Local and systemic effects of phosphorus and nitrogen on nodulation and nodule function in *Alnus incana*

Francesco Gentili* and Kerstin Huss-Danell

Department of Agricultural Research for Northern Sweden, Crop Science Section, Swedish University of Agricultural Sciences, Box 4097, S-904 03 Umeå, Sweden

Received 14 May 2003; Accepted 22 September 2003

Abstract

Phosphorus (P) and nitrogen (N) effects on nodulation, nitrogenase activity and plant growth were studied in the root-hair-infected actinorhizal plant *Alnus incana* (L.) Moench. A split-root experiment, as well as a short-term experiment with entire root systems and a broader range of P concentrations, showed that P effects were specific on nodulation and not a general stimulation via a plant growth effect. These results indicate that nodule initiation and nodule growth have a high P demand. The split-root assay, comprising seven combinations of two N and two P levels, showed that P could counteract systemic N inhibition of nodulation, but did not counteract N inhibition of nitrogenase activity.

Key words: Actinohiza, *Alnus incana*, *Frankia*, nitrogen, nitrogen fixation, nodulation, phosphorus, split-root.

Introduction

*Frankia* can infect actinorhizal plants in two different ways: root hair (intracellular) infection or intercellular penetration (Berry and Sunell, 1990; Franche et al., 1998). The two infection pathways, which are determined by the host, are different in several respects (Huss-Danell, 1997). The root-hair-infection pathway is characterized by the following steps: root hair deformation and intracellular infection by *Frankia*; cell divisions in the cortex result in a so-called prenodule; cell divisions in the pericycle are the origin of a nodule primordium that will grow and develop into the true nodule (Berry and Sunell, 1990; Akkermans and Hirsch, 1997). *Alnus* represents plants with a root-hair-infection pathway. In the intercellular-infection pathway, root-hair deformation and cell divisions in the cortex (prenodule) are missing, but the true nodule will develop from the pericycle (Berry and Sunell, 1990) as in *Hippophaë rhamnoides*. Furthermore *Frankia* grows inside the cell (intracellularly) in *Alnus* while it grows intercellularly in *Hippophaë* during the entire infection process.

It has been shown that P (phosphorus) has stimulating effects on nodulation, N₂-fixation and plant growth in actinorhizal plants (Ekblad and Huss-Danell, 1995; Yang, 1995; Wall et al., 2000; Gentili and Huss-Danell, 2002; Valverde et al., 2002) as well as in legumes (Gates, 1974; Gates and Wilson, 1974; Jakobsen, 1985; Israel, 1987; Stamford et al., 1997; Hellsten and Huss-Danell, 2000). Also, mycorrhizal fungi stimulated nodulation and nitrogen fixation in actinorhizal plants (Isopi et al., 1994; Tian et al., 2002). In both actinorhizal plants and legumes, P stimulation of nodulation and nitrogenase activity has been ascribed to a general stimulation via plant growth (Robson et al., 1981; Jakobsen, 1985; Yang, 1995; Reddell et al., 1997). On the other hand, P stimulation has been reported to be specific on nodulation (Israel, 1987; Hellsten and Huss-Danell, 2000; Gentili and Huss-Danell, 2002; Valverde et al., 2002). In the intercellularly infected *H. rhamnoides* P had a systemic effect on nodulation but N₂ fixation was not studied (Gentili and Huss-Danell, 2002). There is, however, no corresponding information on any specific or systemic P effect on nodulation and N₂ fixation in root hair-infected actinorhizal plants.

N (nitrogen) inhibits nodulation and N₂-fixation in actinorhizal plants as summarized in Huss-Danell (1997). However, the N inhibition depends on other nutrients. When several N and P levels were combined in a study of...
A. incana, ammonium nitrate inhibited nodulation at N/P ratios >7, but not at N/P ratios ≤7 (Wall et al., 2000). Split-root experiments have been used to distinguish between local and systemic nitrate effects, but have given mixed results. In A. glutinosa grown in liquid culture, nitrate mainly caused a local, but also a systemic, inhibition of the number of nodules per plant (Pizelle, 1965, 1966). In Casuarina cunninghamiana, another root hair-infected actinorhizal plant, nitrate in water culture gave a local inhibition of the number of nodules and nodule biomass per plant while nodule biomass related to plant growth and nitrogenase activity were inhibited systematically (Arnone et al., 1994). In the intercellularly infected H. rhamnoides grown in a split-root system, ammonium nitrate inhibited both number of nodules and nodule dry weight systematically (Gentili and Huss-Danell, 2002).

Actinorhizal plants representing the two different infection pathways regulate nodulation in different ways (Wall and Huss-Danell, 1997; Valverde and Wall, 1999; Wall, 2000; Wall et al., 2003). N and P effects have previously been studied in the intercellularly infected H. rhamnoides, where N had a systemic inhibition and P had a systemic stimulation of nodulation. The P stimulation was specific to nodulation and not simply mediated via plant growth (Gentili and Huss-Danell, 2002). The present work focused on Alnus incana to obtain the corresponding information on N and P effects on a root hair-infected actinorhizal plant.

The aims of this work were therefore (a) to study the effects of P and N and their interaction on nodulation in the root hair-infected actinorhizal plant A. incana; (b) to distinguish between local and systemic effects of the two macronutrients on nodulation and N2-fixation, and (c) to distinguish between specific effects on nodulation and general effects via plant growth. A split-root experiment was designed to identify local and systemic effects on nodulation and nodule function while a second experiment was designed to identify local and systemic effects of the two

table 1. N and P concentrations used in the nutrient solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>N (mM)</th>
<th>P (mM)</th>
<th>N:P ratio</th>
<th>N:P mass ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>0.71</td>
<td>0.1</td>
<td>7.1</td>
<td>3.21</td>
</tr>
<tr>
<td>hNP</td>
<td>6.45</td>
<td>0.091</td>
<td>71</td>
<td>32.06</td>
</tr>
<tr>
<td>NhP</td>
<td>0.71</td>
<td>1</td>
<td>0.71</td>
<td>0.32</td>
</tr>
<tr>
<td>hNhP</td>
<td>6.45</td>
<td>0.91</td>
<td>7.1</td>
<td>3.21</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NvP</td>
<td>0.71</td>
<td>0.01</td>
<td>71</td>
<td>32.06</td>
</tr>
<tr>
<td>NilP</td>
<td>0.71</td>
<td>0.03</td>
<td>23.7</td>
<td>10.70</td>
</tr>
<tr>
<td>NP</td>
<td>0.71</td>
<td>0.1</td>
<td>7.1</td>
<td>3.21</td>
</tr>
<tr>
<td>NhNhP</td>
<td>0.71</td>
<td>0.3</td>
<td>2.37</td>
<td>1.07</td>
</tr>
<tr>
<td>hNhNhP</td>
<td>6.45</td>
<td>0.273</td>
<td>23.7</td>
<td>10.70</td>
</tr>
</tbody>
</table>

and shaven for 15 min in hydrogen peroxide (30% v/v) followed by rinses in sterile water. The seeds were sown in sterile Petri dishes containing perlite and moistened with modified Evans solution (Huss-Danell, 1978) diluted to 1/10 of full strength and with 0.71 mM of N added as ammonium nitrate, hereafter referred to as NP solution. Seedlings grew for approximately 3 weeks on the perlite before transfer to 6 x 6 cm pots with sterile perlite. For the next 5 weeks the N concentration of the nutrient solution was raised from 0.71 mM to 3.5 mM to support the growth of the seedlings. The seedlings were then transplanted into split-root pots taking care to divide the root system into two parts as equally as possible. Split-pots were made by taping together two 6 x 6 cm pots. A slot (c. 1 cm) was made in the middle wall to support the plant at the point where the roots were divided. The surface of the perlite was a few mm below the slot. About 1 week later the N concentration of the nutrient solution was returned to 0.71 mM to avoid nitrogen accumulation in plant tissues.

Seedlings were inoculated 3 weeks after transfer to split pots (11-week-old plants) with the ‘local source of Frankia’ (Huss-Danell, 1991). A crushed nodule suspension (Huss-Danell, 1991) corresponding to 250 μg fresh weight of nodules was given to each side of the root system. During the whole growth period (mid-December to mid-May) the plants grew in a greenhouse in Umeå, Sweden (63°45' N) and had 17 h of supplemental light at about 25 °C and 7 h of darkness at about 15 °C. Philips HPI/T 400 W lamps were used. Relative humidity was about 40%.

Experiment 1: P and N treatments

Treatments with different P and N concentrations began immediately after inoculation. N was added as different concentrations of ammonium nitrate and P as K-phosphate to the modified Evans solution (Huss-Danell, 1978) at 1/10 of full strength (Table 1). The N:P ratio in the solutions varied from 0.71 to 7.1, which is a normal value in plants (Marschner, 1995), and up to 71 (Table 1). Plants were watered 3–4 times a week with a large volume such that solution drained through all pots. Pots were suspended in a large mesh-size net such that solutions did not move from one side to the other in a split-pot. The pH was monitored in the added solution as well as in the solution draining through the pots. No significant change in pH was noticed. Moreover, the electrical conductivity of the draining solution was measured without a sign of salt accumulation. At the start of the experiment plants had similar shoot height

Materials and methods

Experiment 1: Plant material, split-root design, growth conditions and inoculation

Seeds from a clone of Alnus incana (L.) Moench (Huss-Danell, 1991) were shaken in water with a few drops of detergent, rinsed,
in all treatments. Plants were grown for 10 weeks after inoculation. Each treatment had 7–10 replicate plants.

**Experiment 1: Nitrogenase activity**

Two weeks before harvest ARA (acetylene reducing activity) was measured in three randomly chosen plants in each treatment. Cuvettes were built to cause minimal disturbance to the plants. Each pot containing half a root system was enclosed in a Magenta jar (Teron GmbH, Heidelberg Germany) was used around the stem base. The gas chromatography was as previously described (Valerde et al., 2000).

**Experiment 1: Measurements**

Number of nodules, DW (dry weight) (60 °C for 24 h) of leaves, stem, roots, and nodules were determined at harvest (21-week-old plants). Each half of the root system was measured separately. Any multilobed nodules were counted as one nodule. N and P concentration in leaves and roots were analysed in the plants used for ARA measurements. Nodules from all plants belonging to the same treatment were pooled for N and P analysis. Dried, ball-milled (Retsch, MM2000, Haan Germany) samples were used for the N and P analysis. The N content was analysed in a CN-analysers (Europa Scientific) at the Department of Forest Ecology, Swedish University of Agricultural Sciences (SLU), Umeå, Sweden. Samples were digested in HNO3 and HClO4 at 130 °C of Agricultural Sciences (SLU), Umeå, Sweden.

**Experiment 2: Plant material, growth conditions and inoculation**

Seeds and surface-sterilization were as in experiment 1. The seeds were sown in sterile Magenta jars containing sterile glass beads moistened with NP solution. Seedlings grew for 7 weeks in the jars before transfer to clean 6×6 cm pots with sterile perlite. One week later the N concentration of the nutrient solution was raised from 0.71 mM to 3.5 mM to support growth of the seedlings. After 4 weeks the seedlings were transferred to clean 8×8 cm pots with perlite and the different P and N solutions (see below) were applied. Seedlings were inoculated as in experiment 1 when 14-week-old, 2 weeks after N and P treatment began. The greenhouse conditions were as in experiment 1, but plants grew from April to August.

**Experiment 2: P and N treatments**

Treatments with different P and N concentrations (Table 1) began 2 weeks before inoculation. The range of P concentrations was broader than in experiment 1, from 0.01 to 0.3 mM. The pH was adjusted to 6.8 at solution preparation. Plants were grown for 4 weeks after inoculation. The time chosen was short in order to identify specific P effects on nodulation that in a long-term experiment could be masked by general effects on plant growth. Each treatment had 9–10 replicate plants.

**Experiment 2: Measurements**

The number of nodules and DW (60 °C for 24 h) of leaves, stem, roots, and nodules were determined at harvest. Any multilobed nodules were counted as one nodule.

**Statistical analyses**

In experiment 1 the paired t-test was used to identify statistically significant differences between the two sides of root systems each receiving different nutrient solutions. The t-test was used to identify statistically significant differences between the control plants (NP NP) and all other treatments. As described in the text, but not shown in the figure, the t-test was used to compare plants receiving both high N and P in one root side (NP hNhP) with plants receiving only high N in one root side (NP hNP). In experiment 2 the t-test was used to identify statistically significant differences between all treatments. Statistical calculations were performed with Minitab software (Minitab Inc., State College Pennsylvania, PA, USA, 2000). P ≤0.05 was used as the significance level.

**Results**

**Experiment 1: Nodulation**

Plants given a high P level to one side (NP NhP) or both sides (NhP NhP) produced almost the same number of nodules (Fig. 1a) as the control (NP NP) plants. The nodules were spread all over the root systems. High N level given to one side of the root system (NP hNP) produced a large decrease in the number of nodules on both sides of the root system (Fig. 1a). When the N level was high on both sides (hNP hNP) the number of nodules was decreased further to only one-third of the number of nodules in controls (NP NP). When both N and P were high on one side (NP hNhP) the number of nodules was reduced on that side (NhnP) to about half the number in the control. The other root side, kept at the control level (NP), was barely affected. Thus, within these plants, there was a difference between the two sides of the root system with hN acting locally and hP acting systemically (Fig. 1a). When both N and P were high on both sides (hNhP hNhP), the number of nodules was about half the number in controls.

High P decreased nodule DW, whether P was high on one or both sides of the root system (Fig. 1b). N affected nodule DW in the same direction as number of nodules, but the effects on nodule DW were more pronounced (Fig. 1b). When N was raised on one side (NP hNP), it produced a strong inhibition of nodule DW on both root sides. But, a statistically significant difference was noticed between the two root sides too, indicating that N inhibition was both local and systemic. When both N and P were high on one side (NP NhP) the response was very similar to the response of number of nodules, that is, a reduction only on the side receiving high N and high P. Comparing this root side (hNhP) with the side receiving only high N (hNP) of the treatment NP hNP, the increase of P could reduce the N inhibition on the side receiving high N (hNhP; P <0.05, not labelled on the bars in Fig. 1b). High N and high P on both sides of the root system (hNhP hNhP) produced a strong decrease in nodule biomass, which was 10-fold less than in the control but similar to hNhP hNhP (Fig. 1b).

**Experiment 1: Growth**

The effects of P and N on the DW production of leaves, roots and total plants are shown in Fig. 2a–c. High P on one side decreased plant DW, especially root DW. The high N effect on root DW was only significant for the hNhP hNhP.
Fig. 1. Number of nodules and nodule dry weight in *A. incana* grown with split-root systems (experiment 1) receiving different levels of N and P. The prefix ‘*h*’ stands for the presence of a nutrient at its high concentration. Mean ± SE for 7–10 plants grown for 10 weeks after inoculation. Plants receiving the same N and P levels in both sides of the root system are represented by the mean value of the two sides. (a) Number of nodules per root side; (b) nodule dry weight per root side; (c) number of nodules per root dry weight; (d) nodule dry weight per root dry weight; (e) number of nodules per plant dry weight; (f) nodule dry weight per plant dry weight. The letter *a* on the top of a column stands for no difference, *b* for statistically significant inhibition and *c* for statistically significant stimulation when compared with the control (NP NP), while *s* shows statistically significant difference between the two root sides receiving different nutrient solutions.
treatment. Leaf DW was not influenced. However, some plants in the treatment receiving high N on both root sides (hNP hNP) had lost some leaves before harvest. High P or both high N and high P on both sides of the root system are represented by the mean value of the two sides. These plants showed necrotic leaf margins indicating that the P supply was toxic, as confirmed by P concentration in leaves (see below). On the other hand, when both N and P were high on one side (NP hNhP) there was a high DW in all plant parts.

**Experiment 1: Nodulation in relation to plant growth**

To separate specific effects on nodulation from general effects on growth, the number of nodules and nodule DW were related to root DW (Fig. 1c, d) and plant DW (Fig. 1e, f). High P strongly stimulated the number of nodules per root DW when high P was given to one or both root sides (NP NhP, NhP NhP; Fig. 1c). That high P given to one side stimulated the number of nodules on the other side of the root system shows a systemic P effect (Fig. 1c). When P was increased on both sides of the root system the number of nodules per root DW was 2.5 times as high as in the controls.

The effects of N followed the same trend as previously seen for the number of nodules and nodule DW per root side (Fig. 1a, b). High P had only slight effects on nodule DW per root DW (Fig. 1d). When both N and P were raised on one or both sides their effects (Fig. 1c, d) followed the same trend as previously seen for the number of nodules and DW per root side (Fig. 1a, b). Thus, high P counteracted a systemic inhibition, and could partially counteract N inhibition on the side receiving high N and high P (hNhP), as seen when comparing the hNhP root side.
of the NP hNhP treatment with the side receiving only high N (hNP) of the treatment NP hNP (P < 0.05, not labelled on bars in Fig. 1d).

The number of nodules per plant DW (Fig. 1e, f) follow the same trend as described for nodulation per root DW (Fig. 1c, d).

**Experiment 1: P and N concentration in leaves, root and nodules**

At control P levels in the nutrient solution the P concentration in leaves and roots was fairly similar, about 0.08% of DW, irrespective of N treatments (Table 2). When high P was given to one or both sides of the root system the P concentration in plants increased, especially in leaves. The leaf P concentration was highest when high P or both high N and high P were given to both sides of the root system (NhP NhP, hNhP hNhP). The P concentration in the leaves of these plants exceeded 1%, a value that is usually considered toxic to plant growth (Marschner, 1995; Jones, 1998). The nodule P concentration was fairly constant among treatments except for plants receiving high N (NP hNP, hNP hNP) which had about half the P concentration of the other treatments.

The N concentration (Table 2) in leaves ranged from 2.03% to 2.85% of DW, except for plants given high P and high N on both root sides where leaf N concentration was much higher, almost 4% of DW. In roots the N concentration ranged from 1.36% to 2.45% of DW with the highest values in plants given high N or both high N and high P on both root sides. The N concentration in nodules was always higher than in roots and leaves, except for leaves in plants receiving high N on both root sides (hNP hNP, hNhP hNhP). The highest N concentration in nodules was in plants receiving high P on one root side (NP NhP).

**Experiment 1: Nitrogenase activity**

ARA was measured only on a limited number of plants. When P was increased in one root side (NP NhP), the side receiving high P had the highest activity (Fig. 3a). However, ARA was similar when expressed per nodule DW (Fig. 3b). High P to both root sides slightly inhibited nitrogenase activity (Fig. 3a, b). High N inhibited nitrogenase activity whether expressed per root side or per nodule DW (Fig. 3a, b). In plants receiving high N on one root side (NP hNP), ARA per root side was inhibited locally (Fig. 3a) while ARA per nodule DW was inhibited systemically (Fig. 3b). P could not counteract N inhibition when the two nutrients were supplied at high level (NP hNhP, hNhP hNhP; Fig. 3a, b). N inhibition was almost complete when high N was given to both root sides, irrespective of P level.

**Experiment 2**

Increasing P up to the control level (NP) clearly increased both the number of nodules and nodule DW per plant by about three times (Fig. 4a, b). The P stimulation on nodule DW was statistically more pronounced than on the number of nodules. Increased N at moderately high P level (hNmhP) drastically decreased both the number of nodules and nodule DW (Fig. 4a, b).

When P was supplied at a very low level, leaf DW (Fig. 5a) and plant DW (Fig. 5c) were impaired. Otherwise, different P levels did not influence leaf and plant DW. Root DW did not vary among P treatments.

### Table 2. Concentration of N and P in leaves, roots and nodules on each root side in A. incana grown with split-root systems receiving different levels of N and P (experiment 1)

The prefix ‘h’ stands for the presence of a nutrient at its high concentration. Values for leaves and roots are mean ± SE of three plants. Values for nodules are from a pooled sample of all 7–10 plants per treatment. Plants were grown for 10 weeks after inoculation. Concentrations are per cent of DW.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Side of split-root system</th>
<th>Leaf N (%)</th>
<th>Root N (%)</th>
<th>Nodule N (%)</th>
<th>Leaf P (%)</th>
<th>Root P (%)</th>
<th>Nodule P (%)</th>
<th>N:P Leaf (%)</th>
<th>N:P Nodule (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP NP</td>
<td>NP</td>
<td>2.03±0.15</td>
<td>1.36±0.11</td>
<td>2.47</td>
<td>0.08±0.001</td>
<td>0.08±0.003</td>
<td>0.20</td>
<td>25.1</td>
<td>12.6</td>
</tr>
<tr>
<td>NP hNP</td>
<td>NP</td>
<td>2.15±0.08</td>
<td>1.54±0.01</td>
<td>2.61</td>
<td>0.08±0.004</td>
<td>0.08±0.003</td>
<td>0.12</td>
<td>21.6</td>
<td>11.8</td>
</tr>
<tr>
<td>hNP hNP</td>
<td>hNP</td>
<td>2.85±0.18</td>
<td>1.67±0.03</td>
<td>2.29</td>
<td>0.08±0.01</td>
<td>0.08±0.005</td>
<td>0.10</td>
<td>34.6</td>
<td>21.4</td>
</tr>
<tr>
<td>NP NhP</td>
<td>hNP</td>
<td>2.57±0.07</td>
<td>1.98±0.05</td>
<td>2.32</td>
<td>0.60±0.12</td>
<td>0.08±0.01</td>
<td>0.11</td>
<td>4.6</td>
<td>2.6</td>
</tr>
<tr>
<td>NhP NhP</td>
<td>hNhP</td>
<td>2.14±0.19</td>
<td>1.72±0.10</td>
<td>2.47</td>
<td>1.29±0.04</td>
<td>0.39±0.01</td>
<td>0.29</td>
<td>17</td>
<td>11.5</td>
</tr>
<tr>
<td>NP hNhP</td>
<td>NhP</td>
<td>2.56±0.05</td>
<td>1.50±0.05</td>
<td>2.52</td>
<td>0.58±0.08</td>
<td>0.18±0.02</td>
<td>0.19</td>
<td>1.7</td>
<td>16.5</td>
</tr>
<tr>
<td>hNhP hNhP</td>
<td>hNhP</td>
<td>3.94±0.04</td>
<td>2.45±0.04</td>
<td>2.50</td>
<td>1.55±0.19</td>
<td>0.32±0.02</td>
<td>0.19</td>
<td>2.6</td>
<td>13</td>
</tr>
</tbody>
</table>
and was related to growth of the plants. A P stimulation on nodulation was evident in both experiments. However, in experiment 1, the highest level of P impaired plant growth and nodule growth when given to both root sides (NhP NhP, Figs 1b, 2a–c). P concentration was very high in leaf tissues, but lower in roots and in nodules (Table 2). This indicates that nodules regulate P acquisition independently from other plant parts. P effects on nodule initiation were independent of plant growth as seen from number of nodules related to root DW or plant DW (Fig. 1c, e). It was concluded that P acts specifically on nodule initiation. This is in accordance with an early single observation that P was needed for nodulation during the first week after inoculation of *A. glutinosa* (Quispel, 1958).

In experiment 2 the number of nodules per plant as well as nodule DW per plant were stimulated by increased P. By contrast, root DW was barely affected by P. Again, the observed P effects on nodulation were thus specific for nodules and not a general below-ground growth effect. In the legume *Trifolium pratense* P specifically stimulated nodulation (Hellsten and Huss-Danell, 2000). In soybean, P was suggested to have a specific effect on the early stage(s) of nodule development (Israel, 1987). Although these data do not permit any conclusion about mechanisms involved, a pattern is emerging from actinorhizal symbioses where *Frankia* has different infection pathways and from legumes that P is essential and is stimulating nodulation.

The time span of an experiment is important to ascribing P effects specifically to nodulation or to general plant growth. Consequently, a short-term experiment such as experiment 2 allows the first response of P stimulation, which was on nodulation, to be identified, while an experiment as described by Reddell *et al.* (1997), where the plants were harvested 20 weeks after inoculation, showed P effects to be largely mediated via plant growth.

By contrast to what was found for *Casuarina cunninghamiana* (Arnone *et al.*, 1994), a high N level at a control level of P (NP hNP) gave a systemic inhibition of the number of nodules. N inhibition on nodule biomass was systemic and was particularly strong on the side receiving high N (hNP; Fig. 1a–d). This suggests that internal N inhibited nodule initiation and nodule growth, which is in agreement with results from the intercellularly infected *H. rhamnoides* (Gentili and Huss-Danell, 2002). Also in *A. glutinosa* it has been hypothesized that nodule growth is sensitive to the N status of the plant and that the level of amino acids returning to the nodules may feedback regulate nodule growth and activity (Baker *et al.*, 1997).

High P could counteract systemic N inhibition of the number of nodules and nodule biomass (Fig. 1a–d). P could even reduce the N inhibition of nodule biomass in the side receiving hNhP in the treatment NP hNhP when compared to the side receiving hNP in the treatment NP hNP (Fig. 1b, d). This partial counteraction of local N
inhibition was not seen in *H. rhamnoides* (Gentili and Huss-Danell, 2002). However, high P could not alleviate N inhibition when both nutrients were high in both root sides (hNhP hNhP; Fig. 1a–d).

The two actinorhizal plants, having different infection pathways, *A. incana* and *H. rhamnoides*, did respond differently to a high P level. That a high P level did not impair plant growth in *H. rhamnoides* (Gentili and
Huss-Danell, 2002) but did so in *A. incana* indicates that *H. rhamnoides* has a higher need for P, or a greater tolerance for high P levels. By contrast, *H. rhamnoides* was more sensitive than *A. incana* to high N when given to both root sides. This treatment completely inhibited nodulation in *H. rhamnoides* (experiment 1 in Gentili and Huss-Danell, 2002).

The method developed for nitrogenase activity measurements was successful and allowed ARA to be measured separately in the two root sides without disturbing the plants. Nitrogenase activity was measured only once and on a relatively small number of plants; however, it allowed a distinction to be made between local and systemic effects of N and P on nitrogenase activity. The high N level inhibited nitrogenase activity per nodule DW (Fig. 3b) systemically, as for *Casuarina cunninghamiana* (Arnone et al., 1994). Plants receiving the high level of P on one side of the root system (NP NhP) had the highest nodule N concentration (Table 2), which may be a result of stimulated N$_2$ fixation. By contrast with the effects of high P on nodulation, high P could not counteract local or systemic N inhibition of nitrogenase activity. Nor could increasing P alleviate the inhibitory effects of high nitrate on nitrogenase activity in legumes (Leidi and Rodriguez-Navarro, 2000).

In the present work, P concentration in nodules, but not in roots or leaves, was reduced at high N (NP hNP, hNP hNP) (Table 2). This reduction was systemic as shown by plants receiving high N on one root side (NP hNP, Table 2). A low nodule P concentration could result from a reduced bacterial amount in the nodules and, consequently, lower amount of membranes, nucleic acids and other P-rich compounds as was proposed for *H. rhamnoides* (Gentili and Huss-Danell, 2002). Reduced amount of *Frankia* in nodules could thus explain low nitrogenase activity as well as low nodule P concentration in response to high N treatment.

N inhibition can occur at different stages of the nodulation process (Wall, 2000). In the case of the number of nodules, N inhibition must occur during the early stages of nodulation. A certain nodule DW can be comprised of many small or fewer but larger nodules and inhibition of nodule DW by N can thus occur from the early stages of nodulation until harvest of the plants. Parsons et al. (1993) and Baker et al. (1997) suggested that the concentration of reduced N compounds, probably amino acids, are involved in an internal feedback mechanism. Recently it was found in legumes that systemic shoot control of nodulation is mediated by a receptor-like kinase (Krusell et al., 2002; Nishimura et al., 2002). In this work it has also been shown that, in actinorhizal plants, shoot controls nodulation (systemic effects) and it can be speculated that a similar receptor is present in actinorhizal plants. Future work is needed to identify the receptor and the signal molecules.
In conclusion, in the root-hair-infected actinorhizal plant *A. incana*, P stimulation of the number of nodules was systemic, and it was specific for nodules and not a general stimulation via plant growth. When P was increased in one root side it could counteract the systemic N inhibition of nodulation, but not N inhibition of nitrogenase activity.

**Acknowledgements**

We thank Ann-Sofie Hahlin and Tina Johansson for valuable technical help, Dr Ari Jumpponen for valuable statistical advice, and Georg Carlsson and Dr Luis Wall for stimulating discussions. This work was financially supported by the Swedish Natural Science Research Council, the Swedish Council for Forestry and Agricultural Research, and EC FAIR-BM-972009.

**References**


