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Nitrogen, Phosphorus, and the Ratio Between them Affect Nodulation in *Alnus incana* and *Trifolium pratense*

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Abstract

Nodulation of *Alnus incana* by *Frankia* was studied at three levels of N (ammonium nitrate, 0.071–7.1 mM N) combined with three levels of P (0.01–1.0 mM) in a factorial design. Nodulation of *Trifolium pratense* by *Rhizobium leguminosarum* bv. *trifolii* was studied in a partial factorial design. Plants were in growth pouches for 10.5 weeks. In general, the degree of N inhibition depended on the P level. In *A. incana*, high P level stimulated nodule number and nodule dry matter per plant and per plant dry matter or per root dry matter. High P also stimulated nodule size and nitrogenase activity. Effects on nodule number seemed to be largely explained by plant growth whereas P had more of a specific effect on nodule dry matter. The N/P ratio was important, and increased N levels inhibited nodulation at N/P ratios >7 but not at N/P ratios ≤7. In *T. pratense*, high P level counteracted the inhibition of high N on nodule number and nitrogenase activity. The fact that N

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effects on nodulation and nitrogenase activity depend on P level should encourage more detailed work on effects of nutrient interactions on nodulation, both in actinorhizal plants and in legumes.

Keywords: *Alnus, Frankia,* N/P ratio, nitrogen, nitrogenase activity, nodulation, phosphorus, *Rhizobium, Trifolium*

Abbreviations
ARA = Acetylene reducing activity
DM = Dry matter

1. Introduction

Nitrogen is reported to inhibit nitrogen-fixing symbioses in legumes, from the early stages of interactions and infection through nodule development and nitrogen fixation (Streeter, 1988; Carroll and Mathews, 1990). Since the first report by Quispel (1954), nitrogen inhibition of nodulation and nitrogen fixation has been documented in several actinorhizal species (Quispel, 1958; MacConnell and Bond, 1957; Pizelle, 1966; Huss-Danell et al., 1982; Huss-Danell and Hahlin, 1988; Kohls and Baker, 1989; Thomas and Berry, 1989; Arnone et al., 1994). Split-root experiments in *Alnus glutinosa* indicated that nitrate had a strong local effect on nodule number, and a less strong systemic effect on nodulation time (Pizelle, 1966). In *Casuarina cunninghamiana,* nodule initiation and development were inhibited locally whereas specific nitrogenase activity was inhibited systemically (Arnone et al., 1994). Not only external nitrogen but also nitrogen status of plants affect nodulation (Thomas and Berry, 1989). Actinorhizal plants infected via root hairs (*A. glutinosa, Casuarina cunninghamiana, Myrica cerifera*) were considered more sensitive to nitrate than a plant (*Elaeagnus angustifolia*) infected via intercellular penetration (Kohls and Baker, 1989). Although there were different experimental designs in the above cited studies, inhibitory concentrations of N ranged from 2 to 6.5 mM.

Phosphate affects nodulation and nitrogen fixation in legumes (e.g. Robson, 1983; Jakobsen, 1985; Israel, 1987; Israel, 1993) as well as in actinorhizal plants (e.g. Russo et al., 1993; Yang, 1995). Beneficial effects of P on nodulation in legumes are often ascribed to improved plant growth, and consequently increased nodulation and N_2 fixation are seen as a response to meet the higher demand for N in the plant (Robson et al., 1981). Likewise, in *C. cunninghamiana,* P operated indirectly via growth of the host plant (Yang,

1995; Reddell et al., 1997). On the other hand, experiments with soybean showed a direct effect of P on nitrogen fixation and nodulation which, in turn, caused improved plant growth (Israel, 1993). In *A. glutinosa* Quispel (1958) reported that P was necessary during the week following inoculation for good nodulation to occur. Positive effects of P were obtained when P concentrations were 0.2 to 2 mM in the cited studies.

Information on N effects on nodulation comes largely from studies where N alone was added. Such an experimental design means that not only was the level of N altered but also the ratio between N and other macronutrients. A corresponding situation holds of course for experiments where only P additions were performed. Multivariate analyses of effects of all six macronutrients on an *A. incana-Frankia*-ectomycorrhiza association showed however that N, P and to some extent K had important effects on nodulation and nodule activity, as single elements or because of interactions between N and P and between P and K (Ekblad and Huss-Danell, 1995). In fact, data by Quispel (1958) suggest that inhibition of nodulation by N was counteracted by P, but this P effect seems to have been overlooked. In the present work a factorial design was used to study in more detail the effects of N, P and N/P ratio on nodulation in an actinorhizal symbiosis (*Frankia-Alnus incana*). A partial factorial design was used to study N, P and N/P ratio effects on nodulation in a legume symbiosis (*Rhizobium-Trifolium pratense*).

2. Material and Methods

Plant growth and inoculation

Seeds of grey alder, *Alnus incana* (L.) Moench, were surface sterilised and germinated as previously described (Wall and Huss-Danell, 1997). Seeds of red clover, *Trifolium pratense* (L.) cv. Bjursele, were treated similarly. Seedlings were grown for approximately 1 week on perlite and then transferred to sterile growth pouches (Mega, Minneapolis, MN, USA). Each pouch with four seedlings contained 12.5 ml of nutrient solution (Huss-Danell, 1978) diluted to 1/10 of full strength but with differing levels of N and P (see below). Pouches were watered with the same solution before and after inoculation. Plants were inoculated four weeks after transfer to pouches. At 2 to 3 weeks after inoculation, the bottom of the pouches was cut off and all pouches belonging to the same treatment were placed into a slightly larger sized plastic bag. Nutrient solution was added to a level of about 0.5 cm and was refilled as necessary or renewed at least once a week. The pH of fresh nutrient solutions was adjusted to 6.8. All experiments were done in a growth chamber with 16 h light at 25°C and 8 h darkness at 15°C. Relative humidity was approximately

75%. The photosynthetic photon flux was 150 to 200 μmol $m^{-2}s^{-1}$ provided by Osram (Berlin, Germany) Power Star HQJ-T 400 W lamps.

A crushed nodule inoculum from the so-called "local source of *Frankia*" with the symbiotic phenotype $Spore^+$ and Hup^- (Huss-Danell, 1991) was used. This source of *Frankia* gives efficient nodules on *A. incana*. To standardise the infectivity of the inoculum in different experiments, the same batch of crushed nodules was used in all experiments. Stock inoculum was obtained by crushing 3.3 g nodules in a mortar with sterile water, the suspension was filtered through a 100 μm nylon net, and the filtrate was diluted to an equivalent of 25 mg nodules per ml. Aliquots of the filtrate were immediately stored in liquid nitrogen. As needed, inoculum aliquots were thawed and diluted in sterile water. An amount corresponding to 250 μg nodules per plant was added with a micropipette, covering the root region from the root tip in basipetal direction. Uninoculated plants received only sterile water as "inoculum". These plants never formed nodules.

Trifolium pratense was inoculated with *Rhizobium leguminosarum* bv. *trifolii* strain 298 (Baljväxtlaboratoriet, Uppsala, Sweden) grown in YMB medium (Vincent, 1970) at 28°C on a shaker at 120 rpm. A culture grown to 0.39 optical density (500 nm) was diluted 100 times and 200 μl were spread over each root system.

Treatments

A factorial design with three levels of each nutrient was used. Nitrogen was added as ammonium nitrate at low, medium and high levels (equal to 0.071, 0.71 and 7.1 mM of N, respectively) and phosphorus as K-phosphate was used at low, medium and high levels (0.01, 0.1 and 1 mM P, respectively). Thus the design had nine different combinations of N and P. Concentrations of N and P varied 100-fold but the molar N/P ratio varied 10,000-fold and ranged from 0.071 to 710.

A preliminary experiment with *A. incana* was performed using a partial factorial design with only five treatments: low N low P, low N high P, medium N medium P, high N low P, high N high P. Data shown for *A. incana* are from the complete experiment. For *T. pratense* only the partial factorial design was used.

Measurements

Nitrogenase activity (ARA) was measured 1 day before harvest. Each pouch with intact plants was incubated in a gas-tight 650-ml cuvette under 10% (v/v) of C_2H_2. To facilitate gas diffusion to the roots, the plastic pouch was cut open

along its sides. Gas samples of 0.4 ml were taken at 5–10 min intervals within a total assay time of 30 to 60 min and analysed by gas chromatography (Huss-Danell and Sellstedt, 1985).

Plants were harvested at 10.5 weeks after inoculation. Nodule number was carefully recorded. Multilobed nodules were counted as single nodules. In *A. incana*, DM of shoots and roots was determined for each plant, but nodule DM was determined per pouch. In *T. pratense*, root DM was comprised of both root and nodule DM. Plant parts were dried at 70°C.

One representative nodule per plant was taken from each of three to five arbitrarily chosen *A. incana* plants of each treatment. Nodules were fixed in 2.5%(v/v) glutaraldehyde in 45 mM phosphate buffer, pH 7.2, washed in phosphate buffer, dehydrated in an ethanol series and embedded in resin. Longitudinal nodule sections were mounted on microscope slides and stained with toluidine blue. Sections were examined at 100 to 1000 times magnification in a light microscope.

Data presentation and statistical analysis

Each treatment of *A. incana* comprised ten pouches. Any damaged plants were removed from the pouch. At the end of the experiment, more than 20 plants per treatment were analysed. Data were calculated as average per plant within each pouch, and each pouch was used as one replicate in the statistical analyses (n = 10). All results have been observed in at least two similar independent experiments. Data were studied with ANOVA and Tukey HSD multiple comparison (SYSTAT, 1990-1993). For *T. pratense*, each treatment in the partial factorial design comprised 13–17 pouches and a total of 46–54 plants. Data were calculated as average per plant for each treatment and were analysed using "2^3" test (Bergman, 1992).

3. Results

Alnus incana

All plants were nodulated, except for plants receiving high N and low P, which grew very poorly. Microscopy of nodule sections from all eight nutrient combinations revealed a typical nodule anatomy and normal development of *Frankia* with hyphae, vesicles and spores, similar to earlier observations on this symbiosis (e.g. control plants in Huss-Danell et al., 1982, and unpublished observations).

The number of nodules per plant decreased at high N level when P was at medium level, but at high P level, nodule number per plant increased with

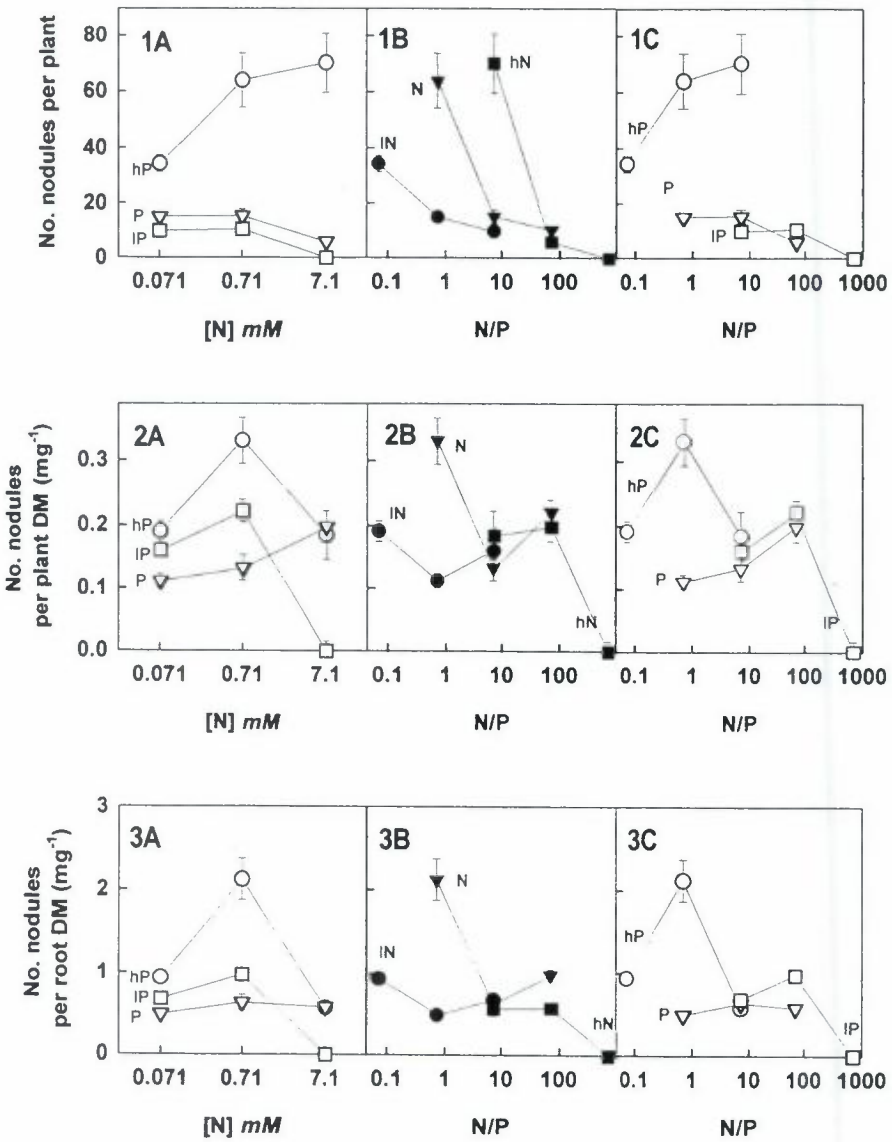

Figure 1. Number of nodules per plant in *A. incana* grown at different N and P levels. Data from 10.5 weeks after inoculation are shown in relation to (A) N level, and (B and C) N/P ratio in the nutrient solution. lN, low N level; N, medium N level; hN, high N level; lP, low P level; P, medium P level; hP, high P level. Mean ± SE (unless smaller than the symbol) for n = 10 growth pouches, each containing 2–4 plants.

Figure 2. Number of nodules related to plant DM in *A. incana* grown at different N and P levels. For symbols and experimental details, see Fig. 1.

Figure 3. Number of nodules related to root DM in *A. incana* grown at different N and P levels. For symbols and experimental details, see Fig. 1.

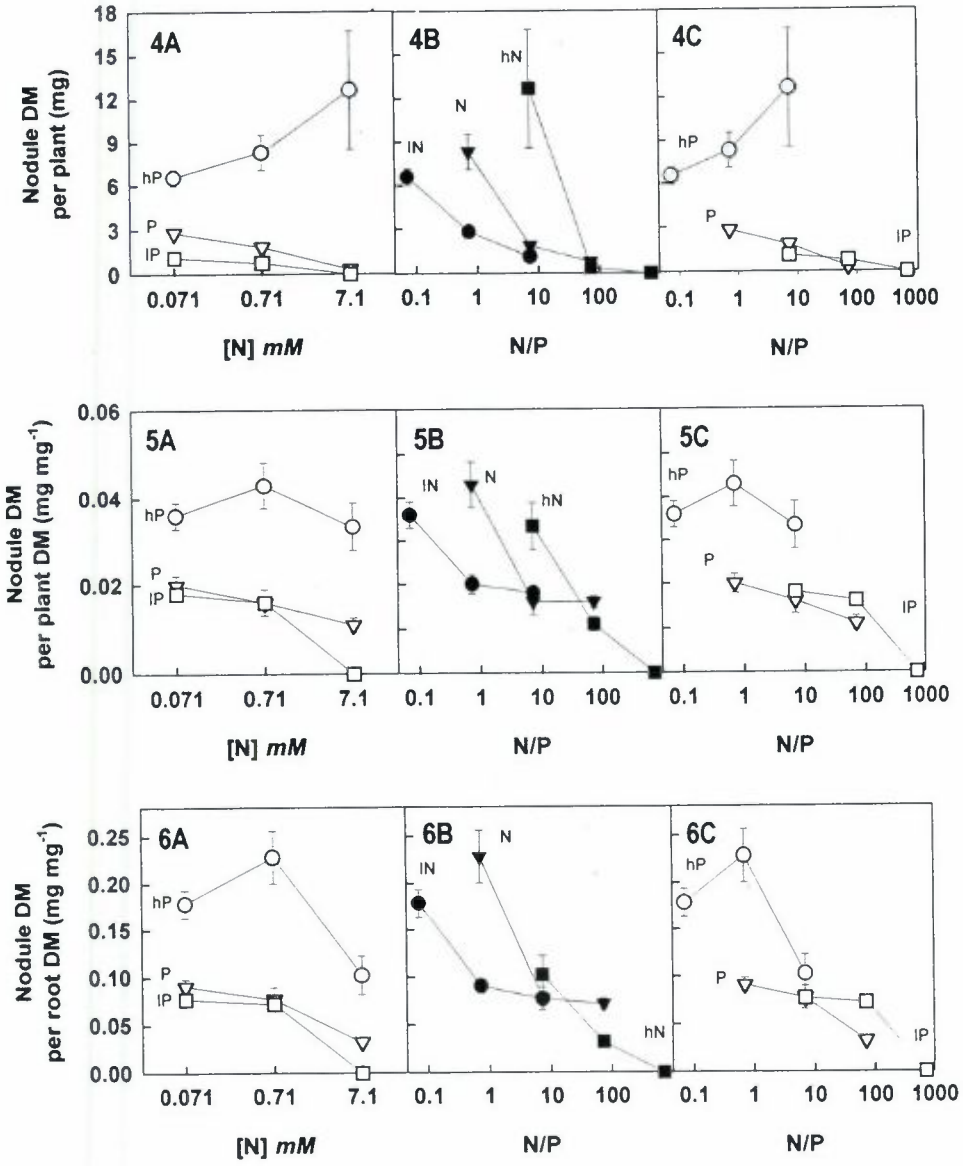

Figure 4. Nodule biomass per plant in *A. incana* grown at different N and P levels. For symbols and experimental details, see Fig. 1.

Figure 5. Nodule biomass related to plant DM in *A. incana* grown at different N and P levels. For symbols and experimental details, see Fig. 1.

Figure 6. Nodule biomass related to root DM in *A. incana* grown at different N and P levels. For symbols and experimental details, see Fig. 1.

increased N levels (Fig. 1A). At high N the increase from medium to high P level increased the number of nodules per plant about 10-fold and at medium N level the increase was more than 4-fold. The interaction between N and P was seen more clearly when nodulation was presented in relation to the N/P ratio. High N inhibited nodulation only when the N/P ratio was >7, that is, ratios 71 and 710 (Fig. 1B). At N/P ratios ≤7 nodulation was determined mainly by P level but also by N level (Fig. 1B–C).

The response to N and P by nodulation measured as nodule DM per plant (Fig. 4) resembled the response seen for number of nodules per plant. When nodule DM per plant was related to N/P ratio, it was obvious that N inhibited nodulation only when the N/P ratio was >7 (Fig. 4B). At N/P ratios ≤7 nodulation was, again, determined mainly by P level (Fig. 4B–C).

Being macronutrients, N and P influenced the growth of the plants. It was therefore necessary to consider the plant size to distinguish between N and P effects specifically on nodulation and N and P effects being general growth effects. Nodule number per plant DM (Fig. 2) as well as nodule number per root DM (Fig. 3) varied no more than 2-fold among treatments (except for the combination medium N and high P) and there was no pronounced effect of N/P ratio. Thus, compared to nodule number per plant (Fig. 1), the effects of N and P on nodule number per plant DM or per root DM were small. It seems that nodule number per plant was to a large extent reflecting the size of plants.

When nodulation measured as nodule DM was related to plant DM (Fig. 5) or to root DM (Fig. 6) effects of N and P were more pronounced than for nodule number related to plant DM or to root DM (Fig. 2–3). The nodule DM per plant DM (Fig. 5) or per root DM (Fig. 6) were stimulated by high P at all N levels, and they were dependent on N/P ratio (Fig. 5B–C, 6B–C). Thus, the effects of N and P on nodule DM was not only an effect of general plant growth but rather a more specific effect on nodule growth. Analyses of variance for the number of nodules per plant, nodule DM per plant, or nodule DM per plant DM, show a significant general positive effect for P ($P<0.0005$) and for N*P interaction ($P<0.0005$), and a general effect for N ($P<0.005$), which can be negative or positive depending on P level.

A more pronounced effect of N and P on nodule growth than on nodule number was further seen from the calculated individual nodule DM (Fig. 7). At all P levels, nodule size decreased when N was increased from low to medium N level. Medium and high P stimulated nodule size at all N levels (Fig. 7A). Interactions between N and P are clearly seen when nodule size is plotted against N/P ratio of the nutrient solution (Fig. 7 B–C). Nodule size was linearly correlated to logN/P ratio with $r^2 = 0.896$.

Nitrogenase activity per plant was inhibited by increased N levels at medium and high P levels and was always stimulated by increased P levels

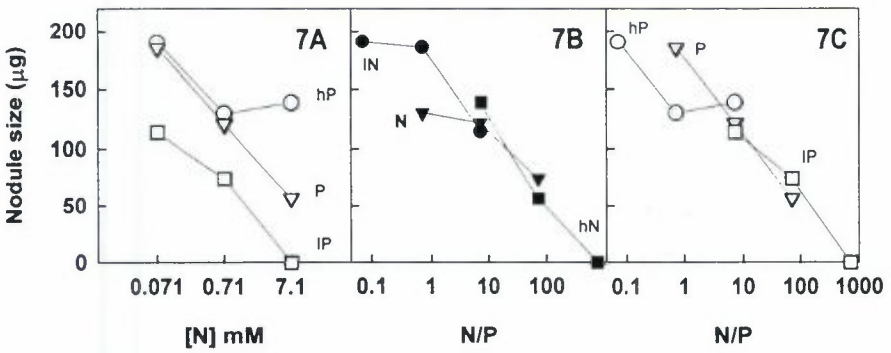

Figure 7. Calculated individual nodule size in *A. incana* grown at different N and P levels. For symbols and experimental details, see Fig. 1.

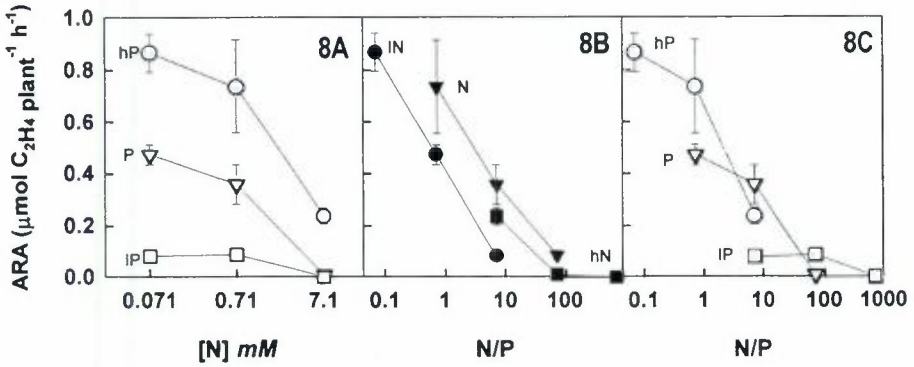

Figure 8. Nitrogenase activity (ARA) in *A. incana* grown at different N and P levels. For symbols and experimental details, see Fig. 1.

(except for medium P at high N) (Fig. 8). However, increased P levels did not counteract N inhibition completely. When N/P ratio was >7, nitrogenase activity was very low or not detectable (Fig. 8B–C). A linear correlation between ARA and logN/P ratio was found, $r^2 = 0.829$.

Specific ARA (µmol C_2H_4 mg nodule^{-1} h^{-1}; cf. Fig. 8A and 4A) at all N levels increased when P was raised from low to medium level. At high N level effects of medium P and high P were similar, but at low and medium N levels the stimulation by high P was less pronounced than stimulation by medium P. Although nodule DM per plant was highest at high P level (Fig. 4A), nodules at different treatments may have had different proportions of active tissue.

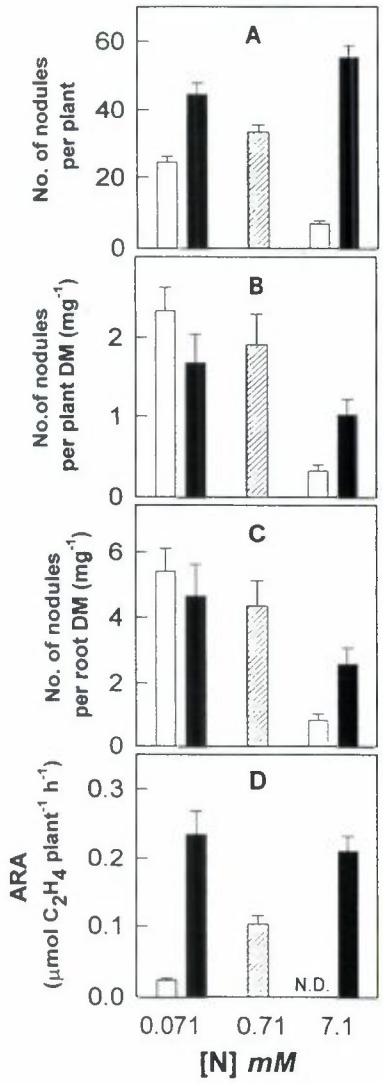

Figure 9. Number of nodules per plant (A), number of nodules per plant DM (B), number of nodules per root DM (C) and nitrogenase activity per plant in *T. pratense* grown at different N and P levels (D). Data are from 10.5 weeks after inoculation. Open bars, low P level; hatched bars, medium P level; filled bars, high P level. N.D. = not detectable. Mean ± SE for n = 13–17 pouches, each containing 2–4 plants.

Trifolium pratense

Plants were nodulated in all treatments. The number of nodules per plant (Fig. 9A) was inhibited by high N when P was low, but this inhibition was

completely counteracted by high level of P. Similar to *A. incana* (Fig. 1), the highest nodule numbers were obtained at high P. For the three nutrient combinations having an N/P ratio of 7.1 (low N and low P; medium N and medium P; high N and high P) the nodule number per plant increased with increased N level and with increased P level. Both P and N*P interaction had a positive significant effect ($P<0.05$) on nodulation in *T. pratense*. Nodule number related to plant DM (Fig. 9B) or to root DM (Fig. 9C) was decreased at high N. This decrease was counteracted by high P, but not as strongly as the effect on nodule number per plant (Fig. 9A).

Nitrogenase activity per plant (Fig. 9D) was strongly stimulated by P at both low and high N. Nodules formed at high N and low P did not show detectable activity but those formed at high N and high P had activities similar to the treatment low N and high P.

4. Discussion

Nodulation expressed as number of nodules per plant is the number of infections that have successfully developed into nodules. Nodulation expressed as nodule DM reflects the growth of nodules, and can be composed of few and large nodules or numerous but small nodules. In the present work both nodule number and nodule DM were recorded to analyse the effects of N, P and N/P ratio on nodulation in *A. incana*. Furthermore, nodule number as well as nodule DM were related to DM of total plant and to DM of roots to distinguish between nutrient effects on nodulation and nutrient effects on plant and root growth. The degree of N inhibition depended on the P level. In general, high P stimulated all nodulation parameters; nodule number and nodule DM per plant, nodule DM per plant DM, nodule DM per root DM, individual DM of nodules, and also ARA per plant. When nodule number and nodule DM were related to plant DM (Figs. 2 and 5) or to root DM (Figs. 3 and 6) the effect of high P remained more clearly for nodule DM than for nodule number. This suggests that effects on nodule number were largely explained by plant growth and root growth whereas, in contrast, effects on nodule DM appeared to be more of specific effects on nodules.

For the N and P levels used in the present work there was an interaction between N and P, and the N/P ratio in the nutrient solution was important. When the N/P ratio was >7 there was inhibition of nodule number and nodule DM per plant, nodule DM per plant DM and per root DM, nodule size and nitrogenase activity. In plant tissues an N/P ratio of 7–10 is often considered adequate (Salisbury and Ross, 1992; Marschner, 1995), and probably a proper N/P ratio in the plant tissues was hard to achieve when N/P ratio in the nutrient solution was >7 (ratios 71 and 710 in the experimental design).

Increased P levels counteracted N inhibition of nodulation in *A. incana* (Figs.

1 and 4), but an increased P level could not fully prevent N inhibition of ARA (Fig. 8). Also in *T. pratense* nodulation was stimulated by high P, and in this species the inhibition by high N was fully counteracted by high P (Fig. 9A). High P had a very strong effect on nitrogenase activity and counteracted completely the inhibition by high N (Fig. 9D). The observed N inhibition of ARA is in accordance with the hypothesis of nitrogenase activity being feedback regulated by plant N (Parsons et al., 1993; Baker et al., 1997), but the mechanism for P stimulation is not known. A specific role of P in nodulation was proposed already by Quispel (1958) who noticed that lack of P inhibited nodulation in *A. glutinosa* but only during the week that immediately followed inoculation. The present data on *T. pratense* together with consistently enhanced nodulation by P addition in a supernodulating, nitrate tolerant mutant of soybean (Gunawardena et al., 1993) suggest an important role of P also in legume nodule formation.

In addition to the symbioses studied here, interactions between N and P have also been noted in the actinorhizal plant *Discaria trinervis* (Valverde, 2000). It seems, therefore, that interactions between N and P occur in actinorhizal plants representing infection via root hairs (*Alnus*) and via intercellular penetration (*Discaria*; Valverde and Wall, 1999) and in both actinorhizal plants and legumes (*Trifolium*). Only a partial factorial design was used for *T. pratense*, but *T. pratense* and *A. incana* were studied under the same conditions to facilitate comparisons between the two symbioses.

The fact that N inhibition of nodulation and nitrogenase activity was modulated by P level and N/P ratio is an important factor that may have been overlooked previously. Depending on which nutrient solution was used, a similar N concentration might have given different N/P ratios in different studies. Therefore, not only plant species, forms of N, time for N additions and differences in growing conditions (Thomas and Berry, 1989; Benoit and Berry, 1990; Huss-Danell, 1997) but also different N/P ratios should be considered when N effects on nodulation are evaluated. In the present work N and P were varied, but it may be that other macronutrients as well as micronutrients will influence nodulation (Ekblad and Huss-Danell, 1995). Our results should encourage further and detailed studies in actinorhizal as well as legume symbioses to understand the mechanisms behind interactions between N and other nutrients in nodulation and nitrogen fixation.

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