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Regulation of Nodulation and Nodule Mass in Relation to Nitrogenase Activity and Nitrogen Demand in *Discaria trinervis* (Rhamnaceae) Seedlings

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Abstract

A time course experiment of nodulation, N_2 fixation and growth was conducted on *Discaria trinervis* seedlings after inoculation with *Frankia* isolate BCU110501, to study the regulation of nodulation in relation to nitrogenase activity. Nodulation started at 1.3 weeks after inoculation and reached a plateau at 7 weeks after inoculation. Nodules were mainly restricted to tap roots. Nitrogenase activity was first detected at 3.6 weeks after inoculation and increased throughout the 9-week experimental period. No H_2 evolution could be detected, thus the symbiosis appeared to be phenotypically Hup^+. Plant growth increased after onset of nitrogenase activity, except for root length growth. Nitrogenase activity expressed on either plant dry matter basis, nodule dry matter basis, or leaf area basis, did not change significantly during the study. N content of all plant parts increased to a maximal

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value at 7 weeks after inoculation At this time formation of new nodules stopped and nodule dry mass relative to total plant dry mass did no longer increase. The results support the idea of a feedback regulation of nodule growth and activity through internal N level.

Keywords: Actinorhiza, *Discaria trinervis*, *Frankia*, N_2 fixation, nodulation, feedback regulation

Abbreviations

DM = dry matter
RT = mark denoting the position of the root tip at the moment of inoculation

1. Introduction

Discaria trinervis (Hooker et Arnot) Reiche is an actinorhizal shrub (Rhamnaceae) from south western South America (Tortosa and Medán, 1983). *Frankia* BCU110501, which was isolated from field collected *D. trinervis* nodules, effectively nodulates *D. trinervis* and *D. chacaye* (Chaia, 1998). *D. trinervis* root infection occurs by means of intercellular penetration (Valverde and Wall, 1999a). *D. trinervis* seems to display mechanisms that control nodule initiation and growth in a way similar to legumes (Valverde and Wall, 1999b). A few days after root inoculation, an autoregulatory response (Pierce and Bauer, 1983) is systemically elicited throughout the root and prevents infection of young developing root regions. This early response is triggered before nodule primordia can be detected on the root surface. A second mechanism, which involves mature and N_2 fixing nodules, operates in keeping the root system under a non susceptible status for nodulation (Valverde and Wall, 1999b). Nodule biomass, rather than nodule number, seems to be regulated by a homeostatic mechanism, when the symbiotic plant is growing in the absence of soluble N sources (Valverde and Wall, 1999b).

Nodulation is also affected by environmental factors. In particular, inhibition of nodule formation and activity by external N has been extensively studied in several N_2 fixing root nodule symbioses (reviewed by Streeter, 1988; Huss-Danell, 1997). But, nodule growth and N_2 fixation could also be affected by recycling of N within the plant (Parsons et al., 1993; Baker et al., 1997a; 1997b; Neo and Layzell, 1997).

The aim of the present work was to study the regulation of nodulation in relation to growth, nitrogenase activity and N content of *D. trinervis* in the absence of external N. A time course experiment was carried out in young seedlings of *D. trinervis* following inoculation with *Frankia* isolate BCU110501.

2. Materials and Methods

Plant material

Mature fruits of *Discaria trinervis* (Hooker et Arnot) Reiche were collected in field in 1996 from Pampa de Huenuleo (41°10' S, 71°12' W, Bariloche, Rio Negro, Argentina). Seeds were separated from dry fruits, sun exposed for 2 h and stored at –20°C. No loss of germination occurred during storage. Surface sterilisation was performed by scarification (3 min immersion in concentrated H_2SO_4, with occasionally shaking by hand), followed by exhaustive rinsing with sterile distilled water (Chaia, 1997). Seeds were blotted dry with filter paper, transferred to perlite moistened with the modified Evans nutrient solution (Huss-Danell, 1978) diluted to 1/10 of full strength (hereafter referred to as 1/10 E) and supplemented with 10 mg l^{-1} of N as NH_4NO_3. Seeds were kept at 4°C for 5 days.

Growth conditions and experimental design

For experiments 1 and 2, seed germination and plant growth took place in a greenhouse in Umeå (63°3' N, 20°6' E, Sweden), during the period April–July 1997. Average minimum and maximum temperatures were 14 and 28°C, respectively, while average relative humidity was 51%. Light : dark cycles were 19 : 5 h and metal halogen lamps (Philips HPI-T 400W, photosynthetic active radiation 130 µmol m^{-2} s^{-1}) supplemented natural light during short days. Four seedlings at the cotyledonary stage (12 d after start of germination), were aseptically transferred to each growth pouch (Mega International, Minneapolis, USA) moistened with 1/10 E solution containing 1 mg N l^{-1} as NH_4NO_3. Two days after inoculation with *Frankia*, the bottom of each growth pouch was cut open and groups of pouches were transferred into slightly bigger plastic bags to give seedlings access to a larger volume of nutrient solution. Nitrogen-free 1/10 E was renewed in the plastic bags every 3–4 days. Each experimental unit consisted of one growth pouch with 3 to 4 plants. Although data are presented on a plant basis, means and standard errors were calculated for pouches as units.

Experiment 1: A set of 10 pouches was used to follow nodulation and nodule distribution throughout the 9-weeks experimental period. An additional set of pouches was left non-inoculated to serve as a control for N content.

Experiment 2: At each experimental time (once a week), 3 to 5 pouches were randomly chosen.

Experiment 3 was carried out in a greenhouse in Bernal (34°7' S, 58°3' W, Argentina) during 1999, where average temperature was 24°C and relative humidity in the range 40–85%. Light: dark cycles were 16 : 8 h and metal

halogen lamps (Osram 400W) supplemented natural light to complete the photoperiod during short days. Seedlings were planted in pots with sterilised perlite and irrigated with 1/10 E containing either 1 or 20 mg N l^{-1} as NH_4NO_3. Two sets of 20 seedlings each were grown for 10 weeks. At time of inoculation with *Frankia*, leaves were randomly sampled from each set of plants for N content. Six weeks later, both groups of plants were harvested.

Frankia strain and inoculation

Frankia BCU110501, isolated from *D. trinervis* nodules, is infective (Nod^+) and effective (Fix^+) in *D. trinervis* plants (Chaia, 1998). Inoculum was prepared from bacteria grown in static BAP minimal medium with 55 mM glucose as C source (Chaia, 1997) at 28°C during 4 weeks. Cells were harvested by centrifugation (11000xg, 20 min), and washed with 1/10 E + 1 mg N l^{-1}. The cell suspension was homogenised by three passages through a 0.8 mm gauge needle and three more passages through a 0.5 mm gauge needle (Valverde and Wall, 1999a; 1999b). The amount of *Frankia* biomass in the homogenate was estimated as packed cell volume and total protein by the bicinchoninic acid method using BSA (Sigma, St. Louis, USA) as standard (Nittayajarn and Baker, 1989).

At the moment of inoculation (3 weeks after seedling transfer to pouches), the position of each root tip was marked on the plastic pouch (hereafter referred to as RT). Each seedling was inoculated by dripping 200 µl of inoculum (which contained 0.8 µl of *Frankia* packed cells containing 4.8 µg of total proteins) from the root tip towards the shoot (Valverde and Wall, 1999a; 1999b).

Nodule scoring

Nodules on all roots were counted. Nodule position on tap roots was marked on the pouch, and their distance from RT was measured. Position of nodules relative to RT on lateral roots was not considered.

Nitrogenase activity

Nitrogenase activity was estimated as acetylene reduction activity (ARA) of intact plants in pouches. Measurements were done weekly starting at 3 weeks after inoculation. Each pouch was incubated in a 2 l gas-tight cuvette. Glass beads (0.5 mm) were used to reduce the gas volume to ca. 600 ml. Pouches were cut open along their sides to facilitate gas diffusion, and shoots were gently bent to fit into the cuvette. C_2H_2 was injected to a final concentration of 10% (v/v). Incubations were done in the greenhouse in the morning. A gas sample of

0.6 ml was taken at 12, 18, 24 and 30 min after C_2H_2 injection, and analysed for C_2H_4 production in a Varian GC 3400 gas chromatograph with a Haysept-T mesh range 80-100 column run at 90°C. Carrier gas was N_2 at a flow rate of 30 ml per minute. The flame ionisation detector was operated with air at a flow rate of 300 ml per minute and H_2 at a flow rate of 30 ml per minute.

Measurement of H_2 evolution was done on 10 pouches with intact plants kept in a 2-l air tight cuvette as above. Gas samples of 0.5 ml were taken at 10 min intervals within a period of 60 min, and analysed by gas chromatography. A Molsieve 5A mesh range 80–100 column was run at 60° C. Carrier gas was Ar at a flow rate of 25 ml per minute. The filament temperature in the thermal conductivity detector was 200°C.

Plant size and dry matter

Shoot height was measured to the nearest 1 mm. Root systems were separated from pouches and a digital image was obtained with a HPIICX scanner and processed with the software Delta-T SCAN Image Analysis System, from which root length could be derived. Leaf area per plant was measured in an area meter (LI-COR LI-3000A). Leaves, stem, roots and nodules were dried separately for 48 h at 55°C and then weighed to estimate dry matter (DM). The relative growth rate (R) of shoot height, root length, leaf number, leaf area and plant DM was calculated assuming an increment of each variable X with time according to [$\ln X = (e^R - 1) \times t$] (Cheeseman, 1993). R value was obtained from the slope of a plot of the logarithm of each variable as a function of time.

N and C content

Dried plant parts were milled in a Retsch mixer mill MM2000 and C and N content were determined in a Perkin-Elmer 2400 element analyser separately for stem, leaves and root + nodules. In Experiment 3, Kjeldahl analysis (Jackson, 1958) was used. Data are expressed as % (w/w) of DM.

Light microscopy

Root segments (Experiment 2) bearing developing nodules were fixed in 2.5% (w/v) glutaraldehyde in 45 mM K-phosphate buffer, pH 7.2, for 30 min at reduced pressure and then for at least 3–4 h at atmospheric pressure. Samples were stored at 4°C for ca. 4 months. Fixed root pieces were pre-stained with osmium tetroxide 2% (w/v) and dehydrated with ethanol (50, 70, 80, 95 and 100% (v/v)). Dehydrated samples were embedded in Epon-Araldite and

polymerisation was carried out for 3 days at 70°C. Transverse or longitudinal sections (1–1.5 µm) were mounted on glass slides, stained with methylene blue-Azur II and then examined in a Zeiss Jenaval light microscope.

3. Results

Nodules were first seen on seedling tap roots at 1.3 weeks after inoculation while nodulation on lateral roots was first detected at 3 weeks after inoculation (Fig. 1A). At the end of the experiment, 100% of the inoculated plants were nodulated. Plants developed an average of 16.4 ± 1.7 nodules, and of these 82% were located on tap roots. Nodule number increased until 7 weeks after inoculation. In parallel, nodule DM fraction increased up to a final level of near 5% of the plant biomass (Fig. 1B). Distribution of nodules along tap roots was restricted to the region spanning from 40 mm above RT through 50 mm below RT (Fig. 2). This zone of nodulation was defined already at 4 weeks after inoculation (Fig. 2), when ca. 50% of the final number of nodules had developed (Fig. 1A).

The time course of nodule development was similar to what we have previously described for this species (Valverde and Wall, 1999a). Nodule primordia with few infected cells were seen at 1.3 weeks after inoculation but *Frankia* filaments had not differentiated into vesicles. At 2.3 weeks after inoculation a higher number of hypertrophied and infected nodule cells were seen and vesicles, surrounded by "void space", had developed (microscopy data not shown).

Nitrogenase activity, estimated as ARA, was first detected at 3.6 weeks after inoculation and then increased throughout the study period (Fig. 3). No H_2 evolution was detected at 9 weeks after inoculation (10 pouches, 39 plants) indicating that *Frankia* had an active uptake hydrogenase. Non-inoculated control plants did not develop any nodules and did not reduce C_2H_2.

The relative increase in leaf number, leaf area and shoot height was significantly higher after than before onset of ARA ($P<0.005$, Figs. 4A, B and C). On the other hand, the relative increase of root length (0.036 d^{-1}) was not different after ARA onset ($P>0.005$, Fig. 4D). Relative growth rate of plants (DM basis) was significantly higher from 5 weeks after inoculation than before (0.030 d^{-1} to 0.051 d^{-1}, $P<0.005$; Fig. 4E). Shoot : root ratio was at minimum value at 3–4 weeks after inoculation (Fig. 4F), i.e. at the same time as N began to become available through N_2 fixation. Then, shoot : root ratio increased up to a maximum at 7–9 weeks after inoculation (Fig. 4F), when nodule number and nodule fraction (Fig. 1) had also reached a maximum. C content in all nodulated plants was similar throughout the study, ranging from an initial value of 45.9 up to a final value of 48.5% (data not shown).

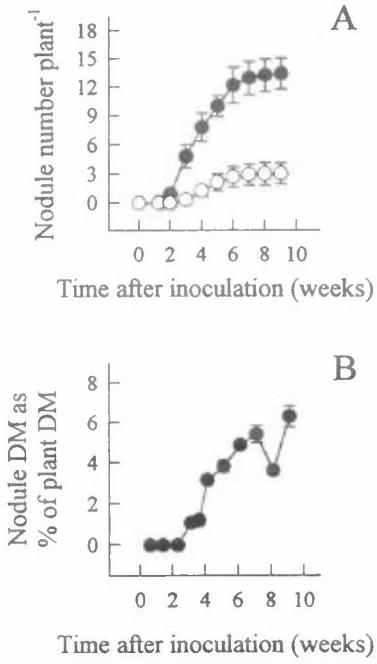

Fig. 1.

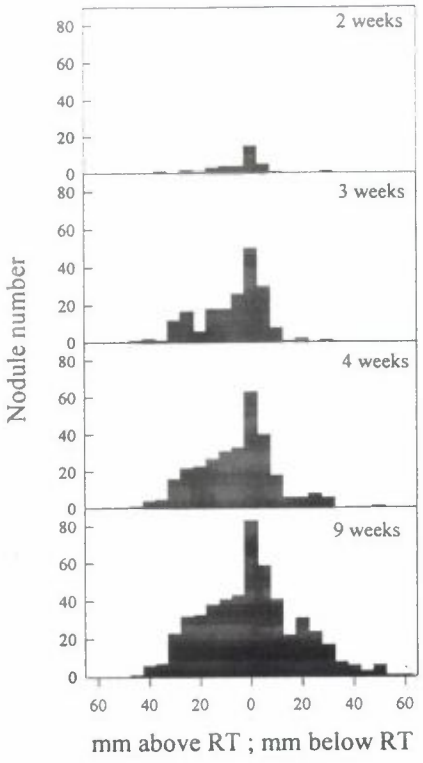

Fig. 2.

Figure 1. Nodulation of *D. trinervis* seedlings inoculated with *Frankia* isolate BCU110501. A) Nodule number. ●, nodules on tap roots; O, nodules on lateral roots. The same plants were studied throughout the experiment (Experiment 1). Mean ± SE for 10 pouches (39 plants). B) Nodule DM as fraction of plant DM. Separate pouches were studied at each experimental time (Experiment 2). Mean ± SE for n = 3 to 5 pouches.

Figure 2. Nodulation profiles along *D. trinervis* tap root at different times after inoculation. At the moment of inoculation, RT was marked on the plastic pouch. Nodule position on tap root was marked on the pouch, and the distance from RT was measured. Nodules located within every 5 mm segment of tap root above and below RT were counted. Observations were done on the same plants throughout the experiment (10 pouches, 39 plants; Experiment 1).

Although ARA per plant increased throughout the experiment (Fig. 3), ARA in relation to plant DM, leaf area or nodule DM remained relatively constant (Fig. 5). N concentration in all plant parts reached a maximum at 7 to 8 weeks after inoculation (Fig. 6). Leaves had the highest N concentration throughout

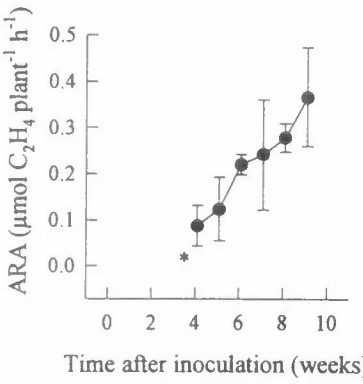

Figure 3. Time course of nitrogenase activity (ARA) in *D. trinervis* after inoculation with *Frankia* isolate BCU110501. Data are presented on a plant basis. Measurements were made on separate pouches at each experimental time (Experiment 2). Mean ± SE for n = 3 to 5 pouches. *, ARA was detected when 5 pouches were incubated together but activity was too low to be measured in a single pouch.

the study. Plant N concentration doubled between 3.6 and 7 weeks after inoculation. Non-nodulated plants had an N concentration of 1.0 % at the end of the experiment.

Following inoculation plants were grown with no other N source than N_2 fixation (Experiments 1–2). From our data on N content and plant growth, it was therefore possible to calculate the N increment in a single plant and thus to validate the ARA data (Table 1). Total N increment for the period between 5 and 7 weeks after inoculation, i.e. when plants increased both their biomass and N concentration, equals 0.38 mg or 13.6 µmoles of N_2 per plant. In the same period, average ARA was 183 nmol h^{-1}, which represents a hypothetical production of 183 nmol h^{-1} during 14 days and 24 h per day = 61.5 µmoles of C_2H_4. For a 4:1 ratio between ethylene production and N_2 reduction of nitrogenase, this amount of ethylene is equivalent to 15.4 µmoles of N_2 reduced in 2 weeks. Similar results were obtained for calculations made for the period between 7 and 9 weeks after inoculation, when plants increased biomass but did not modify their N concentration (22.9 µmoles of fixed N_2 from N content data and 25.5 µmoles from ARA data). Using the same ARA during 24 h per day is probably an overestimation in our calculations, but, in general, our calculations support the reliability of the method used to estimate nitrogenase activity in young nodulated *Discaria*.

When *D. trinervis* seedlings were pre-treated with different N supplies for a 10-weeks period (Experiment 3) different leaf N concentrations were obtained

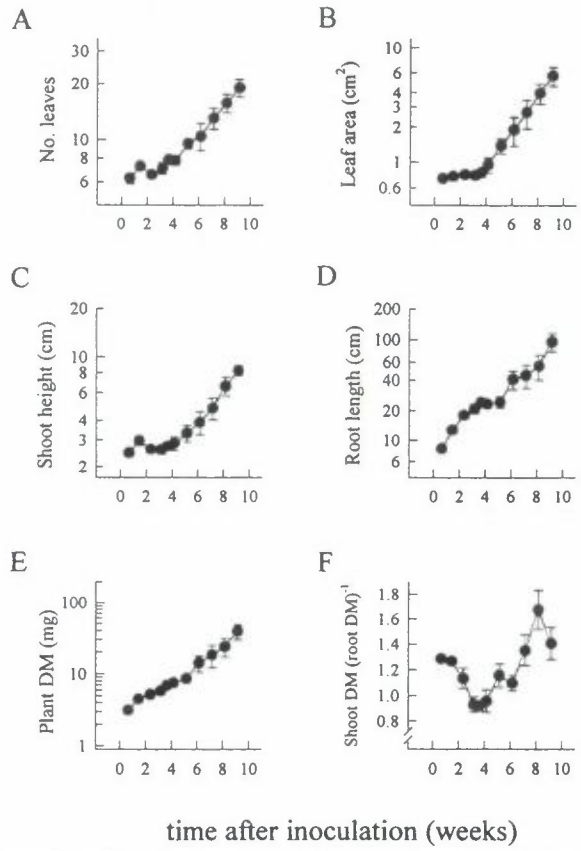

Figure 4. Growth of *D. trinervis* seedlings inoculated with *Frankia* isolate BCU110501. Measurements were made on separate pouches at each experimental time (Experiment 2). Mean ± SE for n = 3 to 5 pouches. All ordinates are logarithmic, except for shoot : root ratio (F), which is linear.

(Table 2). Inoculation of plants having a N concentration in their leaves of 4.4% failed to induce nodulation (Table 2). The same inoculum induced an average of 9.3 ± 1.2 nodules (Table 2) in *D. trinervis* plants with a leaf N concentration of 2.1% at time of inoculation.

4. Discussion

The establishment of the symbiosis between the rhamnaceal *Discaria trinervis* and the homologous isolate *Frankia* BCU110501 resulted in the growth of effectively nodulated plants, in the absence of N in the nutrient

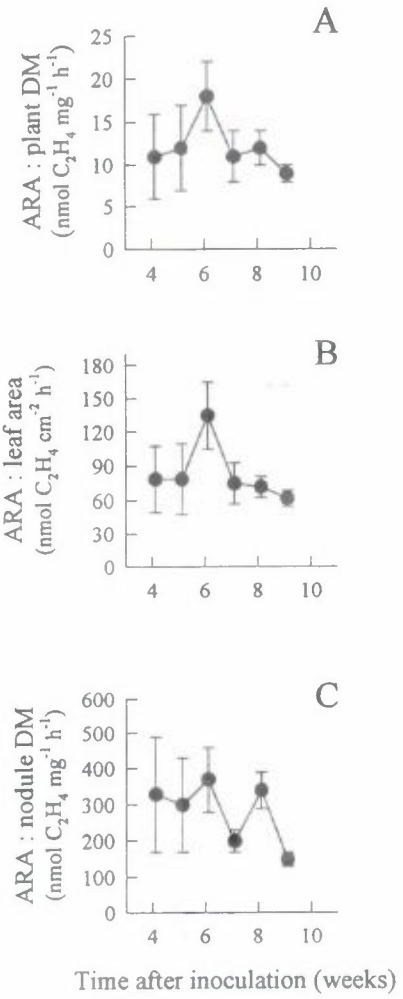

Fig. 5.

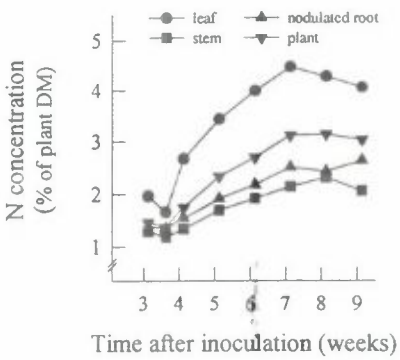

Fig. 6.

Figure 5. Nitrogenase activity in *D. trinervis*, inoculated with *Frankia* isolate BCU110501, in relation to plant size. A) plant DM basis, B) leaf area basis and C) nodule DM basis. Data are from separate pouches at each experimental time (Experiment 2). Mean ± SE for n = 3 to 5 pouches.

Figure 6. N content of *D. trinervis* plant parts after inoculation with *Frankia* isolate BCU110501. Each point represents all plants from 3 to 5 pouches studied at each experimental time (Experiment 2).

solution. Nitrogen fixation activity during the study period could explain the increment in plant growth rate and N content.

Nodulation was confined to a defined region of the tap root. The limit of the nodule distribution profile was defined already 3 days after inoculation (Valverde and Wall, 1999b) and was almost definite at 4 weeks after inoculation (Fig. 2). Reduction of nodulation in younger parts of the tap root (below RT) was not due to lack of infective *Frankia* since reinoculation did not result in new nodule formation (Valverde and Wall, 1999b). *Frankia* vesicle differentiation within *D. trinervis* nodule cells were detected at 2 weeks after inoculation (this study; Valverde and Wall, 1999a), but nitrogenase activity was first detected, at a very low level, at 3.6 weeks after inoculation (Fig. 3). The delay in the ARA detection could be due to either vesicle immaturity or extremely low nitrogenase activity. This result confirms that the early control of nodule formation in *D. trinervis* is independent of N_2 fixation activity, as was previously suggested (Valverde and Wall, 1999b).

Steady-state growth of plants can be characterised by both constant shoot : root ratio and relative growth rate (R). According to a simple model for plant growth (Cheeseman, 1993), plants show an increment rate in their structures defined as: $\delta W/\delta t = W(e^R-1)$, where W is the mass of a plant compartment, and R is its actual relative growth rate. Growth of symbiotic *D. trinervis*, expressed as plant DM, fitted this model and an R-value of 0.051 d^{-1} ($r^2 = 0.98$) was obtained for the period between 5 and 9 weeks after inoculation. After onset of ARA, growth rate of shoots, but not root length, increased (Fig. 4). Prior to onset of ARA, the shoot : root ratio of *D. trinervis* decreased (Fig. 4F). During this period plant N was limited to seed N plus N added to the nutrient solution until inoculation, and reduced shoot : root ratio reflects resource re-allocation within plant to favour root development in order to seek N. After onset of ARA, shoot growth increased faster than root growth and the shoot : root ratio increased.

Nitrogenase activity was measured as acetylene production from ethylene (ARA). Some drawbacks with ARA as an estimation of N_2 fixation have been identified in different N_2 fixing systems, mainly related to a decay in nitrogenase activity shortly after acetylene injection in the assay system (Winship and Tjepkema, 1990). In this study, however, ARA proved to be a useful method to estimate nitrogen fixation in nodulated *Discaria* seedlings (Table 1). The fact that no H_2 evolution could be detected in N_2 fixing *Discaria* root nodules, indicates that *Frankia* BCU110501 expresses an uptake hydrogenase which should make the symbiosis more energy efficient in the reduction of N_2.

The increments in nodule number and biomass were down regulated at 6–7 weeks after inoculation (Figs. 1A and 1B) as we have previously observed (Valverde and Wall, 1999b). Nodule DM fraction appeared to reach a stable level of 5% of the plant DM (Fig. 1B). This means that nodule growth and plant growth were closely related to each other, which is reasonable for a root nodule

Table 1. Data for calculation of nitrogen fixation in *D. trinervis* based on N content and nitrogenase activity (ARA)

Weeks after inoculation	Dry matter (mg)	N concentration[1] (% of dry matter)	N content[1] (mg)	ARA (nmol C_2H_4 h^{-1})	Number of pouches
5	8.8 ± 1.1	2.35	0.21	124 ± 69	4
7	18.8 ± 6.4	3.13	0.59	242 ± 119	4
9	40.2 ± 9.7	3.05	1.23	366 ± 107	3

Values are expressed on a plant basis. [1]Data from a single analysis on plant material pooled at this time (see Materials and Methods).

Table 2. Leaf N concentration and nodulation in *D. trinervis*

	At time of inoculation		6 weeks after inoculation	
N supply (mg N l^{-1})	1	20	1	20
Leaf N concentration (% of dry matter)	2.1	4.4	3.5	4.1
Nodules per plant	0.0	0.0	9.3 ± 1.2	0.0

Seedlings were grown with different N supply for 10 weeks prior to inoculation and for 6 weeks more after inoculation (Experiment 3). Mean ± SE for 18–20 plants.

symbiosis where all gain in N comes from N_2 fixation in the absence of other N source. Acetylene reduction activity data expressed on plant or nodule DM or per leaf area basis (Fig. 5) suggest that nitrogenase activity is adjusted to plant growth. Concentration of N in most plant parts was maximal at 7 weeks after inoculation (Fig. 6). Nodule formation was depressed at the same time as N concentration was maximal in tissues. Thus, our interpretation of the results on the *Discaria-Frankia* symbiosis is that the plant is susceptible to infection when the N concentration in plant tissues is below a threshold value. For *D. trinervis* leaves this threshold value appeared to be in the range between 3.5 and 4.0% (Fig. 6, Table 2). Furthermore, we have observed that prolonged N starvation of *D. trinervis* seedlings led to almost twice the number of nodules developed per plant, when compared to non-starved seedlings (Valverde and Wall, 1999b). This observation suggests that the number of nodule initials is inversely related to the N status of the plant. The proposed inhibitory level of N could be reached either by root absorption of soluble N (NO_3^- and/or NH_4^+)

or later through N_2 fixation, or a combination of both. If a susceptible plant root (i.e. N content below the threshold value) is infected by *Frankia*, it is likely that nodule development is first controlled by an autoregulatory response (Caetano-Anollés and Gresshoff, 1991; Wall and Huss-Danell, 1997; Valverde and Wall, 1999a). Therefore, autoregulation quickly limits the extension of the root where nodule formation is further allowed. Meanwhile N content of the plant starts to increase after the onset of nitrogenase activity. Finally, once the plant is nodulated and fixing N_2, a feedback regulation of nodule formation and growth related to the N concentration of certain plant tissues may operate as proposed by Parsons et al. (1993) for legumes. This regulation may operate in tuning the final proportion of nodule dry mass in relation to total plant dry mass.

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