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Phosphorus, nitrogen and their interactions affect N_2 fixation, N isotope fractionation and N partitioning in *Hippophaë rhamnoides*

Francesco Gentili

Department of Agricultural Research for Northern Sweden, Crop Science Section, Swedish University of Agricultural Sciences, 904 03 Umeå, Sweden;
Present address: Department of Plant Physiology, UPSC, Umeå University, 901 87 Umeå, Sweden,
Tel. +46-90-7865627, Fax. +46-90-7866676, Email. Francesco.Gentili@plantphys.umu.se

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Abstract
The interactive effects of varying phosphorus and nitrogen supplies on N_2 fixation, N isotope fractionation during N uptake, and N partitioning among plant parts were studied in the actinorhizal plant *Hippophaë rhamnoides* L. (sea buckthorn). Plants were grown for six weeks after inoculation with the N_2-fixing actinomycete *Frankia* and differences in N accumulation were used to quantify N_2 fixation. ^{15}N natural abundance was analysed to study N isotope fractionation in specific plant parts in plants receiving different levels of N and P. Furthermore, the root system was split to study N isotope fractionation in roots supplied with different levels of N and P. Phosphorus stimulated N_2 fixation by direct effects on nodule dry matter and nodule function, rather than indirectly via plant growth. Phosphorus also stimulated N uptake from solution and influenced N isotope fractionation during N uptake. The inclusion of ^{15}N natural abundance analyses made it possible to detect P effects on N uptake, fractionation and N_2 fixation even though the plants used both N_2 fixation and combined N as N sources.

Keywords: *Hippophaë rhamnoides*, isotope fractionation, nitrogen, ^{15}N, N_2 fixation, phosphorus

1. Introduction

Hippophaë rhamnoides L. has the capacity to form N_2-fixing root nodules when infected by the actinomycete *Frankia* (Huss-Danell, 1997). This actinorhizal species is a multi-purpose plant (Li and Schroeder, 1996) used for forage, food production and land reclamation, especially in Asia (Cifen et al., 2002) and Eastern Europe. More recently, extracts of *H. rhamnoides* have been used for pharmaceutical purposes such as UV protection (Beveridge et al., 1999).

Both nitrogen (N) and phosphorus (P) influence nodulation and N_2 fixation, but in opposite ways. N inhibits nodulation and N_2 fixation in actinorhizal plants (Huss-Danell, 1997; Gentili and Huss-Danell, 2002, 2003) and legumes (Streeter, 1988). For example, N_2 fixation in *H. rhamnoides* was found to be strongly inhibited by addition of nitrate to the nutrient solution by Bond and Mackintosh (1975).

Addition of ammonium to N-free grown *Alnus incana* can also inhibit nitrogenase activity and alter the structure of *Frankia* vesicles, the site of N_2 fixation, in the nodules (Huss-Danell et al., 1982; Huss-Danell and Hahlin, 1988). In split-root cultures of legumes as well as actinorhizal plants nitrogenase activity has been shown to be systemically inhibited by nitrate (Carroll and Gresshoff, 1983; Silsbury et al., 1986; Arnone et al., 1994). In contrast, P stimulates nodulation, N_2 fixation and N accumulation in legumes (Israel, 1987; Stamford et al., 1997; Hellsten and Huss-Danell, 2001; Tang et al., 2001). Stimulation of nodulation by P has also been observed in the actinorhizal *A. incana* (Wall et al., 2000; Gentili and Huss-Danell 2003), *Discaria trinervis* (Valverde et al., 2002) and *H. rhamnoides* (Gentili and Huss-Danell, 2002). However, stimulation of nodulation does not necessarily mean stimulation of N_2 fixation. Phosphorus effects on N_2 fixation in actinorhizal plants have been largely neglected, but Ekblad and Huss-Danell (1995) found a stimulating effect of P in *A. incana*. Stimulation of N_2 fixation by P could be of great importance from both agronomic and ecological perspectives as P fertilization could potentially increase the yield of N_2-fixing plants over a wide range of N concentrations in the soil.

It has been shown that P stimulates N accumulation in N_2-fixing plants (Ledgard, 1989; Stamford et al., 1997) and uptake of mineral N in non-N_2-fixing plants (Högberg et al., 1999). When N_2-fixing plants are grown in N-free solution, P stimulation of N_2 fixation can be easily

quantified since uptake of mineral N from the solution is excluded (Ledgard, 1989). However, when N is present in the nutrient solution, it is more difficult to assess if P stimulation of N accumulation is mediated via uptake of mineral N or N_2 fixation. While uptake of mineral N has been widely investigated by ^{15}N natural abundance analyses (Högberg, 1997) much less is known about the effects of other nutrients such as P on N isotope fractionation in the presence of both N_2 fixation and mineral N.

The ratio between the two stable isotopes ^{15}N and ^{14}N varies due to fractionation in biological processes (Högberg, 1997). Consequently, the ^{15}N values (or 'signatures') found in tissues of N_2-fixing plants are affected both by the signature of the sources used by the plants and fractionation during uptake of mineral N from the soil, N_2 fixation from the atmosphere and N assimilation and remobilization in them (Evans, 2001).

Here, the N difference technique was used to quantify N_2 fixation, while ^{15}N natural abundance (NA) measurements were used to evaluate the interactive effects of P and N on N_2 fixation and uptake of mineral N in young plants of *H. rhamnoides*. A split-root system was used to study N isotopic fractionation and N partitioning between the two parts of the root system receiving different concentrations of N and P. The N forms used were ammonium and nitrate because they are the most abundant sources of inorganic N present in the soil.

The aims of this work were: to study P effect on N_2 fixation, N isotope fractionation and uptake of mineral N; to study N isotope fractionation among plant parts and among roots receiving different nutrients levels.

2. Materials and Methods

Plant material, N and P treatments

Seeds of *H. rhamnoides* L. were collected from plants growing in southern Sweden at Balsgård research station. The seeds were surface sterilised by incubating them in ethanol (70% v/v, shaken for 15 min), rinsing in water, incubating in hydrogen peroxide (30% v/v, shaken for 15 min) and again rinsing in water. The seeds were then sown in Petri dishes containing perlite and moistened with modified Evans nutrient solution (Huss-Danell, 1978) diluted to 1/10 of full strength, with 0.71 mM ammonium nitrate. All materials and solutions were sterilised prior to use. Three weeks after germination, seedlings were transferred to clean 6 × 6 cm pots with sterile perlite, and the N concentration of the nutrient solution was raised from 0.71 to 2.1 mM to support growth of the seedlings during the following five weeks. The seedlings were watered 3–4 times a week.

Eight-week-old plants were transplanted into split-pots, made from two 8 × 8 cm pots taped together, and each root system was divided into two similar parts. Treatment with the different solutions (Table 1) began immediately after transfer to the split-pots. The surface of the perlite was kept low enough to avoid movement of the solutions from one part of the root system to the other. Pots were placed on a large mesh-size net such that solutions could not move from one side to the other in the split-pots. Plants were watered 3–4 times a week with a sufficiently large volume of nutrient solution to ensure that it drained through all of the pots, to avoid salt accumulation.

At the start of the experiment all plants had similar shoot heights, about 9 cm. Plants were grown for almost six weeks after inoculation, and 10 plants were assigned to each of seven treatments (Table 1). The pH of the solutions was adjusted to 6.8±0.2 and it was regularly monitored throughout the experiment. During the entire growth period from late March to mid-July the plants were kept in a greenhouse in Umeå, Sweden (63°45'N), under a diurnal regime of 17-h photoperiods, with supplementary light provided by Philips HPI/T 400 W lamps at about 25°C, and 7-h dark periods at about 15°C.

Frankia *strain and inoculation*

Plants were inoculated when 10 weeks old and two weeks after transfer to split-pots with the effective *Frankia* strain E15b grown in K-medium (Lumini and Bosco, 1996) for 2.5 weeks. Cells of *Frankia* were harvested by centrifugation and resuspended in 1/10 strength modified Evans solution (Huss-Danell, 1978) with 0.71 mM of ammonium nitrate. Each half of the root system received about 9 mg packed cells (fresh weight), added as a cell suspension to its base. Some plants receiving a control solution (NP NP) were not inoculated and served as reference plants.

Harvest, and chemical analyses

At harvest, almost six weeks after inoculation, plants were divided into leaves, stems, roots and nodules. Each half of the root system was carefully cleaned from perlite and measured separately. Plant parts were dried at 60°C for 24 h and weighed. The concentrations of N, P and ^{15}N in the leaves, roots and stems were analysed in five randomly chosen plants from each treatment. Dried plant parts were ball-milled (Retsch, MM2000, Haan Germany). The concentrations of N and ^{15}N were analysed using a Europa Scientific ANCA-IRMS (Ohlsson and Wallmark, 1999) at the Dept. of Forest Ecology, Swedish University of Agricultural Sciences (SLU), Umeå, Sweden. The $\delta^{15}N$ value of the ammonium nitrate salt, similarly analysed, was –2.18 (mean value of five replicates). The $\delta^{15}N$ value of the ammonium N in the ammonium nitrate salt was –3.79 (mean value of four replicates) as analysed by a PDZ Europa Orchid system according to Norlin et al. (2002). Consequently, the calculated $\delta^{15}N$ value of the nitrate N in the salt was –0.57. Plant samples were digested in HNO_3

Table 1. N and P concentrations in the nutrient solutions applied.

Nutrient	Solution NP	hNP	NhP	hNhP
N (mM)	0.71	6.45	0.71	6.45
P (mM)	0.1	0.091	1	0.91
N:P (mM/mM)	7.1	71	0.71	7.1
N:P (mass/mass)	3.21	32.1	0.321	3.21

The solutions were separately supplied to the two parts of the root system of the split-root plants in the seven following permutations: NP NP (control); NP hNP; hNP hNP; NP NhP; NhP NhP; NP hNhP; hNhP hNhP. All other nutrients were as in the modified Evans solution (Huss-Danell, 1978) diluted to 1/10 of full strength.

and $HClO_4$ at 130°C before measurement of their P concentrations using a Tecator autoanalyser in the Environmental Research Laboratory, SLU, Umeå, Sweden.

N_2 fixation

N_2 fixation was calculated using the N difference method, the non-inoculated plants serving as reference plants, by the following formula:

$\%Ndfa = [(N_{inoc} - N_{ref}) / N_{inoc}] * 100$

where %Ndfa is the percentage of N derived from the atmosphere, N_{inoc} stands for total N in inoculated plants, and N_{ref} for total N in reference plants. However, the nodules were not included in the calculation.

Table 2. %Ndfa, total N content, nodule DW, nodule number, root, shoot and plant DW (excluding nodules) in *H. rhamnoides* grown in split-root systems with different parts of the root system receiving different levels of N and P. The prefix "h" stands for the presence of a nutrient at its high level. Reference plants were not inoculated.

Treatment	% Ndfa	Total N content per plant (mg)	Nodule DW per plant (mg)	Nodule number per plant	Root DW (g)	Shoot DW (g)	Plant DW (g)
Reference	0	6.0±0.2	0	0	0.1±0.01*	0.3±0.03	0.5±0.04
NP NP	29	8.4±0.7	17.7±2.5	18.8±4.4	0.1±0.02	0.4±0.04	0.6±0.05
NP hNP	0	27.6±2.9	2.0	5.0	0.6±0.1	1.1±0.1	1.7±0.2
hNP hNP	0	35.9±1.3	0	0	0.5±0.02	0.8±0.01	1.3±0.03
NP NhP	47	11.3±1.6	25.5±1.9	39.8±12.5	0.2±0.01	0.4±0.04	0.6±0.05
NhP NhP	38	9.7±1.3	19.5±3.2	33.8±11.3	0.1±0.01*	0.4±0.1	0.5±0.1
NP hNhP	NA	29.9±2.7	15.9±6.7	7.0±2.8	0.6±0.1	1.0±0.05	1.6±0.1
hNhP hNhP	0	47.8±4	0	0	0.7±0.05	1.4±0.1	2.1±0.2

Values are means ± SE of five plants in each treatment at harvest six weeks after inoculation. NA, not applicable as the N level supplied was higher than for reference plants. * shows statistically significant difference.

Table 3. N concentration (% of dry matter) in roots, stem, leaves and plant (excluding nodules) and P concentration in leaves of *H. rhamnoides* grown in split-root systems with different parts of the root system receiving different levels of N and P. The prefix "h" stands for the presence of a nutrient at its high level (Table 1). Reference plants were not inoculated and received NP NP.

Treatment	Part of split-root system	Root N (%)	Stem N (%)	Leaf N (%)	Plant N (%)	Leaf P (%)
Reference		1.5±0.04	0.9±0.04	1.1±0.1	1.2±0.1	0.1±0.01
NP NP	NP	1.5±0.1	1.2±0.04	1.6±0.1	1.5±0.1	0.1±0.02
NP hNP	NP	1.6±0.1*	1.0±0.02	1.6±0.1	1.6±0.1	0.1±0.005
	hNP	2.3±0.2*				
hNP hNP	hNP	2.9±0.1	1.9±0.1	2.8±0.1	2.7±0.1	0.1±0.003
NP NhP	NP	1.9±0.1	1.4±0.1	2.0±0.1	1.9±0.1	0.5±0.05
	NhP	2.0±0.1				
NhP NhP	NhP	2.0±0.1	1.4±0.1	2.1±0.2	2.0±0.1	0.9±0.1
NP hNhP	NP	1.8±0.04*	1.3±0.05	1.9±0.03	1.9±0.03	0.3±0.03
	hNhP	2.4±0.1*				
hNhP hNhP	hNhP	2.5±0.1	1.4±0.03	2.5±0.1	2.3±0.05	0.6±0.04

Values are mean ± SE of five plants in each treatment at harvest six weeks after inoculation. * marks statistically significant difference between the two parts of the root system supplied with different nutrient solutions.

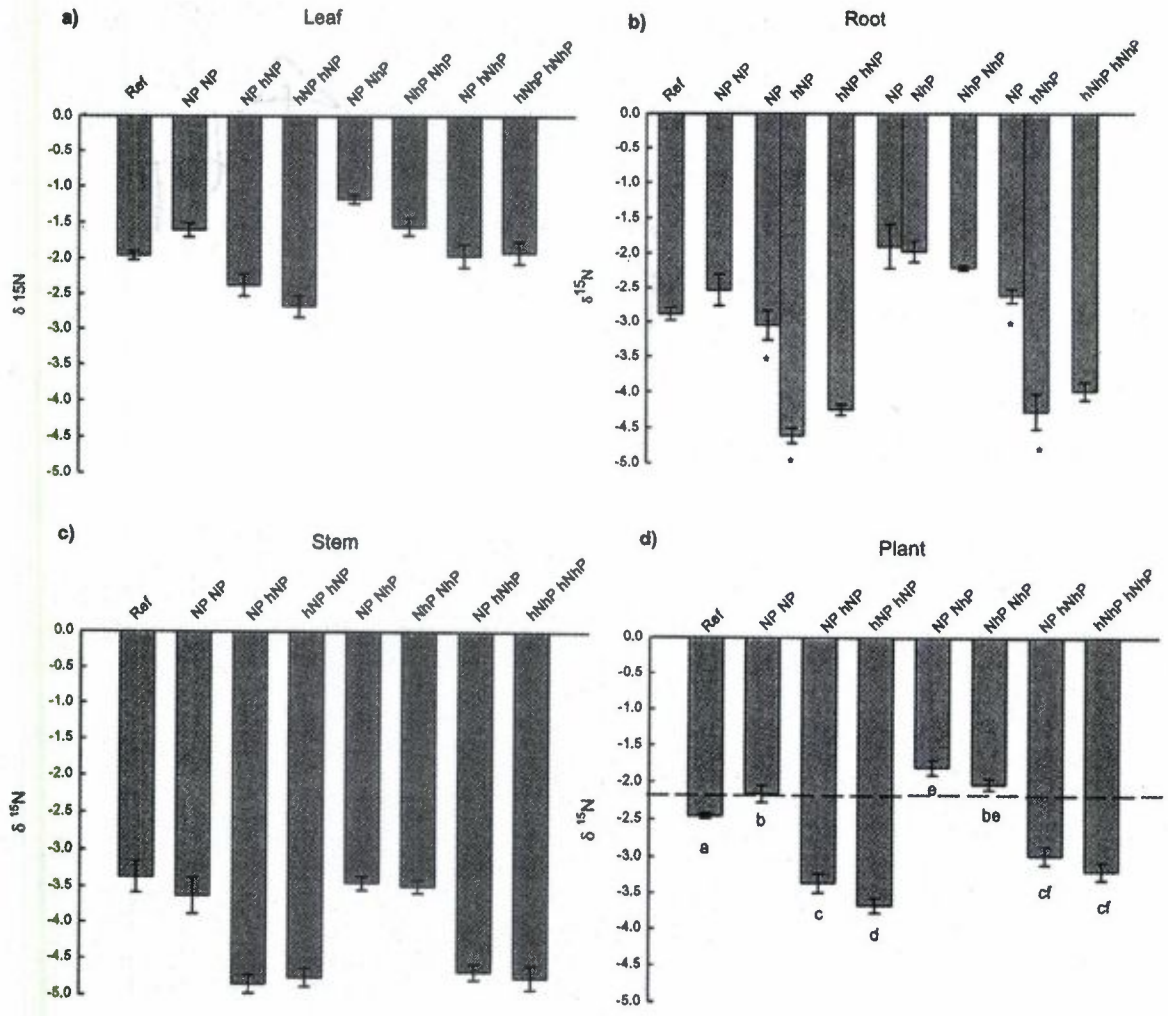

Figure 1. $\delta^{15}N$ values of (a) leaves, (b) roots, (c) stems and (d) whole plants (excluding nodules) of *Hippophaë rhamnoides*. Treatments began two weeks before inoculation. The broken line shows the $\delta^{15}N$ value of the salt ammonium nitrate. Ref indicates non-inoculated reference plants receiving NP NP. Values are means ± SE of five plants in each treatment at harvest six weeks after inoculation. * marks statistically significant differences between the two parts of the root system receiving different nutrient solutions. Columns marked with different letters are significantly different. Differences between means were considered significant when $p \leq 0.05$.

Statistical analyses

One-way ANOVA and paired t tests were performed with Minitab software Release 13.1 (Minitab, 2000 Inc., College State, Pennsylvania, USA), differences between means being considered significant if $p \leq 0.05$.

3. Results

N_2 fixation and N accumulation

The plants used to measure N_2 fixation (NP NP, NP NhP, NhP NhP) had similar shoot masses to the reference plants. Their root masses were also similar, except for those supplied with high P to both parts of the root system (NhP NhP) (Table 2). The highest %Ndfa values were found in plants supplied with high P to one part of the root system at the control N level (NP NhP), with nearly half of their N coming from N_2 fixation. These plants also had significantly higher N concentrations than plants receiving control levels of both nutrients (NP NP; Table 3). In plants supplied with high P to both parts of the root system at the control N level (NhP NhP), 38% of their total N came from N_2 fixation (Table 2). Plants supplied with high P (NP NhP, NhP NhP) had the highest amounts of nodules, in terms of both nodule biomass and (especially) number (indicating nodule initiation; Table 2). In contrast, among plants supplied with high N to one part of the root system (NP hNP), only one plant out of five was nodulated (and even that was poorly nodulated), so the N_2 fixation in this group of plants was designated zero. Irrespective of the P

level, plants supplied with high N to both parts of the root system did not produce any nodules and consequently fixed no N_2 (Table 2).

Plants supplied with high N and high P to one part of the root system (NP hNhP) developed nodules only in the NP part of the root system. However, these plants had a similar N content to plants supplied with high N to one part of the root system (NP hNP) (Table 3) and the $\delta^{15}N$ values (Fig. 1d) were similar in the two treatments. Thus, N_2 fixation also appeared to be negligible in the NP hNhP plants. Use of the N difference method was not appropriate as the N level given to NP hNhP plants was higher than that given to the reference plants (NP NP).

The highest N accumulation (Table 2) and N concentrations (Table 3) were seen in plants supplied with high N to both parts of the root system (hNP hNP, hNhP hNhP). Plants supplied with high P to just one part of the root system (NP NhP) accumulated ca 34% more N than plants supplied with control levels of both N and P (NP NP). This was due to the NP NhP plants having similar dry matter, but higher N concentrations (Tables 2 and 3). Phosphorus stimulated uptake of mineral N significantly, as manifested by the weaker N isotope fractionation in hNhP hNhP plants than in hNP hNP plants (Fig. 1d). The stimulation of uptake of mineral N was accompanied by higher dry matter (Table 2).

N partitioning

Leaves and roots always had higher N concentrations than stems. Differences in the N and P concentrations in the nutrient solution clearly influenced N partitioning between parts of the root system receiving different levels of N and P (Table 3). Plants supplied with high N or high N and high P to one part of the root system (NP hNP, NP hNhP) had a significantly higher N concentration in the part supplied with high N than in the part supplied with the control level (Table 3).

N isotope signatures in specific plant parts

The following assumptions were used to evaluate $\delta^{15}N$ values of inoculated plants. Poorly nodulated plants had to rely on uptake of mineral N from solution. At high N supply they did not have to use all of the available N and could use either nitrate or ammonium as sources of N. Therefore, they were expected to discriminate between the N sources and against the heavier isotope ^{15}N. This would result in the plants in the high N treatment having more negative $\delta^{15}N$ values than those in the control N treatment. Plants fixing N_2 were expected to have $\delta^{15}N$ values approaching 0, since some of the N in N_2-fixing plants would originate from the atmosphere (Table 2), which has a $\delta^{15}N$ value of 0 by definition (Mariotti, 1983); higher than that of the salt (–2.18).

There was great variation in $\delta^{15}N$ values among plant parts (Fig 1). However, in all treatments leaf $\delta^{15}N$ values > root $\delta^{15}N$ values > stem $\delta^{15}N$ values (Figs. 1a–c). There was a significant difference in $\delta^{15}N$ values between the parts of the root system supplied with high N or both high N and high P (hN, hNhP) and those receiving control levels of N and P (NP) in the treatments NP hNP and NP hNhP (Fig. 1b). These results indicate that discrimination against the heavier isotope or a shift from nitrate to ammonium as the source of N occurred in the part of the root system supplied with the high N level.

The plant $\delta^{15}N$ values (Fig. 1d) showed that the non-inoculated reference plants discriminated negligibly against the heavier isotope. The plants with higher $\delta^{15}N$ than the reference plants fixed N_2, while those with lower values discriminated against the heavier isotope. Indeed, when N supplies were increased to one or both parts of the root system at any P level (NP hNP, hNP hNP, NP hNhP, hNhP hNhP) the plants discriminated against the heavier isotope (Fig. 1d).

A replicate experiment, in which N and P treatments began at the time of inoculation, showed essentially the same results (data not shown) as in the experiment presented here. However, the effects in the replicate experiment were always smaller, presumably because N and P exerted their effects during a shorter time.

4. Discussion

In this work ^{15}N:^{14}N analyses were used to evaluate P effects on uptake of mineral N, and $\delta^{15}N$ signatures among plant parts in *H. rhamnoides*. Phosphorus stimulated N_2 fixation even in young plants of *H. rhamnoides*, via direct effects on nodule function rather than indirectly via plant growth. Indeed, plants receiving high levels of P at the N control level did not have higher biomass (Table 2), but they fixed and accumulated more N than plants at the control P level (Fig. 1, Table 2). This supports previous observations that P stimulated N_2 fixation in *A. incana* (Ekblad and Huss-Danell, 1995; Gentili and Huss-Danell, 2003) and in some legumes (Stamford et al., 1997; Hellsten and Huss-Danell, 2001; Leidi and Rodriguez-Navarro, 2000; Tang et al., 2001). However, it has been shown that P effects can vary greatly among bacterial strains (Stamford et al., 1997) and among plant species, sometimes being negligible or even negative (Sanginga et al., 1996). In this study plants gained N from both uptake and N_2 fixation, i.e. from two N sources with different $\delta^{15}N$ values, while in other studies (Ledgard, 1989) legumes gained all their N from N_2 fixation, i.e. one N source. This explains why P effects on $\delta^{15}N$ values have not been reported earlier. When levels of both nutrients were raised in the solution, P stimulated uptake of mineral N, but not N_2 fixation since high N resulted in very poor nodulation. This is in agreement with previous findings in *A. incana* and *Phaseolus vulgaris*, where the inhibitory effect of high

nitrogen concentration on N_2 fixation was not counteracted by increasing the P supply (Leidi and Rodriguez-Navarro, 2000; Gentili and Huss-Danell, 2003).

P clearly stimulated uptake of mineral N from solution, similarly to findings in *Pinus sylvestris* (Högberg et al., 1999). In addition, P influenced N isotope fractionation, as indicated by the differences in N discrimination between plants receiving either high N (hNP hNP) or both high N and P via both parts of their root systems (hNhP hNhP; Fig. 1d).

By using a split-root design it was possible to identify differences in $\delta^{15}N$ values in roots receiving different levels of nutrients (NP hNP and NP hNhP in Fig. 1b). This interesting finding could be due to the fact that in the presence of a large supply of ammonium and nitrate, the plants preferentially take up ammonium, (a hypothesis supported by the observation that the $\delta^{15}N$ values of parts of the root system supplied with high N (hN, hNhP) in the NP hNP and NP hNhP treatments were similar to the $\delta^{15}N$ value in the ammonium). This result is supported by previous finding where *H. rhamnoides* had a preference for ammonium in the presence of both NH_4^+ and NO_3^- (Troelstra et al., 1987).

In a field situation with patchy distribution of nutrients, different root parts can thus be expected to have different ^{15}N signatures. The non-inoculated reference plants, grown at control levels of N and P, were limited in N, as indicated by the low N concentration in all their parts (Table 3) and by the fact that they took up all available N with negligible isotope discrimination (Fig. 1d). This is consistent with findings in analyses of whole plant isotope composition and natural N-limited systems, where all available inorganic N is taken up and consequently there is little or no fractionation (Högberg, 1997; Evans, 2001).

The difference between $\delta^{15}N$ values of leaves and other plant parts (Fig. 1) suggests that we should exercise caution when using leaves as indicators of whole plant $\delta^{15}N$ values, as noted by Kolb and Evans (2002). The ^{15}N natural abundance measurements allowed clearly to identify cases of excess N supply, P stimulation of uptake of mineral N and N discrimination in specific plant parts in a short-term experiment.

Experiments with different plant genotypes and bacteria with differing N_2 fixation efficiencies will help determine levels of N and P supply that are needed to optimise plant growth and N_2 fixation in *H. rhamnoides*. The N difference method is a useful technique for estimating N_2 fixation (Table 2), but only when the reference plant is of the same species, but non-inoculated, and plants are receiving the same level of N.

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REFERENCES

Arnone, J.A., Kohls, S.J., and Baker, D.D. 1994. Nitrate effects on nodulation and nitrogenase activity of actinorhizal *Casuarina* studied in split-root systems. *Soil Biology and Biochemistry* **26**: 599–606.

Beveridge, T., Li, T.S.C., Oomah, B.D., and Smith, A. 1999. Sea buckthorn products: manufacture and composition. *Journal of Agriculture and Food Chemistry* **47**: 3480–3488.

Bond, G. and Mackintosh, A.H. 1975. Effect of nitrate-nitrogen on the nodule symbioses of *Coriaria* and *Hippophaë*. *Proceedings of the Royal Society of London Series B: Biological Sciences* **190**: 199–209.

Carroll, B.J. and Gresshoff, P.M. 1983. Nitrate inhibition of nodulation and nitrogen fixation in white clover. *Zeitschrift für Pflanzenphysiologie* **110**: 77–88.

Cifen, B., Yuanlin, T., Fugui, W., and Zhe, W. 2002. Discussion on the coordinate functions of sea buckthorn in the sustainable use of water and land resources in soft rock area. In: *Proceedings 12th International Soil Conservation Organization Conference. Technology and Method of Soil and Water Conservation*. Beijing, China. Tsinghua University Press. Vol. III, pp. 19–22.

Ekblad, A. and Huss-Danell, K. 1995. Nitrogen fixation by *Alnus incana* and nitrogen transfer from *A. incana* to *Pinus sylvestris* influenced by macronutrients and mycorrhiza. *New Phytologist* **131**: 453–459.

Evans, R.D. 2001. Physiological mechanisms influencing plant nitrogen isotope composition. *Trends in Plant Sciences* **6**: 121–126.

Gentili, F. and Huss-Danell, K. 2002. Phosphorus modifies the effects of nitrogen on nodulation in split-root systems of *Hippophaë rhamnoides*. *New Phytologist* **153**: 53–61.

Gentili, F. and Huss-Danell, K. 2003. Local and systemic effects of phosphorus and nitrogen on nodulation and nodule function in *Alnus incana*. *Journal of Experimental Botany* **54**: 2757–2767.

Hellsten, A. and Huss-Danell, K. 2001. Interaction effects of nitrogen and phosphorus on nodulation in red clover (*Trifolium pratense* L.). *Acta Agriculturae Scandinavica. Section B, Soil and Plant Science* **50**: 135–142.

Högberg, P. 1997. N-15 natural abundance in soil-plant systems. Tansley Review No. 95. *New Phytologist* **137**: 179–203.

Högberg, P., Högberg, M.N., Quist, M.E., Ekblad, A., and Näsholm, T. 1999. Nitrogen isotope fractionation during nitrogen uptake by ectomycorrhizal and non-mycorrhizal *Pinus sylvestris*. *New Phytologist* **142**: 569–576.

Huss-Danell, K. 1978. Nitrogenase activity measurements in intact plants of *Alnus incana*. *Physiologia Plantarum* **43**: 372–376.

Huss-Danell, K. 1997. Actinorhizal symbioses and their N_2 fixation. Tansley Review No. 93. *New Phytologist* **136**: 375–405.

Huss-Danell, K. and Hahlin, A.S. 1988. Nitrogenase activity decay and energy supply in *Frankia* after addition of ammonium to the host plant *Alnus incana*. *Physiologia Plantarum* **74**: 745–751.

Huss-Danell, K., Sellstedt, A., Flower-Ellis, A., and Sjöström, M. 1982. Ammonium effects on function and structure of nitrogen-fixing root nodules of *Alnus incana* (L.) Moench. *Planta* **156**: 332–340.

Israel, D.W. 1987. Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiology* **84**: 835–840.

Kolb, K.J. and Evans, R.D. 2002. Implications of leaf nitrogen recycling on the nitrogen isotope composition of deciduous plant tissues. *New Phytologist* **156**: 57–64.

Ledgard, S.F. 1989. Nutrition, moisture and rhizobial strain influence isotopic fractionation during N_2 fixation in pasture legumes. *Soil Biology and Biochemistry* **21**: 65–68.

Leidi, E.O. and Rodriguez-Navarro, D.N. 2000. Nitrogen and phosphorus availability limit N_2 fixation in bean. *New Phytologist* **147**: 337–346.

Li, T.S.C. and Schroeder, W.R. 1996. Sea buckthorn (*Hippophaë rhamnoides*): a multipurpose plant. *HortTechnology* **6**: 370–380.

Lumini, E. and Bosco, M. 1996. PCR- restriction fragment length polymorphism identification and host range of single- spore isolates of the flexible *Frankia* sp. strain UFI 132715. *Applied Environmental Microbiology* **62**: 3026–3029.

Mariotti, A. 1983. Atmospheric nitrogen is a reliable standard for natural ^{15}N abundance measurement. *Nature* **303**: 685–687.

Norlin, E., Irgum, K., and Ohlsson, K.E.A. 2002. Determination of the $^{15}N/^{14}N$ ratio of ammonium and ammonia in aqueous solutions by equilibrium headspace-gas chromatography-combustion-isotope ratio mass spectrometry. *The Analyst* **127**: 735–740.

Ohlsson, K.E.A. and Wallmark, P.H. 1999. Novel calibration with correction for drift and non-linear response for continuous flow isotope ratio mass spectrometry applied to the determination of $\delta^{15}N$, total nitrogen, $\delta^{13}C$ and total carbon in biological material. *The Analyst* **124**: 571–577.

Sanginga, N., Okogun, J.A., Akobundu, I.O., and Kang, B.T. 1996. Phosphorus requirement and nodulation of herbaceous and shrub legumes in low P soils of Guinean Savanna in Nigeria. *Applied Soil Ecology* **3**: 247–255.

Silsbury, J.H., Catchpole, D.W., and Wallace, W. 1986. Effects of nitrate and ammonium on nitrogenase (C_2H_2 reduction) activity of swards of subterranean clover, *Trifolium subterraneum* L. *Australian Journal of Plant Physiology* **13**: 257–273.

Stamford, N.P., Ortega, A.D., Temprano, F., and Santos, D.R. 1997. Effects of phosphorus fertilization and inoculation of *Bradyrhizobium* and mycorrhizal fungi on growth of *Mimosa caesalpiniaefolia* in an acid soil. *Soil Biology and Biochemistry* **29**: 959–964.

Streeter, J. 1988. Inhibition of legume nodule formation and N_2 fixation by nitrate. *CRC Critical Reviews in Plant Sciences* **7**: 1–23.

Tang, C., Hinsinger, P., Drevon, J.J., and Jaillard, B. 2001. Phosphorus deficiency impairs early nodule functioning and enhances proton release in roots of *Medicago truncatula* L. *Annals of Botany* **88**: 131–138.

Troelstra, S.R., Blacquière, T., Wagenaar, R., and Van Dijk, C. 1987. Ionic balance, proton efflux, nitrate reductase activity and growth of *Hippophae rhamnoides* L. ssp. *rhamnoides* as influenced by combined-N nutrition or N_2 fixation. *Plant and Soil* **103**: 169–183.

Valverde, C., Ferrari, A., and Wall, L.G. 2002. Phosphorus and the regulation of nodulation in the actinorhizal symbiosis between *Discaria trinervis* (Rhamnaceae) and *Frankia* BCU110501. *New Phytologist* **153**: 53–61.

Wall, L.G., Hellsten, A., and Huss-Danell, K. 2000. Nitrogen, phosphorus, and the ratio between them affect nodulation in *Alnus incana* and *Trifolium pratense*. *Symbiosis* **29**: 91–105.