Effects of different nitrogen sources on growth, chlorophyll concentration, nitrate reductase activity and carbon and nitrogen distribution in *Araucaria angustifolia*

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The southern Brazilian highland plateau is a mosaic of two contrasting plant communities, *Araucaria* forests and grasslands, both differing in the relative abundances and spatial patterns of soil nitrate and ammonium. However, we still do not know the inorganic N preferences of one key species in this mosaic, *Araucaria angustifolia*, the dominant tree species in the *Araucaria* forests and an important tree species invading the adjacent grasslands. Growth responses measured in a greenhouse study demonstrated that the species prefers NH_4^+ over NO_3^- as an inorganic N source. When provided alone, NO_3^- induced N deficiency symptoms: increases in root: shoot ratio, root branching and leaf mass per area, thickening of the shoot apexes and decreased mass-based chlorophyll and N concentrations of the young leaves. Nitrate-based nutrition also affected the whole plant N and carbon (C) distribution: young leaves accumulated less N and showed a larger C:N ratio than mature leaves. The nitrate reductase activity (NRA) followed the pattern of root: shoot partitioning expected for temperate climate conifers, i.e. activity concentrated in roots. However, the presence of NRA even under sole NH_4^+ nutrition indicates that plants may show constitutive levels of the enzyme, or that low levels of NO_3^- (possibly formed by contamination of the growth media) can induce leaf NRA. We suggest that *A. angustifolia* uses ammonium as a preferential inorganic N source, and that this preference may favor a more successful establishment in grassland than in forest areas.

Key Words: Araucariaceae, ammonium, inorganic nitrogen, nitrate, nitrogen deficiency.

Efeitos de diferentes fontes de nitrogênio no crescimento, concentração de clorofila, atividade de nitrato redutase e distribuição de carbono e nitrogênio em *Araucaria angustifolia*: o planalto sul-brasileiro apresenta um mosaico de duas comunidades vegetais contrastantes, florestas com *Araucaria* e campos, que diferem em suas abundâncias relativas e nos padrões espaciais de nitrato e amônio no solo. Entretanto, ainda não se conhecem as preferências quanto ao N inorgânico de uma espécie-chave neste mosaico, *Araucaria angustifolia*, a espécie arbórea dominante nas florestas com *Araucaria* e uma importante espécie florestal que invade os campos adjacentes. Respostas de crescimento medidas em um estudo em casa de vegetação demonstraram que a espécie prefere o NH_4^+ ao NO_3^-, como fonte de N inorgânico. Quando fornecido isoladamente, o NO_3^- induziu sintomas de deficiência de N: aumentos na razão raiz:parte aérea, ramificação radicular e massa foliar por área, espessamento dos ápices caulinares e redução

nas concentrações por unidade de massa de clorofila e N, nas folhas jovens. Uma nutrição baseada em nitrato também afetou a distribuição de N e C na planta como um todo: as folhas jovens acumularam menos N e mostraram uma maior razão C:N do que as folhas maduras. A atividade da redutase do nitrato (ARN) seguiu o padrão de partição entre raiz e parte aérea esperado para coníferas de clima temperado (atividade concentrada nas raízes). No entanto, a presença de ARN mesmo sob nutrição exclusivamente com NH_4^+ indica que as plantas devem ter níveis constitutivos da enzima ou que baixos níveis de NO_3^- (possivelmente gerado pela contaminação do meio de cultivo) podem induzir ARN. Sugere-se que *A. angustifolia* tem no amônio sua forma preferencial de N inorgânico e que esta preferência pode favorecer um melhor estabelecimento nos campos do que nas florestas.

Palavras-chave: Araucariaceae, amônio, deficiência de nitrogênio, nitrato, nitrogênio inorgânico.

INTRODUCTION

The southern Brazilian highland plateau is a mosaic of two contrasting plant communities, *Araucaria* forests and grasslands ("campos"), which differ not only in terms of vegetation structure and species composition, but also in terms of relative abundances and spatial patterns of inorganic nitrogen forms, nitrate (NO_3^-) and ammonium (NH_4^+) (Garbin et al., 2006). Thus, different strategies of nitrogen acquisition will affect the relative ability of plants to successfully establish in these contrasting soil environments. However, the inorganic N preferences of plant species which inhabit these plant communities are still poorly understood.

There are differences in the form of N that plants preferentially use (Bijlsma et al., 2000), and both NO_3^- and NH_4^+ may comprise about 70% of the total cations and anions taken up by plants (Beusichem et al., 1988). The form of N preferred by a tree is not necessarily the most easily absorbed one (Arnold and Diest, 1991), such that plants which grow better with NO_3^- may absorb more NH_4^+ instead (Kronzucker et al., 2003). For this reason, dry mass accumulation may be considered a more robust indicator of N preference than the rate of absorption (e.g. Gigon and Rorison, 1972; Rorison, 1985; Falkengren-Grerup and Lakenborg-Kristensen, 1994), even though dry mass accumulation alone does not inform us about the plasticity required by a plant to cope with different N forms. Other variables related to N metabolism, such as nitrate reductase activity (NRA), chlorophyll concentration, root morphology and resource allocation are important for understanding the growth regulation processes of a given plant species when exposed to different forms of inorganic N.

Simões (1973) demonstrated the major impact of N availability on the initial growth of *A. angustifolia*, and Franco et al. (2005) quantified carbon (C) and N in leaves, roots and stems of young and mature individuals and showed that, particularly for young plants, roots are important reservoirs of these elements. Duarte and Dillenburg (2000) detected very low NRA in young plants and found that the activity was concentrated in the roots, suggesting a NH_4^+-N preference by the species. These studies indicate an important role of roots in the C and N metabolism of the species. In the present study, we aimed to establish whether *A. angustifolia* shows a preference for a given form of inorganic N, by comparing growth and other selected physiological processes among young plants growing under sole or combined nutrition with NO_3^--N and NH_4^+-N. Given the existing evidence that N metabolism is mostly concentrated in roots, it is hypothesized that ammonium is the N form that promotes the greatest growth of *A. angustifolia*.

MATERIALS AND METHODS

Growth conditions: The experiment was conducted in a glass greenhouse of the Soil Department, Federal University of Rio Grande do Sul (30º01' S, 51º13' W, 4 m a.s.l.), Brazil, without temperature and humidity controls. Commercially obtained seeds from São Francisco de Paula (29º24' S, 50º22' W, 912 m a.s.l.), Rio Grande do Sul State, Brazil, were disinfested with a 2.5% solution of sodium hypochloride for 30 min, and thoroughly rinsed in distilled water thereafter. The upper third (radicle-protruding region) of the seed had its external covers removed to accelerate germination (Áquila and Ferreira, 1984). Germination took place in plastic trays filled with wet, autoclaved (120ºC for 1 h) vermiculite, under laboratory conditions. After 10 d, germinated seeds (radicle length with 6 ± 0.58 cm; mean ± SD) were planted in PVC tubes (height = 45 cm; diameter = 10 cm), which allowed

free drainage through the bases. Sand (36% fine - $\varnothing = 0.05$ – 0.2 mm - and 64% coarse - $\varnothing = 0.2 – 2.0$ mm – sand, according to analyses performed by the Soil Department of Federal University of Rio Grande do Sul, Brazil) was used as substrate for plant growth to prevent NH_4^+ adsorption to solid particles (Arnold and Diest, 1991). Sand was washed thoroughly and sequentially using tap water, distilled water, HCl solution (pH 2.8) and again with distilled water. Each pot was filled with 3.8 kg of sand.

Experimental design: Growing plants were fed with three different nutrient solutions, all based on a modified Hoagland and Arnon (Taiz and Zeiger, 2004) nutrient solution (Table 1). These solutions differ from each other in terms of the supplied N form(s): combined supply of NO_3^--N and NH_4^+-N ($NO_3^- + NH_4^+$ treatment); only NO_3^--N (NO_3^- treatment); and only NH_4^+-N (NH_4^+ treatment). In all cases, concentration of total N was kept the same (4 mM) and pH was adjusted to 5.1. Ionic strength, concentration and activity of each component, and charge differences of the nutrient solutions were checked using Visual Minteq V. 2.30 (Allison et al., 1991; Gustafsson, 2004).

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Table 1. Composition and concentrations (mM) of each of the three nutrient solutions used in the study. Micronutrients (mg L^{-1}): B (H_3BO_3) 0.541; Cu ($CuSO_4 \cdot 5H_2O$) 0.032; FeEDTA ($FeSO_4 \cdot 7H_2O$ + NaEDTA) 65; Mn ($MnSO_4 \cdot H_2O$) 0.11; Mo ($H_2Mo_4O_4$ [85% MoO_3]) 0.048; Zn ($ZnSO_4 \cdot 7H_2O$) 0.131. Other salts used in the nutrient solutions: KNO_3, $NH_4H_2PO_4$, KCl, $CaCl_2$, KH_2PO_4, and $MgSO_4$.

Nutrients	Treatments		
	NO_3^-	NH_4^+	$NO_3^- + NH_4^+$
NO_3^--N	4	-	2
NH_4^+-N	-	4	2
P	4	4	4
K	8	4	4
Ca	2	2	2
Mg	1	1	1
S	1	1	1
Cl	4	8	4

Treatments were assigned to 45 experimental units (15 plants per treatment), following a randomized complete block design (five blocks, each consisting of nine plants, grouped together on the greenhouse bench). Pots were watered on a weekly basis (400 mL of distilled water) in the period between planting and first application of the nutrient solutions (starting 54 d after planting). Nutrient solutions were applied to the sand surface on a weekly basis, alternating one application of 750 mL (vessel capacity) with another of 100 mL (winter) or 250 mL (summer), sufficient amounts to bring substrate to vessel capacity. Before each application of 750 mL, the substrate was washed with 400 mL of distilled water, applied to the surface, in order to minimize salt accumulation (Pereira et al., 1996) and nitrifying effects in those treatments that received NH_4^+.

Growth measurements: On a weekly basis, starting on the first day of nutrient solution application, plant height and total shoot length (height plus length of lateral branches) were measured. After 290 d (from planting day), the experiment ended and plants were harvested. Oven-dried (50°C) mass (total, shoot, and main and lateral roots), basal and apical stem diameters, and main (tap) root length were evaluated. The number and length of first order lateral roots per unit of main root length, and the number of second order roots per unit of first order root length were also measured. A sample of 20 mature leaves of each plant was collected for leaf mass per area (LMA) determination, using a portable leaf area meter (LI-3000, LI-COR, Lincoln, USA) for the leaf area measurements.

Chlorophyll concentration: Samples of 20 (NO_3^- treatment) or nine (NH_4^+ treatment) apical leaves from the top-most lateral branches were collected for chlorophyll (*a*, *b* and total) measurements. Young leaves were selected because they appeared much paler than the others, particularly in the NO_3^- treatment. Chlorophyll was extracted with 100% ethanol (Knudson et al., 1977). The analyses of absorbance (649 and 665 nm) were made with a spectrophotometer (Cintra 5 GBC UV-VIS, Victoria, Australia). Chlorophyll concentration was calculated according to Wintermans and De Mots (1965), and expressed on a leaf dry mass basis.

Nitrate reductase activity: The *in vivo* NRA was measured 2 d after the last nutrient solution application, according to Klumpp et al. (1989) and Duarte and Dillenburg (2000). Light conditions prior to analysis were the same for all sample units. Briefly, from each plant, 0.4-g-samples of both fresh mature leaves and root tips were taken. Plant material was sliced (ca. 4 mm^2 of area), placed in test tubes and mixed with 5 mL of Sörensen phosphate buffering solution (pH 7.5, KNO_3 0.1 M and isopropanol 1%). Tubes were then incubated at 27°C in a vacuum oven (VT 5042 EK, Heraeus, Hanau, Germany) for 2 h. To 1 mL of solution from each tube, 1 mL of 1% sulfonilamide in 1 N HCl and 1 mL of a 0.01% *N*-(1-naphtyl) ethylenediamine dihydrocloride were added. Thirty minutes later, the absorbance of the resulting solution was read at

540 nm in a spectrophotometer (SQ300, Merck, Darmstadt, Germany). The remaining solution in each tube was incubated under the same conditions for an additional hour and the above procedure repeated. Enzyme activity was then determined by quantifying the amount of nitrite produced by nitrate reduction after 2 and 3 h of vacuum incubation (Klumpp et al., 1989).

Carbon and nitrogen measurements: Carbon and N in mature leaves were quantified in samples of five leaves collected from lateral branches of the second (from the top) whorl of branches. For young leaves, determinations were made in several apical leaves (~50 g) collected from young branches. Carbon and N were also quantified in lateral roots (all roots from a given plant making up one sample). Samples were oven-dried (50°C), ground, and then homogenized. Analyses were performed by the Chemistry Institute of the Federal University of Rio Grande do Sul, using a CHN analyzer (Perkin-Elmer 2400, Waltham, USA).

Statistical analyses: Data were submitted to two-way analysis of variance, with randomization test (Pillar and Orlóci, 1996), using the statistical program MultivMinor v. 2.3.17

(Pillar, 2004). Euclidean distance was used as a dissimilarity measure for all data. Means were compared through orthogonal contrasts ($P \leq 0.05$).

RESULTS

After 200 d, total shoot length (height plus length of all lateral branches) did not differ between NH_4^+-fed (88.2 ± 5.2 cm; mean ± SE) and $NO_3^- + NH_4^+$-fed (85.2 ± 5.0 cm) plants. However, it was significantly less (65.8 ± 5.4 cm) in NO_3^--fed plants than in the other two treatments. This pattern remained unchanged until the end of the experiment (199.9 ± 13.8 cm for NH_4^+-fed plants; 187.9 ± 9.4 cm for $NO_3^- + NH_4^+$-fed plants; and 99.8 ± 7.8 cm for NO_3^--fed plants; $P \leq 0.05$). Plants receiving NH_4^+ had greater shoot and total biomass accumulation (Figure 1) than those that only received NO_3^-. Plants receiving NO_3^-, on the other hand, accumulated more root biomass than the others, because of their greater mass investment in the main tap root (mass of lateral roots did not differ among treatments).
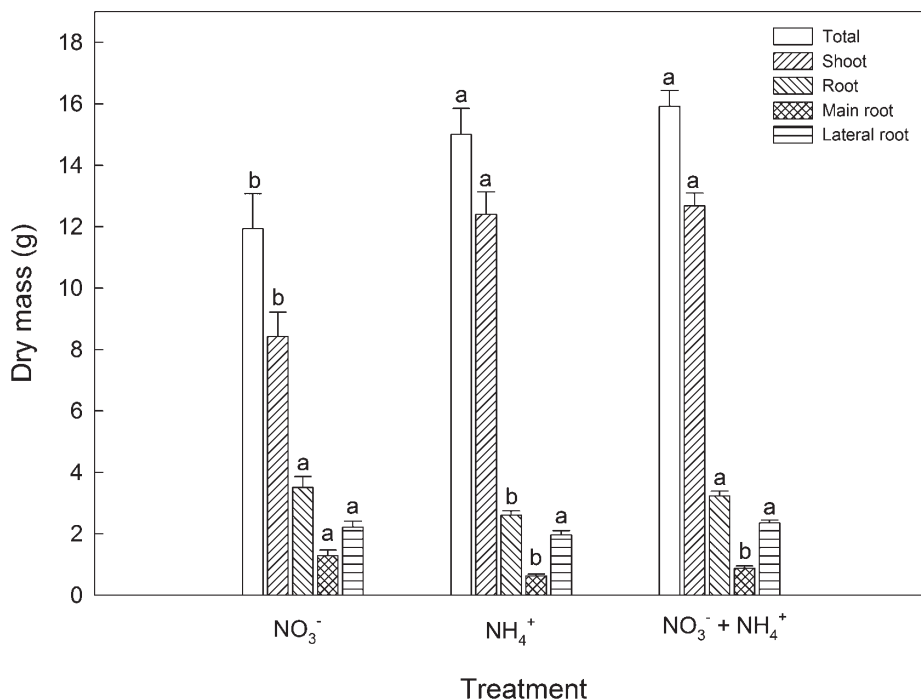

Figure 1. Dry mass accumulation in *Araucaria angustifolia* plants growing under different inorganic nitrogen sources. Vertical bars and lines are means and standard errors, respectively. For a given measurement, different letters indicate significant differences between treatment means ($P < 0.001$ for shoot biomass; $P < 0.05$ for the other parameters).

Nitrate-fed plants had a higher root:shoot mass ratio than plants from the other two treatments, and a lower mass ratio between lateral roots and the main root than the NH_4^+-fed plants. When ratios of root length and number were considered, however, plants of the NO_3^- treatment clearly showed a higher degree of root ramification, particularly when compared to plants fed only with NH_4^+ (Table 2). Plants fed only with NO_3^- also displayed a pronounced thickening of the stem apexes, smaller leaves, as well as higher LMA of both mature and young leaves, when compared to plants receiving NH_4^+ (Table 2), giving them a highly scleromorphic appearance. Differences in LMA were much more pronounced for young leaves, to the point that young leaves of NO_3^--treatment plants reached a higher LMA than the corresponding mature leaves (Table 2). Plants from this treatment also showed visible chlorosis of apical young leaves, as attested by their lower chlorophyll concentrations, especially when compared to young leaves of plants in the NH_4^+-treatment (Table 3). Additionally, NO_3^--treated plants also accumulated less C and N in young leaves than did plants of the other treatments.

Because differences in N were much greater than differences in C concentration, young leaves of NO_3^--treatment plants had a higher C:N ratio than the corresponding leaves of the other two treatments. These treatment differences in C and N did not extend to mature leaves. Feeding of plants with NO_3^- (singly or not) resulted in lower N concentration and higher C:N ratio of lateral roots, compared to plants receiving only NH_4^+ (Table 3). Interestingly, plants that received only NH_4^+ had similar C and N concentrations in all measured plant fractions (leaves and roots), in strong opposition to those that received only NO_3^-, which showed less C and N in young leaves and lateral roots than in mature leaves.

In all treatments, NRA was much higher in roots than in leaves (only about 3 to 13% of total NRA was expressed in leaves). As expected, root NRA was much higher in plants that received only NO_3^- than in the others. Surprisingly, plants that received NH_4^+, either singly or in combination with NO_3^-, showed higher levels of NRA in leaves than those that were fed only with NO_3^- (Table 3).

Table 2. Additional morphological parameters of *Araucaria angustifolia* plants growing under different inorganic nitrogen sources. Data are means ± SE. Different small letters indicate significant differences in the rows ($P \leq 0.05$; $P < 0.001$ for root:shoot ratio). For leaf mass per area (LMA), different capital letters indicate significant differences in the columns ($P \leq 0.003$); $n = 14$ for NO_3^- treatment, and $n = 15$ for NH_4^+ e $NO_3^- + NH_4^+$ treatments. However, for N1, N2, CRP and C1, $n = 10$ for NO_3^- treatment, and $n = 11$ for NH_4^+ and $NO_3^- + NH_4^+$ treatments; and for LMA of young leaves, $n = 11$ for NO_3^-, $n = 13$ for NH_4^+, and $n = 15$ for $NO_3^- + NH_4^+$ treatments. N1 = total number of first order lateral roots; MRL = main root length; N2 = total number of second order lateral roots; L1 = total length of first order lateral roots.

Parameters	Treatments		
	NO_3^-	NH_4^+	$NO_3^- + NH_4^+$
Root : shoot (g g^{-1})	0.46 ± 0.02 a	0.21 ± 0.01 b	0.26 ± 0.01 b
Lateral root : main root (g g^{-1})	1.99 ± 0.19 b	3.64 ± 0.51 a	2.91 ± 0.24 ab
N1 : MRL (nº cm^{-1})	1.51 ± 0.11 a	1.18 ± 0.07 b	1.26 ± 0.06 ab
N2 : L1 (nº cm^{-1})	0.33 ± 0.06 a	0.10 ± 0.03 b	0.17 ± 0.03 b
L1 : MRL (cm cm^{-1})	9.36 ± 0.86 a	6.69 ± 0.66 b	7.45 ± 0.39 ab
Apical stem diameter (mm)	6.90 ± 0.35 a	4.49 ± 0.15 b	5.24 ± 0.23 b
Basal stem diameter (mm)	6.31 ± 0.22 a	6.47 ± 0.13 a	6.48 ± 0.13 a
LMA mature leaves (g m^{-2})	102.6 ± 2.7 aB	90.8 ± 1.9 bA	92.1 ± 1.4 bA
LMA young leaves (g m^{-2})	120.9 ± 4.5 aA	63.3 ± 4.8 cB	82.6 ± 2.7 bB
Size mature leaves (cm^2)	0.57 ± 0.05 b	0.67 ± 0.02 a	0.70 ± 0.03 a
Size young leaves (cm^2)	0.20 ± 0.01 b	0.80 ± 0.13 a	0.62 ± 0.04 a

Table 3. Chlorophyll, carbon (C) and nitrogen (N) concentrations, and nitrate reductase activity (NRA) in *Araucaria angustifolia* plants growing under different inorganic N sources. Data are means ± SE. Different small letters indicate significant differences in the rows ($P \leq 0.05$; $P < 0.08$ for NRA). Different capital letters indicate significant differences in the columns ($P \leq 0.05$); $n = 14$ for NO_3^- treatment, and $n = 15$ for NH_4^+ and $NO_3^- + NH_4^+$ treatments; however, $n = 4$-5 for C, N e C:N determinations.

Parameters	Treatments		
	NO_3^-	NH_4^+	$NO_3^- + NH_4^+$
Chlorophyll young leaves (mg g^{-1})			
a	1.78 ± 0.11 b	3.29 ± 0.18 a	2.34 ± 0.26 b
b	0.76 ± 0.11 b	1.17 ± 0.07 a	0.91 ± 0.12 ab
Total	2.54 ± 0.20 b	4.46 ± 0.22 a	3.25 ± 0.37 ab
a:b ratio	2.75 ± 0.31 a	2.88 ± 0.18 a	2.65 ± 0.18 a
Carbon (%)			
Mature leaves	45.1 ± 0.2 aA	45.9 ± 0.3 aA	45.3 ± 0.4 aA
Young leaves	43.8 ± 0.3 bAB	45.7 ± 0.2 aA	45.2 ± 0.2 aA
Lateral roots	40.7 ± 1.6 aB	42.8 ± 1.8 aA	40.7 ± 1.3 aB
Nitrogen (%)			
Mature leaves	2.46 ± 0.09 aA	2.47 ± 0.23 aA	2.32 ± 0.10 aA
Young leaves	1.79 ± 0.08 bB	2.52 ± 0.07 aA	2.21 ± 0.15 abA
Lateral roots	1.48 ± 0.11 bB	2.60 ± 0.21 aA	1.52 ± 0.09 bB
C:N ratio (%)			
Mature leaves	18.5 ± 0.8 aB	19.0 ± 1.7 aA	19.7 ± 1.0 aB
Young leaves	24.7 ± 1.2 aAB	18.2 ± 0.6 bA	20.8 ± 1.4 bB
Lateral roots	28.3 ± 3.6 aA	16.7 ± 0.9 bA	27.0 ± 1.4 aA
NRA (nmol NO_2^- g^{-1} h^{-1})			
Leaves	6.4 ± 1.5 bB	22.3 ± 5.6 aB	18.2 ± 3.2 aB
Roots	169.4 ± 26.4 aA	91.8 ± 13.8 bA	117.2 ± 27.5 bA

DISCUSSION

Although the species distribution is typically restricted to tropical and subtropical regions, the results show that *A. angustifolia* shares with temperate climate conifers the preference for NH_4^+ as inorganic N source. Nitrate, when provided alone, induced symptoms of N deficiency. These symptoms included overall reduction of plant growth, larger root:shoot mass ratio, root branching and LMA, leaf size reduction, stem thickening, and less mass-based chlorophyll and N concentrations in the young leaves, when compared to plants which received NH_4^+. Many of these aspects were responsible for the scleromorphic appearance shown by those plants. *Araucaria angustifolia* exhibited N deficiency symptoms when submitted to a long period (180 d) of NO_3^--based nutrition. The scleromorphic appearance of the NO_3^--treated plants has similarities to field plants growing under full sunlight conditions. This field scleromorphism may be related to an imbalance in C and N assimilation, mostly due to a high C assimilation not matched by proper N assimilation.

Leaf N deficiency and associated NO_3^--induced responses, such as chlorosis, were more evident in young than in mature leaves, indicating a possible limitation on the process of translocation of organic N from mature to young leaves. Consistent with this scenario is the fact that, in contrast to what would be expected (Mediavilla and Escudero, 2003), young leaves of NO_3^--fed plants had lower N concentrations than mature leaves. Although not tested in this study, this pattern could also be associated with apoplast alkalization caused by possible NO_3^- / H^+ co-transport in the process of leaf cell N absorption, followed by depression in Fe^{3+} reduction and by leaf chlorosis induced by iron deficiency (Kosegarten et al., 1999).

Nitrate is an important regulatory molecule acting on cellular processes related to plant growth (Crawford, 1995). Despite the lower mass ratio between lateral roots and the main root exhibited by the NO_3^--fed plants (forced by a great investment in the main root), these plants had a more intense branched root system than those fed only with NH_4^+, indicating that root architecture, which plays a central role in resource

acquisition by plants (Fitter, 1994), was much more affected by the different treatments than root mass. We suggest that NO_3^- plays an important role in regulating root branching in *A. angustifolia* and that this ramification ability may favor the access to patchily distributed soil resources.

Consistent with the species preference for NH4+, the NRA root:shoot partitioning in *A. angustifolia* was also that expected for temperate climate conifers (Andrews, 1986), with most of the activity concentrated in the roots. These results support the suggestions made by Franco et al. (2005) that most of the N taken up from the soil is metabolized in the roots, and is consistent with the results of Duarte and Dillenburg (2000), who detected NRA only in the roots of *A. angustifolia*. Our study to the best of our knowledge, presents the first evidence of leaf NRA in this species. Leaf NRA is typically low in gymnosperms under natural conditions and was shown to increase when NO_3^- was supplied at 5 mM, thus increasing the importance of the shoot to whole plant N assimilation (Smirnoff et al., 1984; Andrews, 1986; Stewart et al., 1992). The supply of significant amounts of NO_3^- may then explain the leaf activity of NO_3^- reduction reported in this study. *Araucaria angustifolia* may carry out most of its NRA in the roots under natural conditions, where low levels of inorganic N are found (Garbin et al., 2006).

As expected (Oaks and Stevens, 1972; Stewart et al., 1992), NRA in roots was higher when the supply of NO_3^- was also greater. The observed NRA in the NH_4^+-treated plants, however, could indicate contamination of the growth medium by nitrifying agents or constitutive levels of enzyme activity. Sand cultures using NH_4^+-based nutrient solutions may show relatively high levels of nitrifying activity (Padgett and Leonard, 1993), and even a low NO_3^- concentration may induce some NRA (Tischner, 2000). Although nitrification was not evaluated in the NH_4^+ treatment, it is reasonable to expect that the weekly applications of the nutrient solutions ensured the continuous presence of large amounts of NH_4^+ in the system, while the periodic washing of the substrate alleviated any NO_3^- accumulation due to nitrification. Although growth parameters were not likely to be strongly influenced by such nitrification, NRA of NH_4^+-treated plants were expected to significantly respond to any presence of NO_3^- due to nitrification. While both a possible contamination effect and/or a constitutive activity may help to explain the NRA in plants fed only with NH_4^+, it does not account for the greater leaf NRA activity in these plants, when compared to those receiving only NO_3^-. *In vivo* NRA essays with NO_3^- (as used in the present study) only use endogenous NAD(P)H as source of energy for enzyme activity (Andrews, 1986; Li and Gresshoff, 1990). The low leaf NRA of NO_3^--treatment plants could possibly be explained by a lack of energy caused by the NO_3^--imposed N deficiency.

Two additional methodological problems must also be considered in this discussion: (1) N concentration in the applied solutions (4 mM) was probably high, especially considering the intrinsic slow growth of the species, and (2) there was no pH control of the growth medium, only of the nutrient solutions. The N concentration in the applied solutions may appear high when compared to concentrations found in the upper horizons of soils where the species naturally occur (mean concentrations of 3-21 mg kg^{-1} in a native forest; Garbin et al., 2006). However, it is lower than those used in experiments that examined the effects of high N supply in conifers (e.g. Flaig and Mohr, 1992). These concentrations are useful starting points, because, as long as they do not generate serious toxic effects, they ensure that the availability of nutrients will not limit plant growth. Furthermore, other experiments have shown that the species can sustain growth under N concentrations of up to 16 mM (C. Clebsch and L. R. Dillenburg, unpublished results). The pH variation in the growth medium, which could have potentially altered nutrient availability, probably had a small effect, given the high concentration of the solutions and their frequent applications (every week).

Based on growth parameters, we conclude that NH_4^+ was preferred over NO_3^- by young plants of *A. angustifolia*. Nitrate, however, seemed to play an important role in regulating plant growth and N metabolism. Garbin et al. (2006) found strikingly different patterns of spatial distribution of inorganic N in three plant communities of southern Brazil: a native forest, a *Pinus* plantation and a native grassland. Though *A. angustifolia* is able to establish itself in all of these plant communities, the authors found that the native forest differed from the others in that NO_3^-, instead of NH_4^+, was the major inorganic N form. It would be expected that soils which do not have NH_4^+ as a major N form would limit the performance of *A. angustifolia*. However, although the soil under well-developed, mature forests has NO_3^- as the major inorganic N form, it does not exclude the presence of NH_4^+ and it is associated to a low-irradiance understory, which limits overall plant growth. The

two inorganic N forms co-occur in natural soils and only their proportions vary in response to environmental factors, such as temperature, soil pH, humidity, and soil C:N ratio.

Unlike temperate climate forest ecosystems, characterized by an increase in the ratio of NH_4^+ over NO_3^- as succession proceeds (Rice and Pancholy, 1973; Kronzucker et al., 2003), this ratio is greater in grasslands than in the late successional *Araucaria* forests (Garbin et al. 2006). *Araucaria angustifolia*, the dominant plant species in these forests, but also one of the first tree species to invade the adjacent grasslands (Duarte et al., 2006), prefers ammonium over nitrate, at least during initial growth. This preference can make grasslands better suited for the successful establishment of the species than the forest understory itself.

We conclude this report suggesting three different approaches to be taken in the future, in order to better characterize the N nutrition of the species: (1) to measure its relative growth performance when growing in natural environments that differ both in the proportion of these inorganic N forms and in irradiance; (2) to assess the possible use of organic N forms, a use that has already been characterized for other conifers (e.g. Näsholm and Persson, 2001); and (3) to evaluate the impact of inorganic N supply on mycorrhiza, an association to which *A. angustifolia* was found to be highly dependent on (Moreira-Souza and Cardoso, 2002; Zandavalli et al., 2004), and whose effects on plant growth are highly dependent on the form of inorganic N supplied to the plant (Bledsoe and Zasoski 1983).

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