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RESEARCH PAPER

Regulation of nodulation in the absence of N_2 is different in actinorhizal plants with different infection pathways

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Abstract

Root nodulation in actinorhizal plants, like *Discaria trinervis* and *Alnus incana*, is subject to feedback regulatory mechanisms that control infection by *Frankia* and nodule development. Nodule pattern in the root system is controlled by an autoregulatory process that is induced soon after inoculation with *Frankia*. The final number of nodules, as well as nodule biomass in relation to plant biomass, are both modulated by a second mechanism which seems to be related to the N status of the plant. Mature nodules are, in part, involved in the latter process, since nodule excision from the root system releases the inhibition of infection and nodule development. To study the effect of N_2 fixation in this process, nodulated *D. trinervis* and *A. incana* plants were incubated under a N_2-free atmosphere. *Discaria trinervis* is an intercellularly infected species while *A. incana* is infected intracellularly, via root hairs. Both symbioses responded with an increment in nodule biomass, but with different strategies. *Discaria trinervis* increased the biomass of existing nodules without significant development of new nodules, while in *A. incana* nodule biomass increased due to the development of nodules from new infections, but also from the release of arrested infections. It appears that in *D. trinervis* nodules there is an additional source for inhibition of new infections and nodule development that is independent of N_2 fixation and nitrogen assimilation. It is proposed here that the intercellular *Frankia* filaments commonly present in the *D. trinervis* nodule apex, is the origin for the autoregulatory signals that sustain the blockage of initiated nodule primordia and prevent new roots from infections. When turning to *A. incana* plants, it seems likely that this signal is related to the early autoregulation of nodulation in *A. incana* seedlings and is no longer present in mature nodules. Thus, actinorhizal symbioses belonging to relatively distant phylogenetic groups and displaying different infection pathways, show different feedback regulatory processes that control root nodulation by *Frankia*.

Key words: Actinorhizal plants, *Alnus incana*, *Discaria trinervis*, feedback regulation, *Frankia*, infection pathway, nodulation, symbiosis.

Introduction

Roots of an actinorhizal plant develop nodules after successful interaction with soil actinomycetes of the genus *Frankia*, thus becoming a N_2-fixing symbiosis. Actinorhizal plants comprise a highly diverse group of angiosperms (Huss-Danell, 1997; Benson and Clawson, 2000) and are ecologically important due to their natural input of N to the soil (Dawson, 1990; Diem and Dommergues, 1990; Hibbs and Cromack, 1990; Wheeler and Miller, 1990; Huss-Danell, 1997).

Actinorhizal plants like *Discaria trinervis* (Rhamnaceae), an intercellularly infected host genus (Valverde and Wall, 1999*a*), and *Alnus incana* (Betulaceae), an intracellularly infected host genus (Berry *et al*., 1986), control root nodule development by feedback mechanisms (Wall and Huss-Danell, 1997; Valverde and Wall, 1999*b*).

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If *Frankia* is not a limiting factor, nodule primordia development can be observed, both in *Discaria trinervis* and in *Alnus incana*, by root clarification about 1 week after inoculation, and the mature anatomy of the nodule is reached at about 2 weeks after inoculation (Valverde and Wall, 1999*a*). N_2 fixation can be detected (as acetylene reduction activity) not earlier than the third week after inoculation (Valverde *et al*., 2000; Huss-Danell, 1997, see p. 381).

A few days after inoculation, and before N_2 fixation starts, a plant autoregulatory response inhibits further nodule development. This occurs as a systemic effect and delimits the region of nodule appearance. A later phenomenon is that nodule development in the entire root system is down-regulated several weeks after inoculation, at the time when N concentration in leaves is maximal (Valverde *et al*., 2000). The excision of mature N_2-fixing nodules, together with reinoculation of the entire root system, results in the appearance of new nodules, both in *Discaria* and in *Alnus* (Valverde and Wall, 1999*b*; Wall and Huss-Danell, 1997). Most of the new nodules develop in previously non-nodulated younger parts of the roots and correspond to new infections. But, a proportion of the new nodules develop in the regions of the root where mature nodules had been excised and these new nodules likely correspond to nodule primordia or infections that had been arrested at early stages of their development (Wall and Huss-Danell, 1997; Valverde and Wall, 1999*b*), similar to what was demonstrated to occur in certain legume–*Rhizobium* symbioses (Caetano-Anollés and Gresshoff, 1991*a*). All together, these observations support the hypothesis that a second systemic signalling process is related to the plant N status (Parsons *et al*., 1993) and/or plant N/P ratio (Wall *et al*., 2000; Gentili and Huss-Danell, 2002; Huss-Danell *et al*., 2002; Valverde *et al*., 2002), which would control further nodulation.

Although mature nodules seem to be involved in the control of root susceptibility to infection by *Frankia* and nodule development, the nature of such a control is not known. Since nodules are organs developed *de novo* where N_2 fixation and N assimilation take place, it is proposed that N_2 fixation and the assimilation of fixed N are the source of such inhibitory effects on nodule formation. This hypothesis is explored in this paper where nodulated root systems of *D. trinervis* plants were incubated in an N_2-free atmosphere, suppressing N_2 fixation. The response of *D. trinervis* nodulation is compared to the effects of an N_2-free atmosphere on nodulation in *A. incana*.

Materials and methods

Discaria trinervis

Plant growth and inoculation: Seeds of *Discaria trinervis* (Hooker *et* Arnot) Reiche were surface-sterilized and germinated as previously described (Valverde and Wall, 1999*a*). Seedlings at the cotyledonary stage (12–14 d after the start of germination), were aseptically transferred to growth pouches (Mega International, Minneapolis, USA). Each pouch with four seedlings contained 12.5 ml of nutrient solution (Huss-Danell, 1978) diluted to 1/10 of full strength with 0.71 mM of N added as ammonium nitrate. During the whole experiment, pouches were kept in a greenhouse (Universidad Nacional de Quilmes, Bernal, Argentina; 34°7′ S, 58°3′ W) where mean maximum temperature was 26 °C, mean minimum temperature was 20 °C and relative humidity ranged from 65–95%. Incandescent lamps (400 W, Osram, Germany) supplemented natural light up to a photoperiod of 16 h. Light intensity on the plants, under the lamps, was 13 000 lux.

Frankia strain BCU110501, isolated from *D. trinervis* (Chaia, 1998), was used as inoculum. Bacteria were grown in static BAP minimal medium with 55 mM glucose as C source (Chaia, 1998) at 28 °C for 3 weeks. Cells were harvested and the inoculum prepared as described elsewhere (Valverde and Wall, 1999*b*). *Frankia* biomass in the homogenate was estimated by the determination of packed cell volume (Nittayajarn and Baker, 1989).

After 3 weeks of seedling growth in pouches, the position of the tap root tip (RT1) was gently marked with a permanent marker pen on the plastic surface of the pouch, and each seedling was inoculated by dripping 200 µl of inoculum containing 8 µl of packed *Frankia* cells, from the root tip to the uppermost zone of the root. Pouches were subsequently watered with N-free nutrient solution as described elsewhere (Valverde and Wall, 1999*b*). Non-inoculated plants served as controls for contamination but never formed nodules.

Incubation of nodulated root systems in an N_2–free atmosphere: Eleven weeks after first inoculation, nodulated *D. trinervis* roots were re-inoculated with 3 µl of packed *Frankia* cells. Pouches were then transferred to gas-tight acrylic containers with 200 ml of nutrient solution containing 0.071 mM of N as ammonium nitrate. Roots were in direct contact with nutrient solution through the cut bottom edge of the pouches. In addition, the lateral edges of the pouches were cut open to facilitate gas exchange to the roots. Shoots protruded through a thin slot in the container lid. The lid was sealed with a non-toxic sealing compound, and connected with PVC tubes to a source of 80% Ar, 20% O_2 and 350 ppm CO_2 (AGA, San Martín, Argentina). The gas flowed through the container by entry into the nutrient solution and exit through independent cotton plugged tubing. Control plants were subjected to a similar treatment with air. Nutrient solution was renewed each 3–4 d during the 6 weeks duration of the experiment. Infectivity of the second inoculum was tested on 3-week-old seedlings, producing normal nodulation (data not shown).

Nodulation scoring, plant biomass and leaf N content: At the beginning of gas treatments, nodule number was recorded and their position relative to RT1 was marked on all roots. Simultaneously, the new position of the growing root tips was marked as RT2. Six weeks after the start of Ar treatment, nodules were again recorded in order to look for new nodules in the whole root in relation to the different RT1 and RT2. After that, the plants were harvested. Shoots, roots and nodules were dried separately for 48 h at 55° C and biomass estimated as dry matter production (DM). Kjeldahl analysis (Jackson, 1958) was used to estimate the N content of dried leaves.

Light microscopy of nodules: Nodulated root segments of *Discaria trinervis* were randomly sampled and fixed in glutaraldehyde 2.5% (w/v) in 45 mM potassium phosphate, pH 7.2, for 30 min at reduced pressure and then for at least 3–4 h at atmospheric pressure. Fixed root pieces were prestained with 2% (w/v) OsO_4 and sequentially dehydrated in ethanol (50, 70, 80, 95, and 100% (v/v)). Dehydrated

samples were embedded in Epon-Araldite and polymerization was carried out for 3 d at 70 °C. Longitudinal sections (1–1.5 μm thickness) were mounted on glass slides, stained with methylene blue-Azur II, and examined in a Nikon EFD-3 light microscope.

Alnus incana

Plant growth and inoculation: Seeds of grey alder, *Alnus incana* (L.) Moench, were surface-sterilized, germinated, and grown in pouches as previously described (Wall and Huss-Danell, 1997). Growth was conducted in a growth chamber with 16 h light at 25 °C, 8 h darkness at 15 °C, and a relative humidity of about 75% (Umeå University, Umeå, Sweden). The photosynthetic photon flux was 150–200 μmol m^{-2} s^{-1} provided by Osram (Berlin, Germany) Power Star HQJ-T 400 W lamps.

A crushed nodule from the so-called 'local source of *Frankia*' (Huss-Danell, 1991) was used as inoculum. Plants were first inoculated 4 weeks after transfer to pouches by adding 100 μl per plant of a suspension containing an amount of nodule tissue equivalent to 250 μg (Wall and Huss-Danell, 1997). The inoculation and recording of the position of RT at the time of first inoculation (RT1), on the main root as well as any lateral roots, was done as described for *D. trinervis.* Non-inoculated plants received only sterile water as 'inoculum', and these plants never formed nodules.

Incubation of nodulated plants in an N_2-free atmosphere, nodulation scoring and plant biomass: Nine weeks after first inoculation, just before gas treatment, nodule number was recorded and their position relative to RT1 was marked on all roots, and plants were inoculated again. At the same time, a group of young (3 weeks growth in pouches) non-nodulated seedlings were inoculated to control infectivity of the second inoculum. Simultaneously, the new position of the growing root tips of all plants was marked as RT2. Immediately after the inoculation, both groups of plants were cultivated either under a gas mixture with 79% Ar, 21% O_2 and 350 ppm CO_2 (AGA, Sweden) or under air as a control. In this experiment the whole plants were enclosed in the gas-tight acrylic containers with 200 ml of nutrient solution containing 0.071 mM of N as ammonium nitrate. Gas flow and the watering of plants was similar to that in the *D. trinervis* experiment describe above. At the end of Ar treatment, nodules were recorded in plants of all treatments. Each nodule could be distinguished in relation to the position of each RT (RT1 or RT2). In the case of control plants, which only received an inoculation with the second *Frankia* inoculum, nodules were recorded in relation to the only root tip mark labelled as RT2.

After re-establishing a normal atmosphere, plants were grown for five more weeks before harvest and then plants were dried separately for 72 h at 60 °C and biomass estimated as dry matter production (DM).

Statistical analysis

Significant differences between treatment means were tested with Number Cruncher Statistical System v5.7 (NCSS Statistical Software, Kaysville, Utah, USA). A significance level of P=0.01 was used throughout the work, unless stated otherwise in the text.

Results

Response of nodulated Discaria trinervis *to the absence of N_2*

Incubation of nodulated plants in an N_2-free atmosphere (Ar) was expected to inhibit N_2 fixation completely, due to the lack of substrate. Argon-treated plants showed a reduced biomass (Fig. 1). Roots grew relatively more than shoots in Ar-treated plants compared with air-treated plants (Fig. 1). Leaf N concentration was significantly lower in Ar-treated than in air-treated plants, probably because internal N was not sufficient to support growth in the absence of N_2 fixation (Fig. 1).

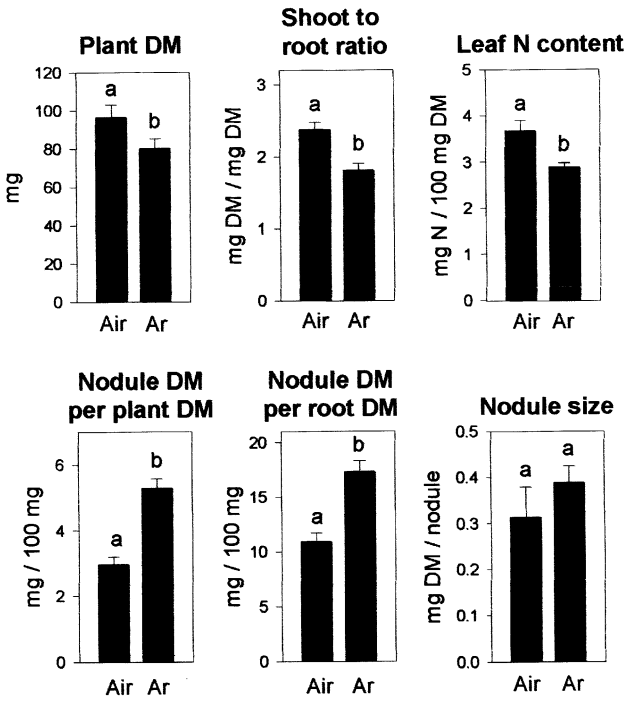

Fig. 1. Biomass, nodulation and leaf N content of symbiotic *Discaria trinervis* under a N_2-free atmosphere. Nodulated root systems were reinoculated with *Frankia* and immediately incubated in an $Ar:O_2:CO_2$ atmosphere for 6 weeks (see Materials and methods). Mean ±SE for n=22–25 plants per treatment. Different letters above each bar denotes that the response was statistically different at the P <0.01 level.

There was almost no new nodules due to re-inoculation of nodulated plants, neither in Ar-treated nor in air-treated plants (Table 1). There was no new nodules at RT2 at all, and nodulation was not increased in the region of pre-existing nodules related to RT1 as recorded before treatment (Table 1).

Nodule biomass was enhanced in Ar-treated plants primarily through the growth of pre-existing nodules (Figs 1, 2). The average of absolute nodule biomass increased with about 25% comparing Ar- versus Air-treated plants (0.39 mg $nodule^{-1}$ and 0.32 mg $nodule^{-1}$, respectively). The effect of Ar became significant when nodule biomass was expressed in relation to plant and root biomass, raising the difference to about 80% and 60%, respectively, compared to air-treated plants (Fig. 1).

The increment in nodule biomass, in the absence of N_2 fixation, was associated with a stimulation of the apical meristem activity which resulted in multilobed nodules in Ar-treated plants (Fig. 2). The transition zone between the

Table 1. *Nodulation and nodule distribution on roots of* Discaria trinervis *before and after a 6-week treatment under an N_2-free atmosphere (Ar)*

All nodules are related to RT1. No nodule at all appeared at RT2 after treatment (see Materials and methods). There was no statistical difference between treatment means (P >0.01) for each nodule score. Mean ±SE.

Nodulated roots grown under		Air ($+N_2$)	Ar ($-N_2$)
Number of nodules before treatment	In tap root	11.4±0.9	10.6±0.9
	In lateral roots	0.3±0.6	1.3±0.5
	Total	11.7±1.1	11.9±1.4
Nodule number after treatment	In tap root	11.7±0.9	10.1±0.8
	In lateral roots	0.7±0.7	1.6±0.6
	Total	12.4±1.0	11.7±1.0
Number of plants		22	25

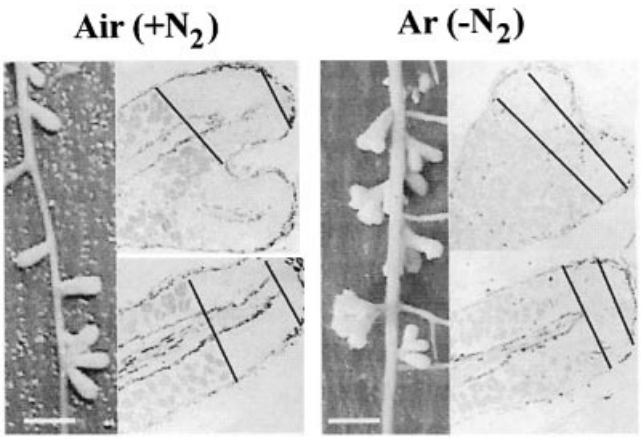

Fig. 2. Stimulation of nodule growth in *Discaria trinervis* plants under a N_2-free atmosphere. Most of the root nodules of plants with roots grown in air ($+N_2$) were single-lobed, whereas roots grown in an $Ar:O_2:CO_2$ atmosphere ($-N_2$) showed multilobed nodules. White line=5 mm. In root nodules grown in an N_2-free atmosphere the transition zone between the nodule apical meristem and the region of cortical cells bearing vesicles of *Frankia* (delimited by solid lines) was shortened, when compared with the corresponding region in air-treated nodules.

meristem and the vesicle containing cortical cells, was shorter in nodules from Ar-treated plants than in air-treated plants (Fig. 2), as if the rate of cortical cell differentiation and subsequent infection by intercellular *Frankia* and vesicle development were stimulated in the absence of N_2 fixation.

Response of nodulated Alnus incana *to the absence of N_2*

Younger Ar-treated plants that were inoculated only once, with their root tips labelled as RT2 (Fig. 3, left), developed a similar number of nodules as air-treated plants, indicating that there were no differences in *Frankia* infectivity and nodule development between treatments. However, plants nodulated at RT1, that had been re-inoculated at RT2 just before being cultivated under different atmospheres, showed a different nodulation pattern at the end of the treatment (Fig. 3, right). Ar-treated plants developed significantly (P <0.02) more nodules in the region of RT2 than did air-treated plants. A lack of N_2 fixation allowed about nine times as many new nodules to develop at RT2. Five weeks after the end of the Ar treatment, plant biomass was reduced in the older group of plants where N_2 fixation had been prevented (Fig. 3, right) but not in the group of younger plants (Fig. 3, left).

Discussion

There is a consistent bulk of evidence supporting the idea that root nodule symbioses between plants and N_2-fixing bacteria regulate the number and biomass of nodules through feedback mechanisms (Caetano-Anollés and Gresshoff, 1991*a*; Wall, 2000). In some cases mature nodules are involved in keeping the root system under a non-susceptible status for infection and nodulation since the removal of mature nodules releases the feedback inhibition of nodule development, allowing new nodules to develop in the growing region of the root (Caetano-Anollés *et al*., 1991; Caetano-Anollés and Gresshoff, 1991*b*; Wall and Huss-Danell, 1997; Valverde and Wall, 1999*b*). We were interested in the role of N_2 fixation in such inhibition of nodule formation in actinorhizal plants.

Different developmental strategies were observed between *D. trinervis,* an intercellularly infected actinorhizal plant (Valverde and Wall, 1999*a*), and *A. incana*, an intracellularly, root hair-infected actinorhizal plant (Berry and Sunell, 1990). *Discaria trinervis* increased nodule biomass mainly due to an increment in nodule growth rate without the significant development of new nodules. In the nodules, the infection zone was expanded towards the nodule apex (Fig. 2). By contrast, *A. incana* formed new nodules both in root regions with and without pre-existing nodules. It is concluded that an inhibitory process was released because of the Ar treatment and it is proposed that the absence of N_2 fixation caused a reduction in the level of a previously proposed N-related signal that is phloem derived to nodules (Parsons *et al*., 1993), and probably modulated by P (Wall *et al*., 2000; Valverde *et al*., 2002).

That removal of mature and N_2-fixing nodules results in the release of the feedback inhibition of nodulation in *D. trinervis* has previously been described (Valverde and Wall, 1999*b*). However, the inhibition of N_2 fixation in *D. trinervis* in the present work did not alleviate the inhibition of infection and new nodule formation, but did stimulate nodule growth (Figs 1, 2). Although nodulation is inhibited when leaf N content is >3.5 mg N per 100 mg leaf DM in *D. trinervis* (Valverde *et al*., 2000) plant N content seems not to be the only factor that controls nodulation in mature *D. trinervis* plants. A sudden stop of N_2 fixation, because of the lack of substrate, which should reduce leaf N content, did not release the inhibition of the

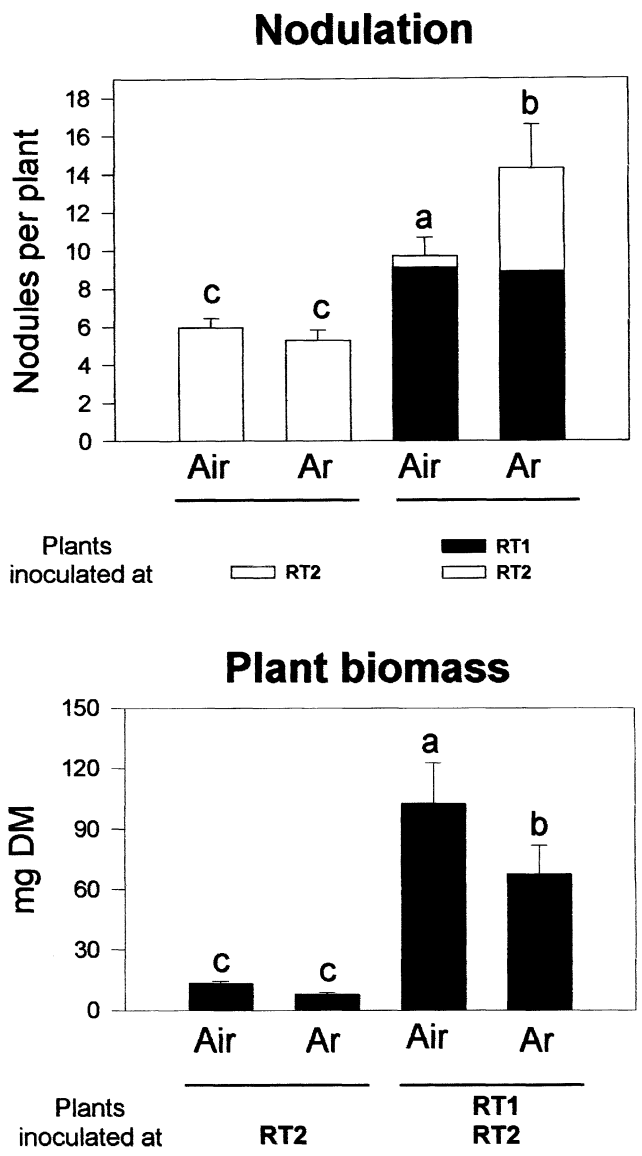

Fig. 3. Nodulation and biomass of symbiotic *Alnus incana* under a N_2-free atmosphere. Control young 3-week-old seedlings were inoculated for the first time with *Frankia* at the same time as pre-nodulated plants, previously inoculated 9 weeks before, were inoculated for a second time with the same *Frankia* as used for the control young plants. Immediately, all plants grew under Ar or air atmosphere for the following 3 weeks (see Materials and methods). At the end of Ar treatment nodulation was recorded related to root tip positions at the time of each inoculation: RT1 refers to the root tip position at the earlier inoculation of pre-nodulated plants, RT2 refers to the root tip position at the later inoculation, (i.e. the only one for control young seedlings or the second one for the pre-nodulated plants). Mean ±SE for n=18–25 plants per treatment. Different letters above each bar denotes that the response was statistically different at the P <0.01 level.

development of new nodules. This suggests that an inhibitory process for infection is still present in inactive (non N_2-fixing) nodules. The persistence of *Frankia* filaments in the intercellular spaces between cortical cells of the post-meristematic region has previously been observed (see Fig. 3 in Valverde and Wall, 1999*a*). The induction of a rapid feedback regulation of infection in *D. trinervis* roots was found to be temporarily associated with the initiation of nodule primordia and the progress of intercellular *Frankia* filaments across the root cortex (Valverde and Wall, 1999*b*). We think that the presence of intercellular *Frankia* in the apex of mature *D. trinervis* nodules can induce a host-derived signal that is responsible for the activation of an autoregulation-like response. This response would sustain the loss of susceptibility for infection of the growing root tip and the blockage of primordia development in nodulated zones of the root system.

When turning to *A. incana* plants, it seems likely that the first signal, which would be related to the induction of the autoregulatory response in *A. incana* seedlings, is no longer present in mature nodules. For that reason, in a situation of suppressed N_2 fixation, *A. incana* nodules would lack not only the signal related to N status of the plant, but also the alternative or additional N-independent mechanism to sustain inhibition of further infection. This hypothesis would explain the appearance of new nodules in Ar-treated alders.

In conclusion, taking into account the observations on *D. trinervis* and *A. incana* plants when either nodules are removed (Wall and Huss-Danell, 1997; Valverde and Wall, 1999*b*) or when only N_2 fixation is impaired (this paper), a difference in the mechanisms that control the symbiotic tissue formation in these hosts is evident. In this work it was not possible to use the same *Frankia* for both plants because of host specificity. Still, these findings add to the list of other host-determined but *Frankia*-independent characters of actinorhizal nodules, such as nodule morphology, nodule anatomy and morphology of symbiotic *Frankia* (Huss-Danell, 1997).

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