

Effects of NO_3^- availability on NO_3^- use in seedlings of three woody shrub species

LINA KOYAMA^{1–3} and NAOKO TOKUCHI⁴

¹ Laboratory of Forest Ecology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

² Present address: Laboratory of Ecology, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan

³ Author to whom correspondence should be addressed (linak@kenroku.kanazawa-u.ac.jp)

⁴ Laboratory of Silviculture, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

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Summary We determined the effects of short-term cultivation with various amounts of available nitrate nitrogen (NO_3^-) on NO_3^- use by woody shrub species. Nitrate concentration ($[\text{NO}_3^-]$) and nitrate reductase activity (NRA) were measured in leaves and roots of seedlings of *Hydrangea hirta* (Thunb.) Siebold, *Lindera triloba* (Sieb. et Zucc.) Blume and *Pieris japonica* (Thunb.) D. Don. Root $[\text{NO}_3^-]$ increased with increasing NO_3^- supply in all species, whereas leaf $[\text{NO}_3^-]$ remained low. There were significant correlations between $[\text{NO}_3^-]$ in roots and leaves in all species, but no correlation was found between root NRA and leaf NRA. The low proportion of leaf NO_3^- assimilation to total NO_3^- assimilation in all species can be ascribed to the lack of NO_3^- transport from roots to leaves. In all species, root NRA increased with increasing NO_3^- supply until reaching a plateau. Species ranking based on maximum root NRA was *H. hirta* > *L. triloba* > *P. japonica*. Root NRA in *P. japonica* was low, even though root $[\text{NO}_3^-]$ increased with NO_3^- supply, indicating that NO_3^- was not an effective N source for this species. The ranking also suggested that *H. hirta* depended more on NO_3^- as an N source than *L. triloba*. The increase in root NRA with increasing NO_3^- supply was greater in *H. hirta* than in *L. triloba*, possibly indicating that a change in NO_3^- availability has a stronger influence on NO_3^- use in *H. hirta* than in *L. triloba*.

Keywords: *Hydrangea hirta*, *Lindera triloba*, nitrate concentration, nitrate reductase activity (NRA), *Pieris japonica*, responsiveness.

Introduction

Species-specific patterns of nitrogen (N) use have been studied to understand how species coexist in N-limited ecosystems, and to predict the responses of ecosystems to additional N inputs (Wedin and Tilman 1990, Högbom and Ohlson 1991, Miller and Bowman 2002). There have been many studies to determine the ability of species to use nitrate nitrogen (NO_3^-) in natural conditions (Stewart et al. 1988, Stadler and Gebauer

1992) and to examine the effects of N addition on plant NO_3^- use (Downs et al. 1993, Truax et al. 1994, Rothstein et al. 1996). The flexibility and responsiveness of plant N use has also been investigated by comparing plants grown with and without N addition (Fredeen et al. 1991, Malagoli et al. 2000). However, to understand the mechanisms underlying species coexistence in N-limited ecosystems, it is necessary to assess species responsiveness to a natural gradient of N availability under field conditions.

There are two major N sources for plants: nitrate (NO_3^-) and ammonium (NH_4^+). After uptake, NH_4^+ is directly assimilated into an organic form, whereas enzymes such as nitrate reductase (NR) are required to reduce NO_3^- to NH_4^+ before organic N can be synthesized (Gebauer and Schulze 1997). Nitrate reductase, which catalyzes the first and rate-limiting step in the NO_3^- assimilation pathway (Beevers and Hageman 1969), is a substrate-inducible enzyme, but the capacity for induced NR synthesis differs markedly among plant species and some species lack the capacity for induced NR synthesis and hence, are unable to use NO_3^- as an N source (Al Gharbi and Hipkin 1984, Smirnov et al. 1984, Gebauer et al. 1988). Therefore, in vivo NRA is an important indicator of plant NO_3^- use, and the presence of NO_3^- in plants is evidence for plant NO_3^- uptake because plants do not have the ability to synthesize NO_3^- .

We investigated NO_3^- use by three shrub species that showed different spatial distributions at the landscape scale. These species, *Pieris japonica* (Thunb.) D. Don (Ericaceae), *Hydrangea hirta* (Thunb.) Siebold (Saxifragaceae) and *Lindera triloba* (Sieb. et Zucc.) Blume (Lauraceae) were dominant on a short continuous slope in a conifer plantation where soil N transformations have been intensively investigated (Hirobe et al. 1998). In this conifer plantation, *P. japonica* was dominant in the area where soil nitrification rate was low and *H. hirta* was dominant in the area where soil nitrification rate was high. In contrast, *L. triloba* grew in both areas, coexisting with each of the other species (Koyama et al., unpublished observations). Relationships were also found between these three species and

soil NO_3^- pool sizes in the plant rooting zone. Soil associated with *P. japonica* had small NO_3^- pools and soil associated with *H. hirta* generally had large NO_3^- pools, whereas both large and small NO_3^- pools were observed in soil from the rooting zone of *L. triloba* (Figure 1). Thus, although there was some overlap, the spatial distributions of the three species differed in a way that reflected variation in soil NO_3^- availability. Based on these relationships between species and soil, we predicted that each species has a specific pattern of NO_3^- use corresponding to the soil NO_3^- pool sizes within their natural range. That is, species occurring only on soils with a low nitrification rate (e.g., *P. japonica*) do not use NO_3^- , whereas species occurring only on soils with high nitrification rates (e.g., *H. hirta*) do use NO_3^- . We also predicted that the extent of distribution of a species is related to its sensitivity and flexibility to changes in NO_3^- availability, i.e., species that occurred regardless of soil nitrification rate (e.g., *L. triloba*) may be less responsive or more flexible, or both, with respect to NO_3^- availability.

The study objective was to elucidate the relationship between the characteristic NO_3^- use pattern of the study species and the soil conditions where they naturally occur. Specifically, we investigated the response of plants to NO_3^- supply based on the following hypotheses: (1) *P. japonica* has little or no ability to take up and use NO_3^- ; whereas (2) *H. hirta* and *L. triloba* both take up and use NO_3^- ; hence (3) their responses to NO_3^- availability differ. To examine these hypotheses, leaf and root nitrate concentration ($[\text{NO}_3^-]$) and nitrate reductase activity (NRA) were measured in seedlings of each species grown in perlite medium and supplied with various amounts of NO_3^- . To investigate NO_3^- use of the species under a range of field conditions, NO_3^- retention in the perlite cultivation medium was taken as an indicator of NO_3^- availability and compared with the NO_3^- pool size in soil from a forest where these species occur naturally.

Materials and methods

Plant cultivation and treatment

Seeds of *L. triloba* were collected from a tree at Mt. Ryuoh in Shiga Prefecture, central Japan (35°10' N, 136°20' E) in September 1997 and stored at ~8 °C until sown in horticultural

soil in April 1998. Because seeds of *H. hirta* and *P. japonica* were unavailable, naturally established seedlings were collected in the vicinity of the *L. triloba* seed tree and transplanted to containers. At the start of the treatments, in September 1999, *L. triloba* seedlings were 1 year old, whereas *H. hirta* and *P. japonica* seedlings were of various ages.

Seedlings were extracted from the rooting medium and the roots were washed with tap water followed by deionized water. Seedlings were then transplanted singly to plastic pots filled with ~600 ml of perlite washed with deionized water. The transplanted seedlings were placed outdoors and supplied with 200 ml of nutrient solution containing: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.35 mmol l⁻¹; KCl 0.63 mmol l⁻¹; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.5 mmol l⁻¹; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 mmol l⁻¹; Fe-EDTA 59.37 mmol l⁻¹; Cu-EDTA 0.43 μmol l⁻¹; Zn-EDTA 0.42 μmol l⁻¹; Mn-EDTA 0.45 μmol mol l⁻¹; H_3BO_3 32.35 μmol l⁻¹; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.41 μmol mol l⁻¹; as well as NO_3^- . Nitrate nitrogen was added to the solution as NaNO_3 at 0, 0.179, 0.714, 1.785, 3.570 and 7.139 mmol l⁻¹. A preliminary experiment indicated that there was a significant correlation between the concentration of supplied NO_3^- and the amount of NO_3^- retained in the perlite (Koyama et al. 2001). The concentrations of NO_3^- retained in the perlite estimated from the regression of NO_3^- supply and NO_3^- retention were 0, 0.069, 0.08, 0.69, 1.38 and 2.76 mmol l⁻¹ in the treatments supplied with 0, 0.179, 0.714, 1.785, 3.570 and 7.139 mmol l⁻¹ NO_3^- , respectively. Ten replicates were prepared per NO_3^- treatment.

Plant analysis

Leaves and roots were collected between 1100 and 1300 h on the day following (i.e., 17–19 hours after) treatment with NO_3^- nutrient solutions. Wilted plants were excluded from the analysis; consequently, the number of replicates was reduced in several treatments. Samples were kept at 4 °C for several hours before measurement of in vivo NRA by a modified version of the in vivo test (Jaworski 1971, Högberg et al. 1986). Storage effects were similar for all samples because, following a decline during the first 30 min after sample collection, changes in NRA occurred very slowly (Högberg et al. 1986). From each individual, 200 2.5-mm-diameter leaf disks were obtained and ~100 mg (dry mass) of fine roots (diameter < 2 mm) were rinsed with deionized water and cut into ~5 mm

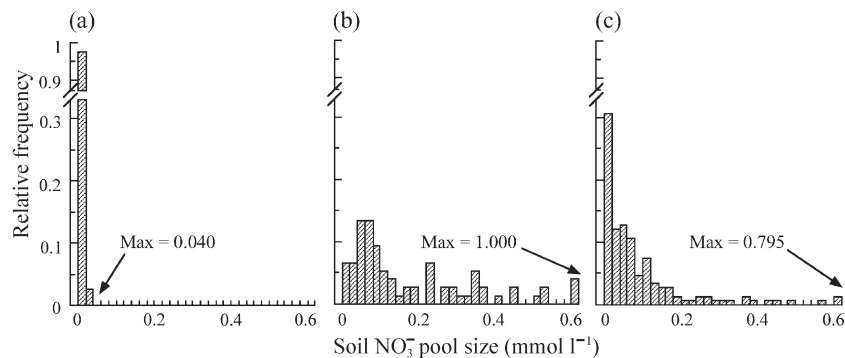


Figure 1. Relative frequency distribution of soil NO_3^- pool sizes associated with three species: (a) *P. japonica*, (b) *H. hirta* and (c) *L. triloba* (modified from Koyama et al. 2001 for *L. triloba*, and from Koyama et al. unpublished data for *P. japonica* and *H. hirta*). Soil samples were collected five times during the 1998 growing season within a 30-cm radius of the base of trees in a conifer plantation. Triplicate soil samples were collected from each sampling area of 10 plants of *L. triloba* and five for the other two species.

lengths. After vacuum infiltration (6 mm Hg; twice for 30 s each) with 5 ml of incubation buffer, the samples were incubated for 1 h at 30 °C in the dark. The composition of the incubation buffer was 0.1 mol l⁻¹ KNO₃, 0.1 mol l⁻¹ KH₂PO₄ and 3% 1-propanol, adjusted to pH 7.5 with NaOH. Enzyme activity was stopped by placing sample vials in hot water (80 °C). Leaves and roots were removed and weighed after oven-drying at 105 °C. The nitrite concentration ([NO₂⁻]) in the incubation buffer was measured colorimetrically by diazotization (Keeney and Nelson 1982). The effect of plant pigments was compensated for by measurement of complete controls lacking *N*-naphthylethylene diamine dihydrochloride (cf. Gebauer et al. 1998).

The remaining leaves and fine roots were dried at 40 °C, and ground with a sample mill (CMT, TI-100, Tokyo, Japan). When sufficient material was available, about 100 mg of ground sample was extracted with 10 ml of deionized water for 1 h at 45 °C. The extract was filtered and [NO₃⁻] in the extract was analyzed by HPLC within 72 h to avoid transformation of nitrate in the extract. Nitrate was separated on an anion exchange column (Shim-pack IC-A1, Shimadzu, Kyoto, Japan) connected to a guard column (IC-GA1, Shimadzu), and electrical conductivity was measured with a potassium hydrogen phthalate solution as the mobile phase.

Statistical analysis

All statistical analyses were conducted with the statistical program SPSS 7.5.1 (SPSS, Chicago, IL). Within-treatment differences among species were analyzed by Kruskal-Wallis one-way analysis of variance and multiple comparisons of mean values among species within a treatment were performed by the sequential Bonferroni test (Rice 1989) after determination of pairwise *P* values by the Mann-Whitney test.

The Michaelis-Menten equation was used to describe the relationship between the quantity of NO₃⁻ retained in the perlite rooting medium and NRA or [NO₃⁻] in the plants (King et al. 1992): $v = SV_{\max}/(S + K_m)$, where *v* is plant NRA or [NO₃⁻], *S* is estimated amount of NO₃⁻ retained in perlite rooting medium, *V*_{max} is maximum value of *v* and *K*_m is the Michaelis constant. Parameters *V*_{max} and *K*_m were calculated in an Eadie-Hofstee plot (i.e., the relationship between the quantity of supplied NO₃⁻ to the quantity of supplied NO₃⁻/NRA; Metzler 2001) and applied as initial values in nonlinear regression analysis in SPSS. Negative *K*_m values were taken to indicate that the Michaelis-Menten equation was inappropriate for relating NO₃⁻ supply to NRA or [NO₃⁻].

Spearman rank correlation coefficients (*r*_s) were calculated to detect pair-wise relationships between (1) NRA and [NO₃⁻] in leaves, (2) NRA and [NO₃⁻] in roots, (3) leaf and root NRA, and (4) leaf and root [NO₃⁻].

Results

Effects of NO₃⁻ availability on plant [NO₃⁻] and NRA

Root [NO₃⁻] increased significantly with increasing NO₃⁻ supply in all species (Figure 2a). Mean root [NO₃⁻] in *P. japonica*

was lower than in *L. triloba* in all treatments except in the 7.139 mmol l⁻¹ NO₃⁻ treatment when *P. japonica* had the higher concentration. The estimated *K*_m value for the relationship between root [NO₃⁻] and NO₃⁻ retained in perlite rooting medium in *P. japonica* was higher than 2.76 mmol l⁻¹, which was the [NO₃⁻] retained in perlite supplied with 7.139 mmol l⁻¹ NO₃⁻ (Table 1).

Unlike the other species, leaf [NO₃⁻] in *H. hirta* showed no increase with increasing NO₃⁻ availability (Figure 2b). When NO₃⁻ supply was low, there was no significant difference in leaf [NO₃⁻] among species. However, when the NO₃⁻ supply was greater than 3.570 mmol l⁻¹, *L. triloba* had higher leaf [NO₃⁻] than *H. hirta*.

Root NRA increased significantly with increasing NO₃⁻ availability, reaching plateaus in all species (Figure 3a). There was no significant difference in root NRA among species when plants received no NO₃⁻, but *H. hirta* had greater root NRA in all other treatments than the other species (Figure 3a). Estimated maximum root NRA was highest in *H. hirta* and lowest in *P. japonica*, differing by one order of magnitude (Table 1). The *K*_m value for root NRA of *P. japonica* was lower than in the other species.

Leaf NRA in *H. hirta* and *L. triloba* showed no significant increase with increasing supply of NO₃⁻ (Figure 3b). Although leaf NRA in *P. japonica* showed a significant relationship with NO₃⁻ availability, estimated maximum NRA was lower in leaves than in roots by an order of magnitude (Table 1). Leaf NRA was higher in *H. hirta* than in the other species irrespective of treatment (Figure 3b).

Relationships between NRA and [NO₃⁻]

Spearman rank correlation analysis between root [NO₃⁻] and root NRA showed significant correlations in *H. hirta* and *L. triloba* (*P* < 0.001), but no relationship in *P. japonica* (Table 2). There was no significant correlation between [NO₃⁻] and NRA in leaves of all species.

Relationships between root and leaf

Spearman rank correlation analysis showed that root [NO₃⁻] was significantly correlated with leaf [NO₃⁻] in all species (*P* < 0.05, Table 3). However, NO₃⁻ concentrations were mostly higher in roots than in leaves; leaf/root ratios of [NO₃⁻] were 0.94 ± 4.13, 0.30 ± 0.20 and 0.43 ± 0.48 (mean ± SD) in *H. hirta*, *L. triloba* and *P. japonica*, respectively. There was no correlation between root NRA and leaf NRA in any species.

Discussion

Partitioning of NO₃⁻ between roots and shoots

Leaf [NO₃⁻] was significantly correlated with root [NO₃⁻] in all species (Table 3), suggesting that NO₃⁻ transport from root to leaf was influenced by root [NO₃⁻]. However, leaf [NO₃⁻] was low compared with root [NO₃⁻] in each species (Figure 2). *Lindera triloba* and *P. japonica* had low leaf NRA and low leaf [NO₃⁻] (Figure 3b). In *H. hirta*, leaf NRA was higher than root

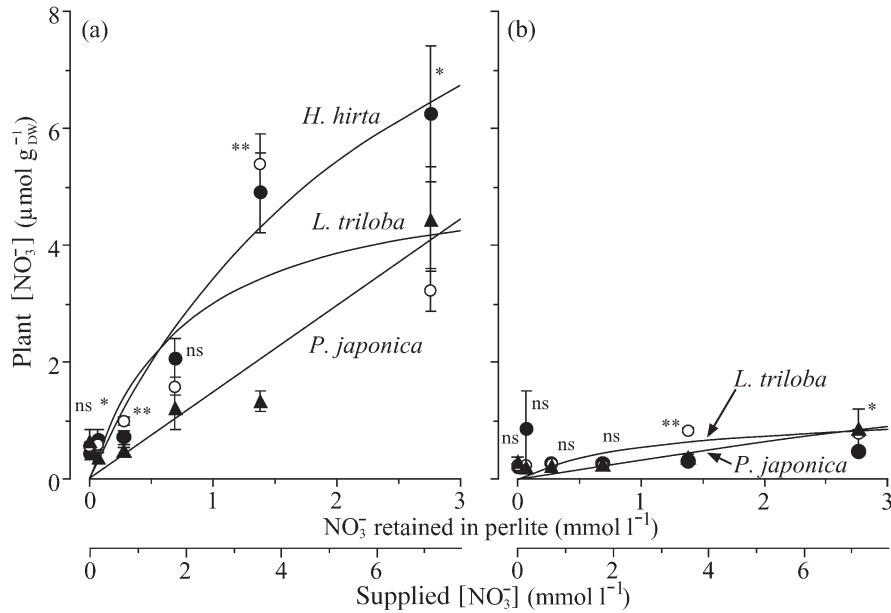


Figure 2. Effect of NO_3^- availability on plant NO_3^- concentration ($[\text{NO}_3^-]$). Mean $[\text{NO}_3^-]$ in (a) roots and (b) leaves of *H. hirta* (■), *L. triloba* (○) and *P. japonica* (▲). The curved lines were described by the Michaelis-Menten equation (see Table 1). The asterisks in figures indicate significant differences between species within treatments (* = $P < 0.05$; ** = $P < 0.01$; ns = not significant). Bars show SE.

NRA when NO_3^- supply was 0, but leaf NRA did not increase with increasing NO_3^- supply (Figure 3). Low leaf NRA in addition to low leaf $[\text{NO}_3^-]$ suggests that the amount of NO_3^- transported to leaves as a proportion of total NO_3^- uptake by roots was low.

For two reasons, it seems unlikely that transport of NO_3^- from roots to shoots requires longer than the 17 to 19 h of NO_3^- exposure provided. First, in the preliminary experiment, leaf $[\text{NO}_3^-]$ in *L. triloba* remained low even when a similar NO_3^- supply was maintained for 42 days (Koyama et al. 2001). Second, in many other woody species, NRA or NO_3^- was detected in leaves within 1 day of exposure to NO_3^- (e.g., Friemann et al. 1992, Min et al. 1998).

In some species, root NRA tends to increase, or the proportion of leaf NO_3^- assimilation tends to decrease in response to several factors including weak light (Li and Oaks 1994), low NO_3^- availability (Gojon et al. 1994), tissue immaturity (Haynes 1986) and high root temperature (Clarkson and Deane-Drummond 1983). According to a field investigation, substantial amounts of NO_3^- and NRA were detected in leaves of *H. hirta* and *L. triloba* in a conifer plantation where these species occurred naturally (Koyama et al., unpublished data).

In that study, the mean canopy openness of the overstory was only about 10% of that in the open-air nursery where the present experiments were conducted. The maximum NO_3^- availability in perlite in our study was higher than the maximum soil NO_3^- pool size in the conifer forest where some species were found to assimilate leaf NO_3^- (Figure 1), indicating that the amount of available NO_3^- was sufficient for NO_3^- transport to the shoots. Therefore, we conclude that interspecific differences, light and NO_3^- availability cannot explain the low $[\text{NO}_3^-]$ and NRA in leaves in our study. An increase in NO_3^- supply had little or no effect on $[\text{NO}_3^-]$ and NRA in leaves of any species (Figures 2 and 3). Accordingly, in our study, the contribution of leaves to whole plant NO_3^- assimilation likely decreased as external NO_3^- supply increased, although NRA in each compartment must be considered (Gebauer and Schulze 1997). In a preliminary observation, the percentages of leaf and root to total biomass were $47.8 \pm 3.3\%$ and $25.5 \pm 3.1\%$ for 1-year-old *L. triloba* seedlings, $5.4 \pm 4.5\%$ and $70.4 \pm 13.5\%$ for field-grown *H. hirta* and $19.1 \pm 9.0\%$ and $57.1 \pm 15.0\%$ for field grown *P. japonica*, respectively (mean \pm SD; Koyama unpublished data). Thus, the proportions of leaf biomass were unlikely to be high enough to compensate for the low NRA in

Table 1. Estimated V_{\max} and K_m for nitrate reductase activity (NRA) and NO_3^- concentration ($[\text{NO}_3^-]$) in roots and leaves of the study species.

Species	$[\text{NO}_3^-]$				NRA			
	Root		Leaf		Root		Leaf	
	V_{\max}	K_m	V_{\max}	K_m	V_{\max}	K_m	V_{\max}	K_m
<i>H. hirta</i>	13.101	2.865	—	ns ¹	0.762	0.178	—	ns
<i>L. triloba</i>	5.381	0.792	1.197	1.189	0.271	0.686	—	ns
<i>P. japonica</i>	2.978×10^7	2.017×10^7	7.131	20.411	0.061	0.032	0.016	0.007

¹ ns = Estimated K_m value was negative, indicating that the Michaelis-Menten equation did not describe the relationship.

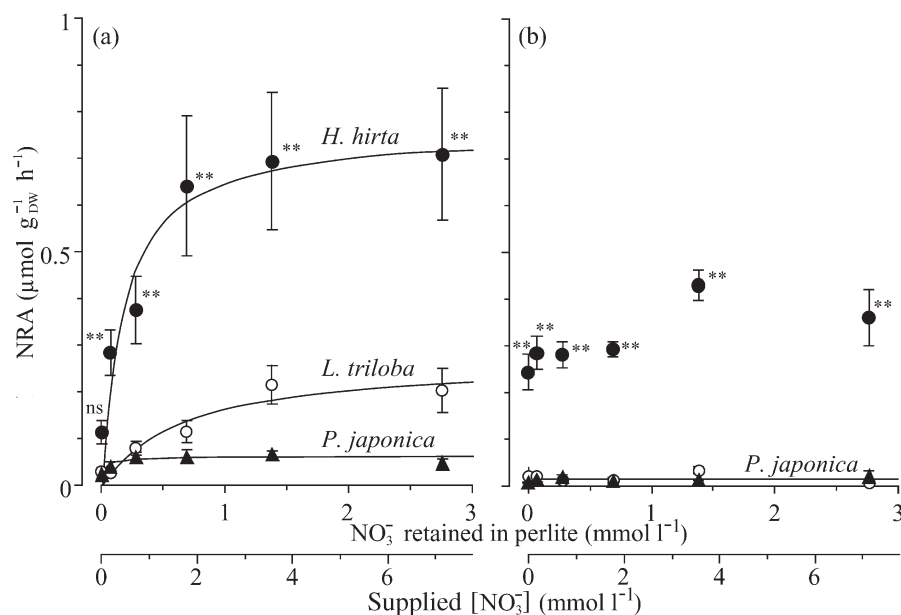


Figure 3. Effect of NO₃⁻ availability on plant nitrate reductase activity (NRA). Mean NRA in (a) roots and (b) leaves of *H. hirta* (●), *L. triloba* (○) and *P. japonica* (▲). The curved lines were described by the Michaelis-Menten equation (see Table 1). The asterisks indicate significant differences between species within treatments (* = $P < 0.05$; ** = $P < 0.01$; ns = not significant). Bars show SE.

leaves. In addition, the percentages of non-photosynthetic aboveground compartments (stems and twigs), which generally had low NRA, were about 25% in all species. Accordingly, the contribution of stems and twigs to whole-plant NO₃⁻ assimilation was unlikely to equal that of roots. Therefore, although further studies are required to elucidate the cause of the small quantity of NO₃⁻ transported from roots to leaves, the results demonstrate that roots played a major role in NO₃⁻ assimilation in seedlings of these species under the study conditions.

Effects of NO₃⁻ supply on plant [NO₃⁻] and plant NRA

Because leaves and stems contributed only slightly to whole-plant NO₃⁻ assimilation in our study, we consider only root NO₃⁻ assimilation when discussing the effects of NO₃⁻ supply on NO₃⁻ uptake and assimilation in the whole plant. Root [NO₃⁻] increased with NO₃⁻ supply in all species, although neither *H. hirta* nor *P. japonica* showed a tendency toward saturation over the range of NO₃⁻ concentrations provided (Figure 2a, Table 1). The amount of NO₃⁻ in a plant organ is not equivalent to plant uptake of NO₃⁻, because plant [NO₃⁻] is the difference between the increase in NO₃⁻ as a result of uptake and the decrease in NO₃⁻ as a result of reduction and translocation (Melzer et al. 1984, Chapin et al. 1990). Thus, in

H. hirta and *L. triloba*, plants with high root [NO₃⁻] may have taken up increased amounts of NO₃⁻ that could not be offset by NO₃⁻ reduction or translocation.

In all study species, the relationships between root NRA and NO₃⁻ supply tended to saturate (Figure 3, Table 1). Possible explanations for the saturation of plant NRA at certain concentrations of NO₃⁻ include: (1) a limit to the uptake of NO₃⁻; (2) a limit to the induction of NR; (3) inhibition of NRA by environmental factors, e.g., light (Lillo 1994), moisture (Sisson and Throneberry 1986, Erskine et al. 1996), temperature (Högberg et al. 1992), molybdenum availability (Yaneva et al. 2000) and pH (Shankar et al. 2000); and (4) inhibition of NRA by N compounds produced in the NO₃⁻ assimilation process (Oaks 1994). When 1.785 mmol NO₃⁻ l⁻¹ was supplied (i.e., the [NO₃⁻] in perlite was 0.69 mmol l⁻¹), NRA in *H. hirta* roots reached ~80% of the saturation point (V_{max}), whereas [NO₃⁻] was less than 20% of the saturation point and increased with further increases in NO₃⁻ supply (Figures 2 and 3, Table 1). These results indicate that, at the saturation point of NRA, the external NO₃⁻ supply did not limit uptake of NO₃⁻ and internal [NO₃⁻] was sufficient to induce NR in root cells of this species; consequently, the first and second explanations are implausible. It is known that environmental factors such as light, tem-

Table 2. Spearman rank correlation coefficients (r_s) between root nitrate concentration ([NO₃⁻]) and root nitrate reductase activity (NRA), and between leaf [NO₃⁻] and leaf NRA in each species.

Species	Root			Leaf		
	<i>n</i>	r_s	<i>P</i>	<i>n</i>	r_s	<i>P</i>
<i>H. hirta</i>	42	0.551	<0.001	50	0.037	0.799
<i>L. triloba</i>	59	0.685	<0.001	56	0.025	0.857
<i>P. japonica</i>	52	0.148	0.294	53	0.061	0.664

Table 3. Spearman rank correlation coefficients (r_s) between nitrate concentration ([NO₃⁻]) in roots and leaves, and between nitrate reductase activity (NRA) in roots and leaves of each species.

Species	[NO ₃ ⁻]			NRA		
	<i>n</i>	r_s	<i>P</i>	<i>n</i>	r_s	<i>P</i>
<i>H. hirta</i>	42	0.377	0.014	54	0.178	0.197
<i>L. triloba</i>	55	0.650	<0.001	60	0.033	0.805
<i>P. japonica</i>	54	0.476	<0.001	53	-0.003	0.983

perature and water availability influence NRA. However, NO_3^- availability has an overriding effect on NRA because NR is a substrate-inducible enzyme. Thus, even when sufficient NO_3^- was available, other environmental factor(s) may repress NRA, leading to saturation. Inhibition of NRA by its products including NH_4^+ and products of NH_4^+ assimilation has been observed in many plant species (Haynes and Goh 1978, Oaks 1994). Thus, the third or fourth explanation could account for the saturation of NRA.

Species differences in NO_3^- use

Because genetic composition influences NRA (Johnsen et al. 1991), effects of seedling sources need to be clarified before interspecific comparisons are made. All seedlings of *L. triloba* were from one mother tree, whereas we studied naturally established seedlings of the other species. We predicted that *L. triloba* would show less variation in NRA than the other species; however, the coefficient of variation showed that this was not the case (data not shown; cf. Figure 3). The higher than expected variance in NRA in the dioecious *L. triloba* might have been caused by the diverse pollen source.

Pteris japonica was unable to use NO_3^- , because it had little or no root and leaf NRA even when supplied with a high $[\text{NO}_3^-]$ (Figure 3). On the other hand, *H. hirta* and *L. triloba* both possessed root NRA and used NO_3^- .

Low NRA in *P. japonica* leaves has also been observed in a field experiment (Koyama et al., unpublished data), and an experiment to induce NR in detached leaves of *P. japonica* showed that they produce little NR even when NO_3^- is present (Routley 1972). Nevertheless, root $[\text{NO}_3^-]$ in *P. japonica* increased with NO_3^- availability, and the estimated V_{max} for root $[\text{NO}_3^-]$ in *P. japonica* was highest among the three species (Figure 2, Table 1). The maximally induced $[\text{NO}_3^-]$ in roots of *P. japonica* could not be determined under field conditions, because K_m for *P. japonica* roots was much higher than the maximum NO_3^- pool size in the soil where *P. japonica* is found (0.040 mol l^{-1} ; Figure 1). However, root $[\text{NO}_3^-]$ in *P. japonica* supplied with $7.139 \text{ mmol NO}_3^- \text{ l}^{-1}$ was not significantly lower than in *H. hirta*, indicating substantial NO_3^- uptake by roots (Figure 2a). On the other hand, NRA in both roots and leaves of *P. japonica* remained low when NO_3^- availability was high, whereas the other species showed an increase in root NRA with increasing NO_3^- supply (Figure 3). These results indicate that *P. japonica* took up NO_3^- when NO_3^- was present, but failed to assimilate it. It is known that many ericaceous species do not use NO_3^- (Smirnov et al. 1984, Gebauer et al. 1988), and we conclude that *P. japonica* is one of them.

Studies of the responses of plant NO_3^- use to NO_3^- supply have revealed marked differences among woody species. For example, Malagoli et al. (2000) showed that the responsiveness and flexibility of NO_3^- use were higher in deciduous European larch than in evergreen Scots pine in a short-term solution culture with and without NO_3^- supply. Truax et al. (1994) found that two deciduous species differing in nutrient status, shade tolerance, shoot growth pattern and root morphology had different N demands: red ash showed higher N demands

than red oak. Downs et al. (1993) reported that responses of plant NO_3^- use to NO_3^- supply differed in different organs of four woody species. Similarly, we found considerable differences in NO_3^- use by species that can use NO_3^- , i.e., *H. hirta* and *L. triloba*. Induced root NRA in *H. hirta* was significantly higher than in *L. triloba*, but constitutive NRA, i.e., NRA in the absence of a NO_3^- supply, did not differ among species (Figure 3a). The estimated K_m value for root NRA was lower for *H. hirta* than for *L. triloba* (Table 1), indicating that the increase in root NRA was greater in *H. hirta* than in *L. triloba* when NO_3^- supply was low.

The soil NO_3^- pool size at the natural growth site of *H. hirta* and *L. triloba* ranged from 0 to 1.0 and from 0 to 0.8 mmol l^{-1} , respectively (Figure 1). Both NRA induced by NO_3^- under a range of field concentrations (0 to 1.0 mmol l^{-1}) and V_{max} for NRA were lower in *L. triloba* than in *H. hirta*. These findings indicate that *L. triloba* is less dependent on NO_3^- as an N source than *H. hirta*; accordingly, NH_4^+ and organic N were more important N sources for *L. triloba* than for *H. hirta*. This is consistent with the pattern of mycorrhizal symbiosis of *L. triloba* in natural conditions. *Lindera triloba* grown at a low NO_3^- site showed high infection rates of arbuscular mycorrhizal fungi (Fujimaki et al. 2001), which may facilitate the uptake of organic N. On the other hand, when NO_3^- in perlite ranged from 0 to 1.0 mmol l^{-1} , root NRA increased more in *H. hirta* than in *L. triloba* (Figure 3a). Thus, *H. hirta* is probably more responsive to changes in NO_3^- availability than *L. triloba* under field conditions.

Our hypothesis that the species distribution is related to the responsiveness of plant NO_3^- use to varying NO_3^- supply was supported by the results. It is possible that species-specific NO_3^- use patterns enable these three species to coexist under natural conditions. However, consideration of temporal differentiation in NO_3^- use may also provide important and interesting information on the mechanisms of species coexistence as suggested by Ohlson and Högbom (1993).

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