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Interactions between *Frankia* BCU110501 (actinorhiza) and *Gigaspora rosea* (arbuscular mycorrhiza) with *Discaria trinervis* studied by spot inoculation

Mariana Obertello[1,2] · **Luis G. Wall**[1]

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Abstract Root endosymbioses are associations between plants and soil microorganisms which contribute worldwide to plant nutrition and fitness. The most common associations is with arbuscular mycorrhizal fungi (AMF) and this is formed by the majority of land plants with fungi belonging to the Glomeromycota. Another important association is that formed by the Gram-positive actinobacteria, *Frankia*, and actinorhizal plants such as *Discaria trinervis*. In this paper we studied the effect of dual symbioses resulting from spot inoculation. This method was developed and tested to ensure actinorhizal nodule development only at the site of inoculation. Our results show that both symbioses can coexist and that AMF and *Frankia* can stimulate plant growth both as a single inoculum and in combination. Moreover, we suggest that there is no interference between the regulation of actinorhizal and arbuscular mycorrhizal symbiosis in *D. trinervis*, and that there is a synergistic effect on plant growth following the development of both symbioses.

Keywords Actinorhizal symbiosis · AMF · Mycorrhizal symbiosis · Root nodulation

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✉ Luis G. Wall
lgwall@hotmail.com

[1] Departamento de Ciencia y Tecnología, Laboratorio de Bioquímica, Microbiología e Interacciones Biológicas en Suelo, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, Bernal B1876BXD, Buenos Aires, Argentina

[2] Present address: Instituto de Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Vuelta de Obligado 2490 Piso 2, Ciudad Autónoma de Buenos Aires C1428ADN, Argentina

1 Introduction

Actinorhizal plants are perennial dicots that develop root nodules in a nitrogen fixing symbiosis with the actinobacterium *Frankia*. All actinorhizal plants are woody shrubs or trees, except for *Datisca glomerata* which is herbaceous. The host species are distributed among 25 genera from 8 different families, and in nature all actinorhizal plants examined have been found to have endo- or ectomycorrhiza (Huss-Danell 1997; Barker and Tagu 2000; Wall 2000). *Discaria trinervis is one of twelve* species of the genus that are native to temperate regions of the Southern Hemisphere, and belong to the family Rhamnaceae. *Discaria trinervis* is an important forage species in some parts of its range (Medan and Devoto 2005). The intercellular infection of *Discaria* sp. roots by *Frankia* and the regulation of root nodulation have been well characterized (Valverde and Wall 1999a, 2002, 2003; Svistoonoff et al. 2014). The ability of these plants develop both symbioses could explain why actinorhizal plants are pioneers on nitrogen-poor open sites and have the capacity to grow well under a range of environmental stresses (after flooding, fires, landslides, glacial activity and volcanic eruptions) (Dawson 1990). These plants accelerate soil development by adding nitrogen and organic matter (Wheeler et al. 1986).

The actinobacterium *Frankia* colonizes roots of actinorhizal plants in two different ways depending on the host: intracellularly by root hair infection or intercellularly by colonization of the intercellular root cortex spaces (Wall and Berry 2008; Svistoonoff et al. 2014).

Arbuscular mycorrhizal fungi (AMF) are all members of the Zygomycota order Glomales. These fungi have the ability to form a mutualistic symbiosis with most terrestrial plant species, producing nutrient exchange structures called arbuscules within root cortical cells of their host plants. These fungi seem to be obligatory biotrophic in nature, and

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it has not been possible to grow them *in vitro* in the absence of a plant host (Bonfante-Fasolo 1984). Little is known about early interactions in mycorrhizal symbiosis and its regulation by the host plant (Smith and Read 2008; Gutjahr 2014) except in relation to feedback inhibition by phosphate in the soil on hyphopodium formation, and to the effects of plant-produced strigolactones on pre-symbiotic growth of AMF.

Both associations can be analyzed as functionally different, complementary mutualistic interactions of microorganisms with their host. The plant supplies the fungus and the bacteria with carbon sources from its fixed photosynthates. In the case of the mycorrhizal symbiosis, the fungi assist the plant in its uptake of phosphate and other mineral nutrients from the soil (Smith and Read 2008); in the case of the actinorhizal symbiosis, the bacteria fix atmospheric nitrogen in nodules which are further assimilated by the plant (Huss-Danell 1997). Knowledge about plant-bacteria or plant-fungi symbioses arose from studies with single associations, but fewer studies have been completed where more than one symbiosis is involved concurrently with the same host (Visser et al. 1991; Tian et al. 2002; Orfanoudakis et al. 2010). Recently, it was shown that the receptor-like kinase gene *SymRK*, which is required for nodulation in legumes, is also necessary both for actinorhizal root nodule formation and for mycorrhization in the tree *Casuarina glauca* (Gherbi et al. 2008).

The regulation of actinorhizal symbiosis has been studied and susceptibility to infection changes with root region. Different levels of nodulation have been observed between young growing root tips and mature zones of the root (Valverde and Wall 1999a). A high concentration of internal and external nitrogen inhibits the development of actinorhizal nodulation by *Frankia* (Wall 2000). In contrast, high external phosphorus levels stimulate root nodulation – even counteracting the inhibitory effect of N (Valverde and Wall 2002). In the mycorrhizal symbiosis an opposite regulation seems to occur; high internal nitrogen benefits the AMF-plant association whereas a high phosphorus concentration inhibits the root colonization by AMF (Smith et al. 2011). These observations as the sharing of some common mechanisms regarding the development of each symbiosis (as in the case of SymRK) prompted us to study what happens to the regulation of both symbioses when they are colonizing the same plant. We investigated how AMF-*D. trinervis* and *Frankia*-*D. trinervis* interactions affect colonization by either or both micro-symbionts, and the consequences for plant growth.

2 Materials and methods

2.1 Collection of seeds and plants growth conditions

Mature fruits of *Discaria trinervis* were collected in Pampa de Huenuleo (41°10′S, 71°21′O, Bariloche, Río Negro, Argentina) and stored at −20 °C in dark until required. Seeds were surface-sterilized with sulfuric acid as previously described (Valverde and Wall 1999a).

2.2 Growth condition and inoculation in pouches

D. trinervis germination was performed on wet perlite in Petri dishes. Three seedlings, at the cotyledonary stage (12–14 days after the start of germination), were aseptically transferred to growth pouches (Mega International, Minneapolis, USA) moistened with $^{1}/_{10}$ Evans medium (E) (Huss-Danell 1978) +10 ppm N as NH_4NO_3. This method offers a number of advantages: it allows a visual check visually for nodule development at any time, and the pouches enable the inoculation of roots inoculation at specific positions. The pouches were kept in a greenhouse at the University of Quilmes throughout the experiment with supplemental artificial light (400 W, K048; Osram, Osasco, Brazil), a photoperiod of 16-h light and 8-h dark, a temperature range between 20 and 26 °C and relative humidity between 65 and 95 %. At the time of inoculation, 3 weeks after the transfer to pouches, the position of the root tip was marked (RT_1) on the plastic bag. Each seedling was inoculated by placing agar squares with either *G. rosea* spores or *Frankia* homogenate, or both, on top of the selected root region as the experimental design indicated (see below). Water agar-squares without microorganisms were used as the control treatment. The pouches were watered twice a week with $^{1}/_{10}$ E +10 ppm N. Between 20 and 30 plants were used per treatment.

2.3 Microorganisms

Frankia strain BCU110501 was isolated from *D. trinervis* nodules of field-grown plants by Chaia et al. 1998. *Frankia* cultures were grown in static BAP minimal medium supplemented with 0.055 M glucose as carbon source (Chaia et al. 1998) and incubated at 28 °C±2 °C in the dark for 3 weeks before use. Cells were centrifuged at 11,000*g* for 15 min. The pellet was washed with $^{1}/_{10}$ E with 10 ppm N as NH_4NO_3 and centrifuged again at 500*g* for 5 min. The supernatant was discarded and packed cells were resuspended in a small volume (2–4 ml) of $^{1}/_{10}$ E. The cell suspension was homogenized by three passages through sterile 0.5 mm needles (21G). The *Frankia* biomass in the homogenate was estimated by the pellet volume after centrifugation at 500*g* for 5 min in Wintrobe tubes to determine the packed cell volume (pcv) (Nittayajarn and Baker 1989). The cell suspension was recovered from the tube with a Pasteur pipette. An inoculum was prepared with equal parts of homogenate with 3 μl/ml of *Frankia* cells and a solution of water-agar (1.5 % Difco Bacto agar) at pH 7. Squares of this agarized inoculum (0.7 % agar and 1.5 μl/ml of *Frankia*) 5×5 mm in size and 2 mm in height were placed at different positions of the root tip (RT) or the

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mature root (MR), and at different times as indicated in Fig. 1a.

For inoculation with the endomycorrhizal fungus, culture traps of *Gigaspora rosea* (BEG 9)-*Discaria trinervis* were set up in pots with a sterile soil-perlite (1:3) mix and supplied with $^{1}/_{10}$ E with 10 ppm N as NH_4NO_3. Spores were collected and stored at 4 °C in dark until required. Spores were surface sterilized with the sterilant solution described by Mosse (1962); briefly: 1 g chloramine T, 10 mg streptomycin plus 2 drops of Tween 80 in 50 ml of sterile distilled water. The sterilant was applied for 20 min. Subsequently, spores were washed several times in sterile deionized water. Four sterile spores were placed onto the surface of squares of water-agar (0.7 % Difco Bacto agar, pH 7) 5×5 mm in size and 2 mm in height and provided the AMF inoculum for placing at on the root tip (RT) or the mature root (MR) as indicated elsewhere (see Fig. 1a).

2.4 Experimental design

Inoculation spots were chosen relative to the position of the root tip, based on the area susceptibility of each part of the root for *Frankia* infection (Valverde and Wall 1999b). Either *Frankia* (F) or *G. rosea* (M) containing agar-squares were gently deposited on top of the root, on a mature (non-susceptible) region of the main root (MR), covering the most susceptible region of the root tip at the moment of the inoculation (RT_1), or at the position of the new root tip at the time of a second inoculation (RT_2). A second inoculation (RT_2) was performed 20 days after the first inoculation (see below and Fig. 1 for details). Control treatment plants were inoculated with an agar square without any microorganism at the same selected root regions, as a control (C) of the agar effect *per se*.

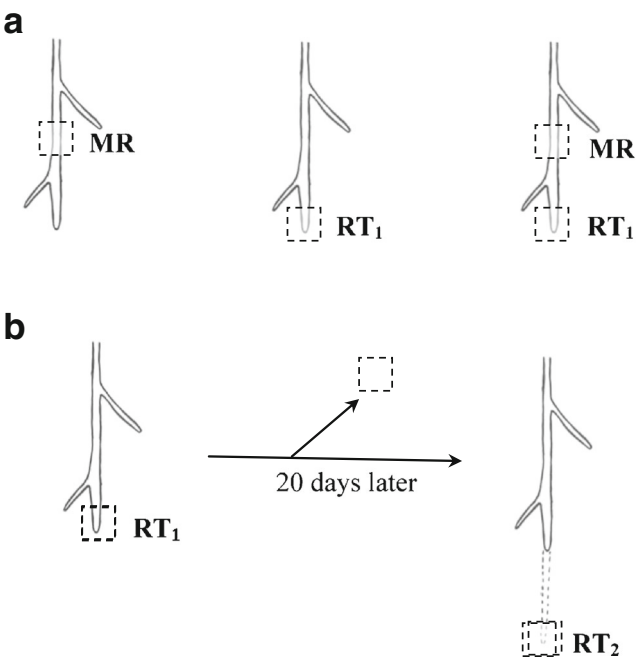

Fig. 1 Experimental design of localized inoculations. **a** Simultaneous inoculations made at a different part of *D. trinervis* main root. Mature zone of the root is indicated as MR, and the root tip position at the moment of the inoculation is indicated as RT_1. **b** Sequential inoculations: 20 days after the first inoculation at root tip (RT_1), the first agar-square is removed and a second agar-square were placed on the position of the root tip of the growing main root (RT_2). Values are the means±SE for 20–30 plants. Final shoot height was measure at the end of the experiment, 47 days after the first inoculation. Statistically significant differences compare to control treatments at §$P \leq 0.001$, ‡$P \leq 0.01$ and *$P \leq 0.05$

In order to study the interactions between these two symbioses, eight treatments of combined inoculations (simultaneous or sequential) were performed considering all possible combinations of root regions (MR, RT_1 or RT_2) and inocula (F or M). The inoculations were performed at the same time for positions MR and RT_1, and with a delay of 20 days for position RT_2 (for more details see Fig. 1). For example, to investigate whether the presence of one symbiosis modified the pattern of colonization by the other symbiont, sequential inoculations were performed. This means that the inoculation with the second microorganism was only performed once the other symbiosis had been established, with a delay of 20 days after the first inoculation.

Altogether, 22 different treatments were performed to study the interaction between the two symbionts.

Controls: C [MR]; C [RT_1]; C [RT_2]; C [MR , RT_1]; and C [RT_1 + RT_2].
AMF inoculations: M [MR]; M [RT_1]; M [RT_2]; M [MR , RT_1]; and M [RT_1 + RT_2].
Frankia inoculations: F [MR]; F [RT_1]; F [RT_2]; F [MR , RT_1]; and F [RT_1 + RT_2].
Simultaneous inoculations with both microorganisms: M/F [MR]; M/F [RT_1]; M[MR] − F[RT_1]; F[MR] − M[RT_1].
Sequential inoculations with both microorganisms: M[RT_1] F[RT_2]; F[RT_1] M[RT_2]

2.5 Symbiosis scoring

The efficiency of mycorrhizal and *Frankia* colonization was assessed in terms of plant growth, and compared to non-inoculated plants. Shoot length and number of leaves were recorded weekly. At harvest, plant biomass was estimated as dry matter (measured after drying at 62 °C for 7 days). The *Frankia-D. trinervis* symbiosis was followed weekly, recording the number of nodules developed on the root system. The AMF-*D. trinervis* interaction was analyzed by staining: Inoculated and non-inoculated roots were harvested at 47 days post-inoculation and washed with sterile water. Mycorrhization was analyzed under the stereomicroscope after root staining with Trypan Blue 0.05 % (Phillips and Hayman 1970). The percentage of total root length colonized

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was estimated by examining stained samples using the grid-line intersect method (Giovannetti and Mosse 1980).

2.6 Statistical analysis

A one-way analysis of variance (ANOVA) was performed, followed by a Tukey *post-hoc* test to identify treatments whose means where different in response to the inocula (GraphPad Prism software).

3 Results

3.1 Single inoculations

To study how the developmental stage of the root is involved in the regulation of both symbioses in *D. trinervis*, localized inoculations at three different positions of the root were assayed. All plants grown in pouches developed a green shoot system and a root system. No mycorrhizal colonization or nodulation was observed in root systems of non-inoculated plants. Leaves of non-inoculated plants or of plants inoculated only with arbuscular mycorrhizal fungi (AMF) were slightly chlorotic in contrast with the dark green leaves of plants that had formed nodules with nitrogen-fixing *Frankia* (data not shown). At the end of the plant growth experiment, which lasted 47 days after the first inoculation, plants with at least one symbiosis showed better growth than the control plants. The statistical analysis indicated that all plants inoculated with *Frankia* at any root region were significantly larger than control plants (Table 1). Since the root systems had to be stained for the analysis of mycorrhizal colonization, AM symbiosis could be recorded only at the end of the experiment using the gridline intersection method, and no time course analysis was possible. All plants inoculated with one or two inocula of *G. rosea* developed fungal colonization (Fig. 2a and b). However, no spatial affinity was found with respect to the position of the agar-inoculum. Intercellular mycelium and arbuscules were found all along the root. Arbuscular mycorrhization levels in roots reached 75–85 %, and there were no significant differences between treatments (M[MR]; M[RT_1], M[MR, RT_1] and M[RT_1 + RT_2]). Despite these levels of root mycorrhization, only the plants inoculated with AMF spores at the root tip (M[RT_1] and M[RT_{1+} RT_2]) showed growth enhancement compared to control plants (Table 1). When we studied the growth of plants treated only with AMF spores, we observed that these plants grew at the same rate as control plants (Figures S1 and S2).

Rather unexpectedly, nodulation occurred only under the agar-squares with *Frankia* (Fig. 3). No nodulation was found in other parts of the root. Nodulation was achieved in 80–90 % of plants inoculated with the actinobacteria. Statistical analyzes showed differences in the total number of nodules between F[MR] and F[RT_1] or F[RT_2] ($P \leq 0.001$) but no differences between F[RT_1] and F[RT_2] (Fig. 4a). Our results with spot inoculation corroborate the finding that the most susceptible zone for nodulation is the youngest part of the root see [RT_1] and [RT_2] (Fig. 4). Plants inoculated in a new zone 20 days after the first inoculation (F[RT_2]) also showed also a better response to the infection with *Frankia* than those inoculated at the beginning of the experiment in the mature region of the root, F[MR]. However, shoot heights of F[RT_2] plants did not reflect this result, probably because the *D. trinervis*-*Frankia* interaction had not reached the nitrogen-fixing state – it takes 3.5 weeks after inoculation and 2.2 weeks after nodulation started to detect Nitrogenase activity (Valverde et al. 2000) –, or because this state was delayed compared to the other earlier inoculated plants (Table 1, F[RT_2] 3.41 cm vs. F[MR] 2.95 cm). It is important to mention that nodules of treatment F[RT_1] were well developed (Fig. 3a and b) at the time of the scoring, while nodules of treatments F[MR] and F[RT_2] were smaller (Fig. 3c and d). When analyzing the growth of these plants, we observed that plants belonging to treatment F[RT_2] showed a similar growth rate as plants inoculated in the mature zone of the root (F[MR]), even when the number of nodules were significantly different between these two treatments (Fig. 4 and Suppl. Figure 3). Plants inoculated with one or two squares of *Frankia* display significant greater shoot height compared with 'non-inoculated' (control plants) and plants 'inoculated only with mycorrhizae' (Table 1).

When combined, simultaneous inoculations were performed with AMF spores and *Frankia*, two different responses were found. When inoculations with both microorganisms were performed at the same time (F[MR, RT_1]), the plants always developed nodules in RT_1 and only four plants

Table 1 Inoculations with either *Frankia* or Arbuscular Mycorrizal Fungi (AMF)

	Shoot height (cm)		
	INOCULANT		
Position of agar	Control	AMF	*Frankia*
MR	2.20±0.19	2.74±0.23	2.95±0.56*
RT_1	2.15±0.16	3.00±0.19‡	4.82±0.54§
RT_2	1.94±0.31	2.23±0.21	3.41±0.39*
MR − RT_1	2.57±0.34	2.78±0.26	6.53±0.37§
RT_1− RT_2	2.10±0.15	2.80±0.16‡	6.46±0.43§

Plant growth heights of *D. trinervis* inoculated with AMF or *Frankia*, using pieces of only agar as control treatments, once or twice, on different places: mature root (MR), root tip at time of first inoculation (RT_1) and position of the new root tip 20 days after the first inoculation (RT_2). For more details see Fig. 1. Values are the means±SE for 20–30 plants. Final shoot height was measure at the end of the experiment, 47 days after the first inoculation. Statistically significant differences compare to control treatments at §$P \leq 0.001$, ‡$P \leq 0.01$ and *$P \leq 0.05$

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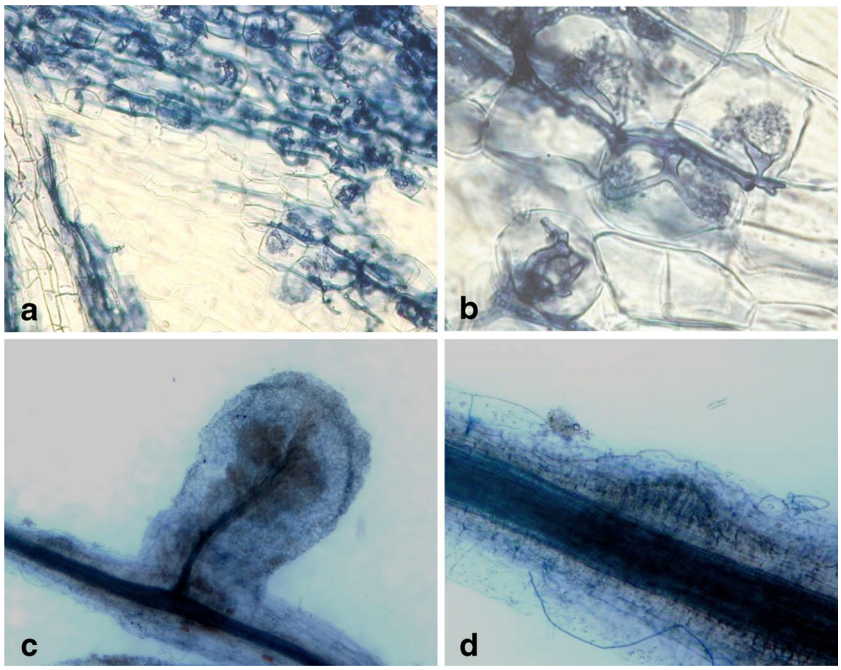

Fig. 2 Nodule of *D. trinervis* stained for AMF visualization. A. Root of *D. trinervis* inoculated with AMF (single inoculations) showing AMF arbuscules (X100). B. Detail of arbuscules (X400). C. Root of *D. trinervis* with combined inoculations with fungal mycelium and a developed nodule (X40). D. Detail of a prenodule surrounded by fungal mycelium (X100)

out of 24 showed nodules in both zones (MR and RT_1). In the latter case, fewer nodules developed in the mature zone of the root (MR) compared to the root tip (Fig. 4b). The same nodulation pattern was found in plants inoculated only with *Frankia* (F[MR] and F[RT_1], Fig. 4). On the other hand, when sequential inoculation was performed with *Frankia* (F[RT_1+RT_2]), no nodules were found at the position of the root tip at the second time of inoculation (RT_2) but we noticed that the presence of the second inoculum led to a positive response in nodule development in the region of the first inoculation (RT_1), see Fig. 4. The analysis of the kinetics of nodulation shows a positive correlation between the number of nodules and plant growth for each treatment (Table 1 and Fig. 4). Higher nodule numbers were found on plants treated with a double *Frankia* inoculation: F[MR, RT_1], F [RT_1+RT_2] (Fig. 3). Consistent with this result, shoots of F[MR, RT_1] and

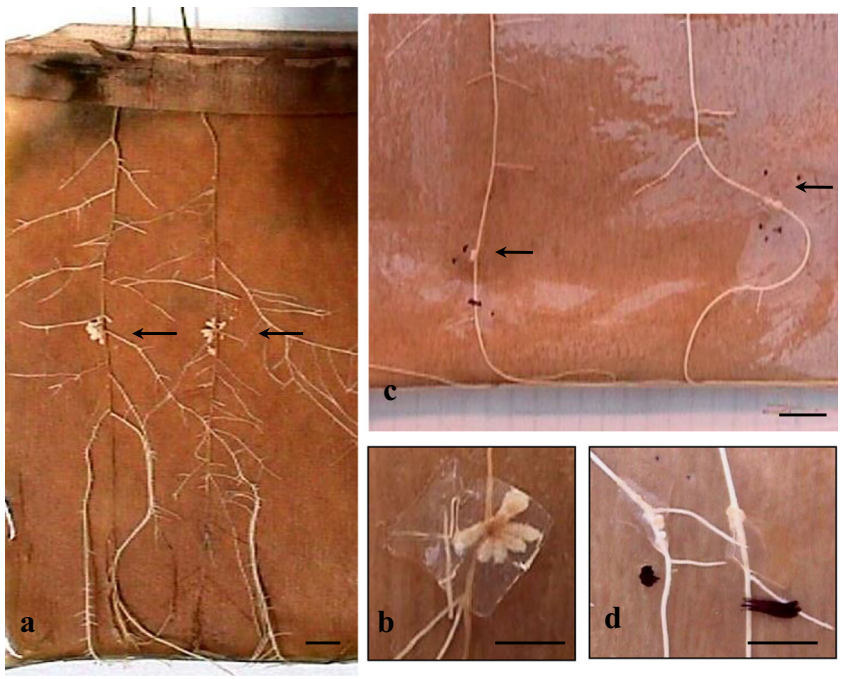

Fig. 3 Plants of *D. trinervis* growing in pouches. **a** and **b** Plants inoculated on the root tip (arrow) at the beginning of the experiment (treatment F [RT_1]). **a** Cluster of nodules only at the inoculated zone. **b** Detail of a nodule cluster under the agar-square containing *Frankia*. **c** Plants inoculated at the new root tip (arrow) 20 days after the beginning of the experiment (treatment F [RT_2]). **d** Cluster of nodules at the mature zone of the root (treatment F [MR]). Scale bars=1 cm

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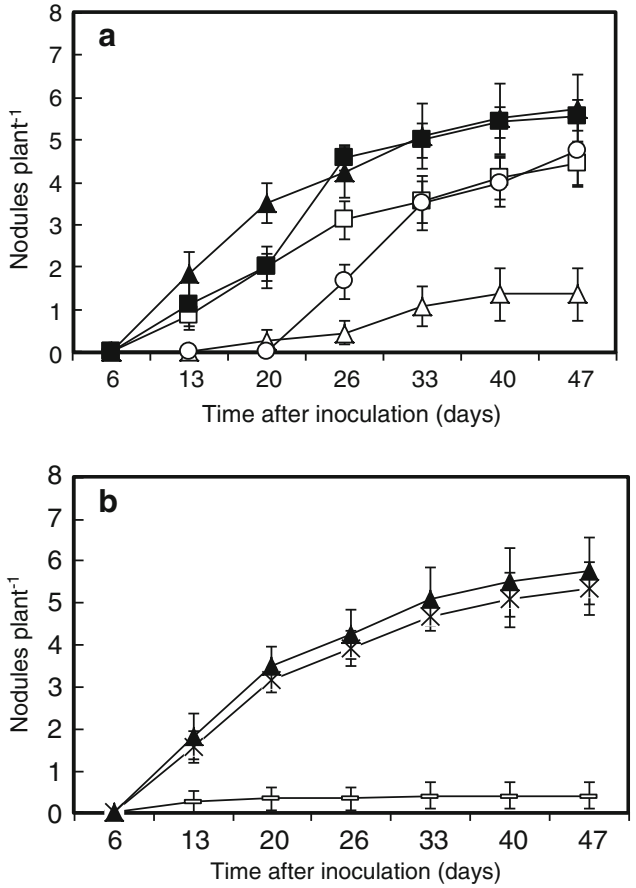

Fig. 4 Nodulation kinetics of *D. trinervis* inoculated with *Frankia*. Values are the means±SE for 20–30 plants. Second inoculation at RT_2 was done 20 days after first inoculation at RT_1. **a** Treatment F [MR] (Δ), F [RT_1] (□), F [RT_2] (○), F [MR , RT_1] (▲), and F [RT_1 + RT_2] (■). **b** For treatment F [MR , RT_1] (▲), nodules in zone [MR] (−) and root tip [RT_1] (X) are shown separate

F[RT_1+RT_2] plants were significantly higher than shoots of plants inoculated only once with *Frankia* ($P \leq 0.01$, ex. F[MR, RT_1] 6.53 cm vs. F[MR] 2.95 cm or F[RT_1] 4.82 cm) and control plants.

3.2 Combined inoculations

With the aim of gaining further insight into the interaction between actinorhizal and AM symbiosis, we performed combined inoculations with both microorganisms.

Mycorrhizal colonization was not affected by the presence of *Frankia* at any place, nor by the development of *Frankia*-induced nodules. As in the single inoculation studies, intraradical mycelium was found all over the root system. Mycorrhizal colonization scored on samples taken at the end of the experiment reached levels of 70–80 % of hyphal and arbuscular colonization with no significant differences between treatments ($P \leq 0.05$). No mycorrhizal colonization was found in nodule tissue (Fig. 2c and d).

As described above, root nodule development was better at the root tip region than at other regions of the root. A detailed analysis of nodulation kinetics shows that early mycorrhizal inoculation did not delay nodulation by *Frankia* (Fig. 5, treatment M[RT_1] F[RT_2]). The pattern of autoregulation, i.e., feedback inhibition of nodulation caused by the first inoculation, was found only with two consecutive *Frankia* inoculations, but not when plants were treated first with mycorrhiza and subsequently with *Frankia* (F[RT_1 + RT_2] vs M[RT_1] F[RT_2]). M[RT_1] F[RT_2]. Plants developed nodules with the same kinetics as F[RT_2] plants (Figs. 4 and 5), suggesting that there were no effects of the previous inoculation with AMF on the susceptibility of RT_2 for *Frankia* infection and nodule development. A higher number of nodules was observed in the presence of AMF inoculum, compared with the single *Frankia* inoculation on the same location; however, this difference was not statistically significant.

Plants inoculated with both microsymbionts showed better shoot growth relative to plants treated with only *Frankia* or *G. rosea* as a single inoculum, which suggests a synergistic effect in these plants (Tables 1 and 2). For example, the shoot height of M[MR] F[RT_1] plants (7.49 cm) is significantly different from M[MR] (2.74 cm, $P \leq 0.001$) and F[RT_1] plants (4.82 cm, $P \leq 0.01$). Surprisingly, the final number of nodules developed on M[MR] F[RT_1] plants did not vary, compared to their single inoculation (M[MR] F[RT_1] vs. F[RT_1]). As mentioned above, when a new inoculation with *Frankia* was performed (FM[RT_1+RT_2]), no nodules developed in response to the new inoculum (F[RT_2]).

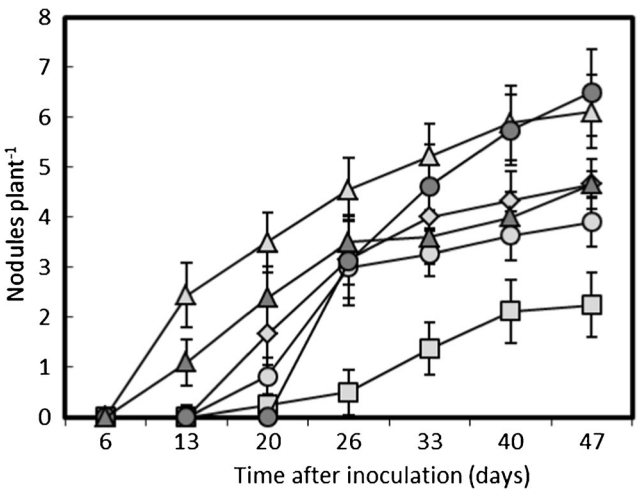

Fig. 5 Nodulation kinetics of *D. trinervis* inoculated with *Frankia* and *G. rosea*. Values are the means±SE for 20–30 plants. Second inoculation at RT_2 was done 20 days after first inoculation at RT_1. Nodules of simultaneous treatments are shown in *light grey* and nodules of sequential treatments are in *dark grey*. Nodules in M/F [MR] (●); M/F [RT_1] (◆); M[MR] F[RT_1] (▲); F[MR] M[RT_1] (●); M[RT_1] F[RT_2] (●) and F[RT_1] M[RT_2] (▲)

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Table 2 Combined Inoculations with *Frankia* and Arbuscular Mycorrizal Fungi (AMF)

		Treatment	Shoot height (cm)
CONTROL	1	C [MR]	2.20±0.19
	2	C [RT_1]	2.15±0.16
	3	C [RT2]	1.94±0.31
	4	C [MR, RT_1]	2.57±0.34
	5	C [RT_1 + RT_2]	2.10±0.15
SIMULTANEOUS	6	M / F [MR]	4.56±0.76§
	7	M / F [RT_1]	5.97±0.39§
	8	M [MR] F [RT_1]	7.49±0.52§
	9	F [MR] M [RT_1]	6.23±0.56§
SEQUENTIAL	10	M [RT_1] F [$RT2_2$]	4.08±0.46*
	11	F [RT_1] M [RT_2]	6.47±0.43§
	12	M / F [RT_1] + F [RT2]	5.75±0.45‡
	13	M / F [RT_1] + M [RT_2]	5.83±0.62‡

Plant growth heights of *D. trinervis* inoculated with *Frankia* (F), AMF (M) or with agar only as a control treatment (C) once or twice on different places: mature root (MR), root tip at time of first inoculation (RT1) and new root tip 20 days after the first inoculation (RT2). For more details see Fig. 1. Values are the means±SE for 20–30 plants. Final shoot height was measure at the end of the experiment, 47 days after the first inoculation. Statistically significant differences compare to control treatments at §$P \leq 0.001$, ‡$P \leq 0.01$ and *$P \leq 0.05$

4 Discussion

Our results show that spot inoculation of *Discaria trinervis* with *Frankia* resulted in nodule formation only in the area where the root was in contact with the agar containing the actinobacteria. This suggests that dispersion of *Frankia* outside the agar pieces was negligible or not enough to start new infections elsewhere.

Previously it has been suggested that combined inoculation with endomycorrhiza and actinobacteria improved plant nutrient status and increased shoot biomass proportionally to C availability (Gavito et al. 2000). For example, when the actinorhizal plant *Casuarina glauca* was inoculated with both *Frankia* and *Glomus*, plants showed a significantly higher dry weight at harvest than plants inoculated with either microbe (Wheeler et al. 2000). Responses were found to vary depending on the *Frankia* strain, the AM fungus and the plant species (Visser et al. 1991; Jha et al. 1993; Sempavalan et al. 1995; Orfanoudakis et al. 2010).

In our experiments, a synergistic effect on shoot height was found when both microsymbionts where present. These results support the conclusion that autoregulation of nodulation is not affected by P, but that nodule biomass is was enhanced by P supply (Valverde et al. 2002). We suggest that the P taken up by the mycorrhiza stimulates nodule growth, but not new infections under our particular experimental conditions, and thereby stimulates subsequent plant growth. The fact that dual colonization was obtained upon simultaneous infection indicates that these microorganisms do not compete for infection sites. We think that the reduced growth observed for plants inoculated with *Gigaspora rosea* (Table 1) could be due to growth conditions with poor nitrogen supply that were suboptimal for this species. It is possible, also, that the physiological status of the young seedlings of *D. trinervis* was not optimal for the establishment of the mycorrhizal symbiosis. These data suggest that the significant difference found in plant growth could result from the benefits caused by the increase in plant nutrition (nitrogen) driven by the establishment of the *Frankia* symbiosis. On the other hand, no significant differences were found in plant growth in F[MR] and F[RT_2] treatments, compared to C[MR] and C[RT_2] respectively. One possible explanation is that F[MR] plants were hardly nodulated, possibly due to the maturation of the root; and in the case of F[RT_2] plants, because nitrogen fixation activity of the nodules were just beginning and no physiological results on plant growth were observed.

It is important to point out that there were nodules at two different regions of the root only when the agar-squares with *Frankia* were placed at those root places at the same time. When nodules had been developed a priori in the root at one region no nodulation was achieved in others parts of the root, in accord with previous studies describing autoregulation of nodulation in *D. trinervis* (Valverde and Wall 1999b). Another remarkable point is that we found neither inhibition of nodulation by mycorrhiza nor inhibition of mycorrhiza by nodulation. The last observation is different from what has been found in legumes using the split root system (Catford et al. 2003, 2006) where rhizobia induced a systemic plant response that inhibit later mycorrization of the plant. This mechanism seems not to be related to nodulation since a similar response was found in barley inoculated with *Rhizobium* NGR234 (Khaosaad et al. 2010). This interesting differential plant response deserves more research, especially with respect to a comparison between actinorhizal plants and legumes. In particular, it would be interesting to analyze the role of the different root infection pathways with respect to systemic effects on mycorrhization. Finally, as observed in the *Glycine-Glomus-Rhizobium* symbiosis (Bethlenfalvay et al. 1985), fungal hyphae did not invade root nodules in *D. trinervis* suggesting the presence of an exclusion mechanism, another aspect worthy of more research.

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Conflict of interest There is no conflict of interest in this work.

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