mesorhizobium loti*); RS: rhizobia+*Streptomyces*; RA: rhizobia+*Actinoplanes*; RM: rhizobia+*Micromonospora*; RSAM: rhizobia+*Streptomyces*+*Actinoplanes*+*Micromonospora*; RSA:

rhizobia+*Streptomyces*+*Actinoplanes*. **a**, Shoot dry weight (mg); **b**, Root dry weight (mg); **c**, Number of nodules per plant; **d**, Nodule dry weight (mg). Values are means (and SE), $n=8$. Different letters indicate significant differences at $p<0.05$ according to the HSD test

showed a limited inhibition of nodulation by N (data not shown).

The inoculation treatments affected all aspects of growth analyzed: shoot length ($F=49.5$; $p=0.0001$); root length ($F=97.4$; $p=0.0001$); number of nodules ($F=5.73$; $p=0.00006$); shoot biomass ($F=19.46$; $p=0.000$); root biomass ($F=6.83$; $p=0.000$) and nodule biomass ($F=7.74$; $p=0.000$). The inoculation with RSA and RA led to the highest values for almost all the growth parameters analyzed and the number of nodules increased, although not significantly when compared with R-inoculated plants ($p=0.149$ and $p=0.163$) (data not shown).

4 Discussion

The enhancement in determinate nodulation of *Lotus tenuis* by *Mesorhizobium loti* following co-inoculation with any of the three strains of actinobacterial genera, *Streptomyces*, *Actinoplanes* and *Micromonospora*, confirms the existence of a general non-specific tripartite interaction between nitrogen-fixing root nodulating plants, their diazotroph microsymbionts and these actinobacteria. The actinobacterial strains were isolated from native actinorhizal nodules and rhizosphere of *O. trinervis* growing in Patagonia soils. They were found to enhance nodulation, and consequently plant growth, in both legumes and actinorhizal plants growing in very different environments (Solans 2007; Solans et al. 2009; this study). The stimulatory effect of actinobacteria on nodulation appears to be of a complex nature since it shows synergism when more than one actinobacterial strain is involved in the interaction. The strongest effects were observed in RSA and RSAM treatments; these effects were similar to those observed in our previous studies with an actinorhizal plant *O. trinervis* and with indeterminate-nodule forming Alfalfa plants (Solans 2007; Solans et al. 2009).

The plant growth promoting effect of the actinobacteria we describe here does not fit into any definition of PGPR bacteria since there is no effect at all after single inoculation of the plant roots with actinobacteria, which excludes a direct plant growth promoting effect by the actinobacteria per se. The positive effect seems to be expressed by affecting other symbiotic plant-microbes as was found in previous studies using *O. trinervis* and Alfalfa (Solans and Vobis 2013).

The analysis of the kinetics of nodulation of co-inoculated plants suggests that the effect of the actinobacteria operates at the beginning of the infection and nodulation process in plant roots, sustaining the initial nodulation rate for a longer time than when only *Mesorhizobium loti* is applied. A positive effect regarding stimulation of nodulation and plant growth was found ($p \leq 0.05$) at low levels of N which do not inhibit the nodulation by rhizobia.

The positive effect of actinobacteria on nodulation in the presence of high levels of N (Solans et al. 2009) suggests that

the actinobacteria could be modulating regulation of nodulation in the plant, most probably by interfering with plant hormone levels. Although there is no evidence that actinobacteria secrete hormones when in interaction with plants, we found that these strains produce hormones in culture (Solans et al. 2011). We know that the positive effect on growth and nodulation occurs when the plant is co-inoculated with the supernatant (mycelium+supernatant or only supernatant) of actinobacteria (Solans 2007, 2008). It is also remarkably that in some replicated experiments we found that plants that showed enhanced nodulation in response to co-inoculation with actinobacteria, also exhibited early flowering compared with plants given other treatments. No flowering was observed all along the experiment in the plants that were not treated with actinobacteria. This suggests a more general plant growth promoting effect probably mediated by phytohormones.

Regarding the compatibility between the rhizobial strain and the actinobacteria, we found a positive effect on rhizobial growth when growing rhizobia and actinobacteria together in the same culture medium, suggesting that part of the stimulation of nodulation by actinobacteria is due to an increase in the population of the rhizobia in the rhizosphere. This hypothesis is supported by the observation of OD600 values increased in the order of 50 % at 24, 48 and 96 h after inoculation, when rhizobia and actinobacteria were cultured together compared to similar cultures of single strains.

Since we still do not know the nature or components of this stimulatory activity, more studies are necessary to understand fully what occurs in this tripartite interaction. We also need to find out which actinobacterial activities are involved in the stimulatory effect on nodulation and whether the actinobacteria are located in the rhizoplane, root cortex or nodule. We observed hyphae and sporangia of the added actinobacteria on the surface of roots and root hairs under a light microscope and are presently working on fluorescent labeling of these actinobacteria to continue the localization studies. Preliminary, the results suggest that actinobacteria have potential agronomic applications that could be further exploited, especially in the Flooding-sensitive Pampa region where *L. tenuis* is an important forage species for cattle.

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