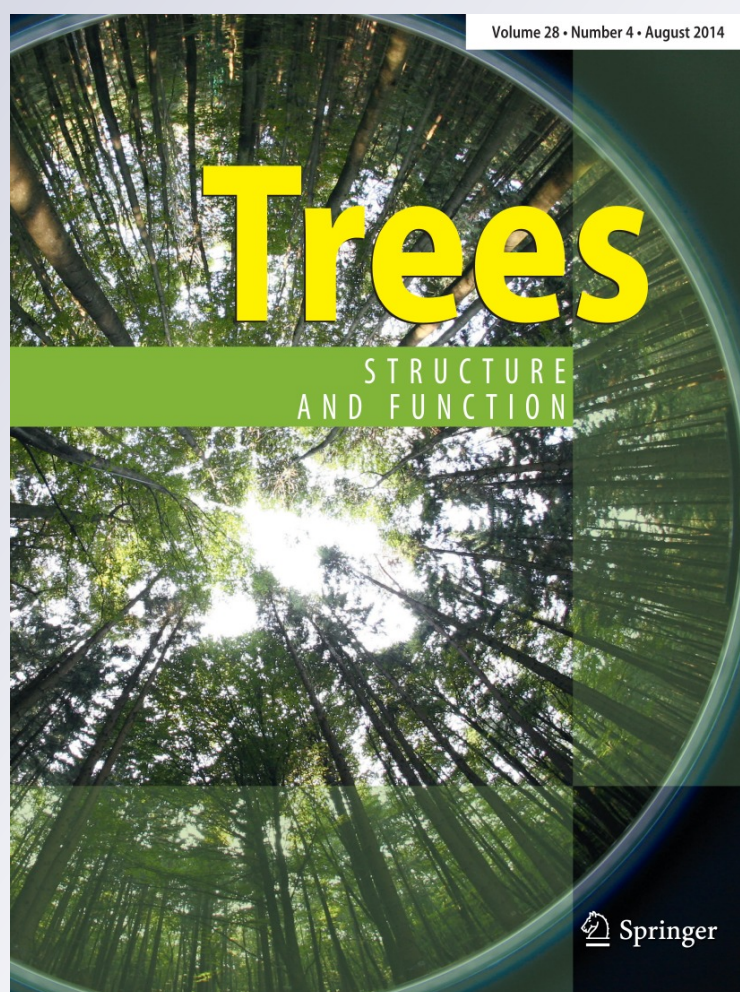# Seedling response of Nothofagus species to N and P: linking plant architecture to N/P ratio and resorption proficiency

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Seedling response of *Nothofagus* species to N and P: linking plant architecture to N/P ratio and resorption proficiency

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Abstract

Key message **As in mature forests, seedlings responded positively to N supply in terms of mass and architecture, especially *N. obliqua*. P became a secondary-limiting nutrient for *N. nervosa* with increased N addition.**

Abstract Previous studies on mature forests of NW Argentinean Patagonia indicated that N is the main growth-limiting nutrient in most dominant tree species, while P limitation is uncommon, despite the soils' volcanic origin. This pattern was inferred from leaf N/P ratios and resorption proficiencies, but has not been experimentally tested. We conducted a greenhouse trial with seedlings of two deciduous species of high timber quality, *Nothofagus nervosa* and *N. obliqua*, and soils characteristic of each species. Seedlings were fertilized with three levels of N (100, 200 and 400 mg kg^{-1} soil) with or without the concurrent application of a single P dose (60 mg kg^{-1} soil) during their second growing season. Response variables were morphological descriptors of shoot and root growth, N and P concentrations in green and senescent leaves and ectomycorrhizal infection. Both species were primarily limited by N: the addition of N resulted in higher shoot and root masses, an increased number of nodes, taller stems and greater basal and root diameters, while no effect of P was found. N/P ratios in green leaves and N and P resorption proficiencies indicate that with increased N availability P can become a secondary-limiting nutrient for *N. nervosa*. This was accompanied by the maintenance of ectomycorrhizal infection and mass allocation to roots in this species. The steep growth response of *N. obliqua* to N addition may signal a strong competitive capacity of this species when growing in soils of high N availability.

Keywords Mass allocation · Architectural descriptors · Ectomycorrhizal infection · Volcanic soils · *Nothofagus* · Nutrient limitation

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Introduction

In temperate forest ecosystems, nitrogen (N) is generally considered the main growth-limiting nutrient, while phosphorus (P) limitation or co-limitation is possible in calcareous, acid or volcanic soils, and in areas affected by high-atmospheric N deposition (Aber et al. 1998; Fisher and Binkley 2000). Several studies indicate that N is the most limiting nutrient in temperate forests of the Andean–Patagonian region of Argentina and Chile, which are characterized by low-atmospheric N deposition and high-plant N conservation (Pérez et al. 1998, 2003; Perakis and Hedin 2002; Diehl et al. 2003, 2008; Godoy et al. 2009). Forests of Argentinean Patagonia occupy a narrow strip of land along the eastern side of the Andes. Soils of these forests are mainly Andisols developed from ejecta from explosive eruptions of Andean volcanoes and carried east into Argentina by the dominant winds (Villarosa et al. 2006; Satti et al. 2007). Although P availability in Andisols is low due to high P retention, no evidence of P limitation was found in nine out of the ten dominant tree species in mature forests of Argentinean Patagonia, a fact that was attributed to high mycorrhizal infection (Diehl

et al. 2003, 2008). The relative importance of N and P in limiting growth in these studies was inferred from indicators of nutrient resorption proficiency (sensu Killingbeck 1996) and the N/P ratio of green leaves (sensu Koerselman and Meuleman 1996).

Nutrient resorption from senescent leaves is considered an adaptation strategy to soil nutrient stress, and exerts strong control on nutrient cycling, as it determines litter quality and the rate of nutrient return to the soil (Vitousek 1982; Aerts and Chapin 2000). Resorption proficiency was defined by Killingbeck (1996) as the ability of a species to reduce N and P concentrations below certain threshold values in senescent leaves. After analyzing a large data set from woody species, he proposed values for ultimate potential resorption (0.3 % N or 0.01 % P) and for resorption proficiency (<0.7 % N or <0.05 % P in deciduous species and <0.04 % P in evergreens). These threshold values have since been widely used in studies of shrubs and trees (e.g., Pérez et al. 2003; Richardson et al. 2005, 2008; Du et al. 2011), and have also been applied to grasses and forbs (e.g. Bertiller et al. 2006; Yuan et al. 2005). Like the classic Redfield ratio in aquatic environments, the stoichiometry between leaf N and P has been suggested as a useful predictor of nutrient limitation in terrestrial ecosystems. Based on experiments with N and P addition in European wetlands, Koerselman and Meuleman (1996) proposed some threshold values for foliar N/P at species and community levels, i.e., N limitation at N/P ratios <14, P limitation at N/P ratios >16 and co-limitation between 14 and 16. Although other authors have suggested lower values (Tessier and Raynal 2003; Knecht and Göransson 2004) or a broader range (Güssewell 2004), the values proposed by Koerselman and Meuleman have been widely adopted as a diagnostic tool of nutrient limitation in different ecosystems and life forms, including tree species (Aerts and Chapin 2000; Tessier and Raynal 2003; Richardson et al. 2004). The generalization of these values, however, is still controversial because in several nutrient-addition experiments they were ineffective predictors of nutrient constraints to plant growth (Drenovsky and Richards 2004; Soudzilovskaia et al. 2005).

In Argentinean Patagonia forests, the validity of the threshold values of nutrient proficiency and leaf N/P ratio used as predictors of N limitation (Diehl et al. 2003, 2008) has not been experimentally tested. Species belonging to the genus *Nothofagus* (Nothofagaceae) constitute 80 % of these forests (Veblen et al. 1996). We selected two deciduous, closely related *Nothofagus* species to investigate N and P limitation on seedling growth. The species were *Nothofagus nervosa* (Phil.) Krasser = *N. alpina* (Poepp. and Endl.) Oerst. and *Nothofagus obliqua* (Mirb.) Oerst., which are included in domestication programs due to their high economic and ecological value (Gallo et al.

2009, Varela et al. 2010). Their distributions in Argentina are limited to areas where annual precipitation usually exceeds 800 mm, and they form either monospecific stands or mixed stands with one another and with *N. dombeyi* (Donoso 2006; Donoso et al. 2006). Both species are ectomycorrhizal, with percentages of infection of around 75 % in mature forests (Diehl et al. 2008). Seedlings of these species are susceptible to water shortages, but *N. obliqua* is less sensitive to drought and has higher photosynthetic rates than *N. nervosa* (Varela et al. 2010). As indicated by leaf N/P ratios and resorption proficiencies, both species are N-limited in mature forests (Diehl et al. 2003, 2008). However, their different patterns of nutrient cycling result in soils under *N. obliqua* having higher N and P availability than *N. nervosa* (Satti et al. 2007), which suggests that they would respond differently to nutrient additions. Architectural studies have identified growth and branching characteristics of *Nothofagus* species growing without apparent environmental restrictions (Raffaele et al. 1998; Barthélémy et al. 1999; Puntieri et al. 2007a, b). This information provides the conceptual framework to evaluate seedling response to nutrient supplies, and to validate the pattern of N limitation based on indicators of N/P stoichiometry and resorption proficiency. In addition, the study enables us to test the commonly reported negative effect of increased nutrient availability on mycorrhizal infection (Smith and Read 1997; Treseder 2004).

We conducted a greenhouse trial over 2 years with seedlings of *N. obliqua* and *N. nervosa* growing in soils characteristic of each species and fertilized during the second growing season with three levels of N, with or without the concurrent application of a single P dose. As response variables, we measured shoot and root masses, morphological descriptors of shoot and root growth, N and P concentrations in leaves and degree of ectomycorrhizal infection. Our main hypothesis was that seedlings of these species growing in their native forest soils are limited by N and not by P. We predicted that: (1) N addition would increase plant growth and cause a shift to non-proficient N resorption and co-limitation of N and P and (2) P addition would not cause a significant response in plant growth. We also predicted that ectomycorrhizal infection would be reduced with increased N and P availability.

Materials and methods

Experimental design

The study was conducted in an unheated greenhouse of a state forestry nursery (Campo Forestal Gral. San Martín,

Table 1 Properties of soils and peat employed in the greenhouse trial

Properties	Soils		Peat of Carex
	N. nervosa	N. obliqua	
pH	5.6	5.7	4.8
Electrical conductivity (mS cm^{-1})	0.32	0.62	0.27
Organic C (g kg^{-1})	81	79	183
Total N (g kg^{-1})	5.9	6.2	4.0
Exchangeable Ca (cmol kg^{-1})	14.5	18.0	5.0
Exchangeable Mg (cmol kg^{-1})	2.6	1.9	0.9
Exchangeable K (cmol kg^{-1})	0.6	0.6	0.5
Olsen-P[a] (mg kg^{-1})	9.5	12.8	3.2
Pot. N miner.[b] (mg kg^{-1})	156	172	–

[a] P extracted with sodium bicarbonate

[b] Potential net N mineralization after 16-week incubation at 25 °C and field capacity moisture

National Institute of Agricultural Technology, INTA) located at Las Golondrinas, Chubut, Argentina (41°59′57.4″ S, 71°31′35.4″ W, 414 m.a.s.l.). Seeds of *N. obliqua* and *N. nervosa* were collected by INTA staff from natural stands and sown in sand beds in October (spring) 2008. On appearance of the first 2–4 true leaves after the cotyledons in November 2008, one seedling of each species was transplanted to removable pots (250 mL each) arranged in trays, containing soil collected from natural stands of that species. These soils were sampled (0–15 cm depth) at about 50 cm from the stems of mature trees in previously studied monospecific stands (Diehl et al. 2003, 2008; Satti et al. 2007), and sieved to 2 mm prior to mixing with *Carex* peat to avoid compaction (50:50 by volume, about 90:10 soil:peat by weight). Both soils are classified as Udivit-rands (Broquen et al. 2005); soil and peat properties are presented in Table 1. Seedlings were grown without nutrient addition during the first season. In November 2009, seven treatments (40 pots per treatment) were established as follows: a control without nutrient additions, three levels of N equivalent to 100, 200 and 400 mg N kg^{-1} soil (hereafter 100 N, 200 N and 400 N) and the same three levels of N plus one level of P (equivalent to 60 mg P kg^{-1} soil) (hereafter 100 N + P, 200 N + P and 400 N + P). Doses of 100 N and 200 N represent about 50 and 100 % of the potential N mineralization rates of each native soil, measured after a 16-week incubation period in optimal conditions (Satti et al. 2007). The 400 N dose was applied as a loading rate to evaluate the allocation of the excess of N to reserves or growth (McAlister and Timmer 1998; Aerts and Chapin 2000). For the P rate, we assumed at least 50 % of P retention in these young volcanic soils (Satti et al. 2007). Nitrogen was

supplied as urea and P as KH$_2$PO$_4$ (analytical grade). The seedlings were irrigated based on the field capacity of the soils. Since previous observations of regional tree nurseries using volcanic soils suggested considerable loss of K under irrigation (Basil et al. 2002), all treatments including controls received 100 mg of K kg^{-1} soil, as KCl. The N, P and K fertilizer applications were made by adding 15 mL of appropriate solution to each pot at four times over the growing season to achieve the total fertilization levels indicated above. Pots of each treatment were randomly placed in trays, which were periodically rearranged in the greenhouse.

Seedling measurements

Aboveground growth was quantified by measuring the size of the well-defined vertical axis that usually does not branch in the first growing season. At the end of the second growing season and before leaf senescence (March 2010), 22–24 seedlings of *N. nervosa* and 16–21 seedlings of *N. obliqua* per treatment were harvested; number or replicates per treatment is shown in Table 3. (Unequal sample sizes were used because we discarded some seedlings exposed to irrigation problems during 1 week.) The following above-ground variables were recorded: length (with a ruler), basal diameter (with digital callipers), number of branches and number of nodes of the vertical stem from soil level up to the distal end. After washing the roots free of soil, root diameter at the point of union with the stem was measured. Since the root systems of both *Nothofagus* species were profusely branched and did not have a main axis, we scanned the root system of each plant and used Image J software to quantify total root area.

Plants were separated into shoot (stem + leaves) and roots and oven-dried at 70 °C for 72 h for dry-mass determination. The root mass ratio (RMR) was calculated by dividing root mass by total plant mass and expressed as percentage, and specific root area as root area per unit root mass.

Nitrogen and P concentrations were determined in green and senescent leaf samples. Three composite samples of green leaves of each treatment were prepared from the complete set of seedlings used to evaluate dry mass (March 2010). Three composite samples of senescent leaves of each treatment were collected during late autumn (May–June 2010) as a composite of about 3–4 seedlings each (until reaching the amount needed for chemical analyses) and oven-dried as described above. Nitrogen was determined with an elemental analyzer (Thermo Electron Corporation Flash EA 1112), and P with an ICP-AES (Plasma 400 Emission Spectrophotometer, Perkin-Elmer) after digestion in nitric-perchloric acid (Jones and Case 1990).

Table 2 Treatment effects on measured variables

	N. nervosa		N. obliqua	
	F, H	p	F, H	p
Shoot mass (g)	20.1	***	98.6	***
Stem length (cm)	9.2	***	84.2	***
Basal diameter (mm)	25.5	***	30.7	***
Number of nodes	13.5	***	30.1	***
Root mass (g)	13.7	***	9.8	***
Root diameter (mm)	18.3	***	13.8	***
Root area (cm^2)	11.7	***	6.6	***
Root mass ratio (%)	1.4	n.s.	74.1	***
Mycorrhizal infection (%)	19.8	**	8.3	***
Stem length/basal diameter (cm cm^{-1})	4.8	***	29.3	***
Specific root area (cm^2 g^{-1})	4.3	***	8.2	***
N green leaves (%)	29.0	***	40.8	***
N senescent leaves (%)	4.1	**	11.4	n.s
P green leaves (%)	65.2	***	105.9	***
P senescent leaves (%)	688.9	***	257.7	***
N/P green leaves	92.8	***	238.4	***

n.s. $p > 0.05$; * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$. Normal characters: Fisher's F test on non-transformed data; italics: Fisher's F test on log-transformed data; underlined: Kruskal–Wallis tests (H)

Mycorrhizal evaluation

Ectomycorrhizal infection was quantified on ten seedlings per treatment by randomly selecting previously dissected fine root segments until 1.30 m per plant was examined. Ectomycorrhizal and non-mycorrhizal root tips were counted under a dissecting microscope to determine the percentage of infected root tips for each plant (Brundrett et al. 1996).

Statistical analyses

For each species, differences among treatments of all variables were evaluated using one-way ANOVA. Comparisons between all pairs of means were performed with the Scheffé method for unequal sample size (growth variables) and with the Tukey–Kramer method (nutrient variables). In order to abide by the normality and/or equal variance pre-conditions for ANOVA, some data were log-transformed (Table 2). When data did not meet such pre-conditions even after transformation, the nonparametric Kruskal–Wallis test was applied (Table 2) (Sokal and Rohlf 1995). Relationships among architectural variables and N, P and N/P in green and senescent leaves were analyzed using Spearman's rank correlation (SRC). For all tests, differences were considered statistically significant at the 0.05 probability level. Statistica 7.0 (StatSoft®) was used for all analyses.

Results

Nothofagus obliqua had higher values than *N. nervosa* for most growth variables in both control and fertilized treatments (Figs. 1 and 2; Table 3). The addition of N resulted in significant increases in both shoot and root masses, as well as stem length, basal diameter, number of nodes and root diameter of both species (Figs. 1 and 2; Tables 2 and 3). However, the number of branches was not affected by the addition of N or N + P and remained close to zero on most individuals (data not shown). The increase in shoot mass, stem length and number of nodes was larger for *N. obliqua,* especially at the highest N application rate. Unlike the responses to N additions, seedling growth was not limited by P: At each rate of N supply, effects of P addition were non-significant for all variables.

The total root area of *N. nervosa,* but not that of *N. obliqua,* increased significantly with added N (Table 3). In both species, the specific root area was lower in the treatments with P addition, but this was only significant with respect to the control. The response of RMR to nutrient addition was different for both species (Table 3). In *N.*
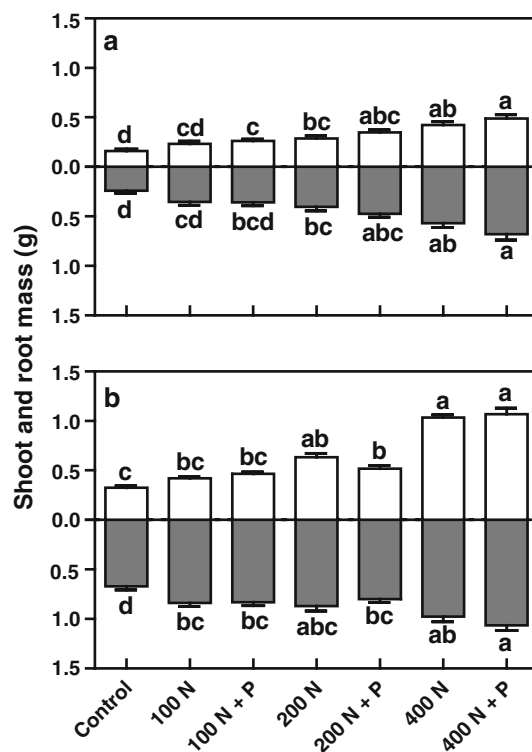

Fig. 1 Changes in shoot and root masses with increased N addition with and without P in a *N. nervosa* and b *N. obliqua*. Shoot mass is represented above the zero line and root mass below the zero line. *Different letters* indicate a significant difference among treatments ($p < 0.05$) for each species and compartment. *Error bars* are standard errors of means. Number of replicates per treatment (n) is given in Table 3
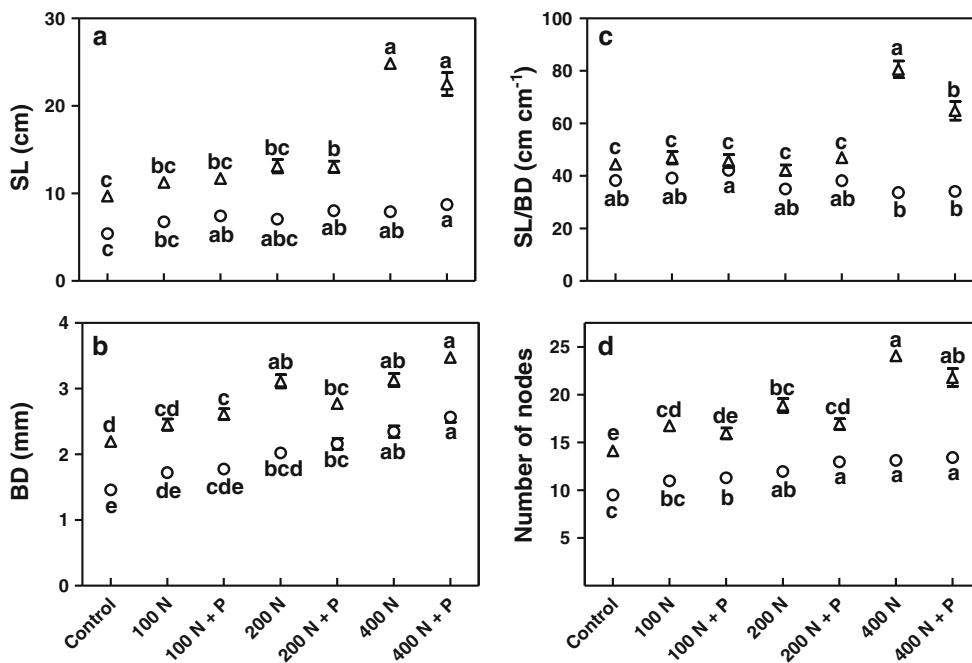
Fig. 2 Changes in **a** stem length (*SL*), **b** basal diameter (*BD*), **c** stem length/basal diameter (*SL/BD*) and **d** number of nodes with increased N addition with and without P in both *Nothofagus* species. *Symbols*: *N. nervosa*, circles, *N. obliqua*, triangles. *Different letters* indicate a significant difference among treatments ($p < 0.05$) for each species and variable. *Error bars* are standard errors of means. Number of replicates per treatment (n) is given in Table 3

Table 3 Mean values for root architectural variables and mycorrhizal infection with increased N addition with and without P

	Root mass ratio (%)	Root diameter (mm)	Root area (cm^2)	Mycorrhizal infection (%)	Specific root area (cm^2 g^{-1})	n
Nothofagus nervosa						
Control	59.3 a (0.78)	2.26 c (0.109)	10.1 c (0.96)	61.1 a (3.75)	44.7 a (2.27)	24
100 N	60.0 a (0.95)	2.77 b (0.124)	14.9 bc (1.58)	58.6 ab (1.59)	44.5 abc (3.08)	23
100 N + P	56.9 a (1.29)	2.79 b (0.099)	14.4 bc (1.21)	60.3 ab (2.56)	42.4 abc (2.35)	24
200 N	58.3 a (1.04)	2.96 b (0.105)	17.0 ab (1.31)	51.8 ab (3.82)	44.8 ab (2.58)	22
200 N + P	57.6 a (0.83)	3.22 ab (0.115)	15.9 b (1.43)	62.5 a (0.98)	32.9 c (2.96)	24
400 N	57.2 a (0.83)	3.52 a (0.113)	24.4 a (1.57)	50.2 b (1.70)	47.4 a (4.04)	24
400 N + P	57.4 a (1.02)	3.65 a (0.122)	22.3 ab (1.93)	54.5 ab (1.51)	33.7 bc (1.52)	24
Nothofagus obliqua						
Control	67.3 a (0.71)	3.24 e (0.090)	31.4 ab (1.77)	66.6 a (3.04)	47.2 a (1.75)	21
100 N	66.3 ab (0.57)	3.41 de (0.073)	33.2 ab (1.71)	57.0 b (2.45)	40.1 ab (1.86)	19
100 N + P	64.0 abc (0.87)	3.56 cde (0.079)	29.8 ab (1.25)	54.3 bc (2.08)	36.1 bc (1.18)	18
200 N	57.7 cd (1.28)	3.70 bcd (0.097)	35.2 ab (2.19)	63.0 ab (1.33)	41.8 ab (3.17)	16
200 N + P	60.7 bc (0.92)	3.98 ab (0.126)	25.6 b (1.90)	58.7 ab (2.33)	31.9 c (1.77)	19
400 N	48.3 d (0.87)	3.97 abc (0.097)	37.9 a (1.25)	57.2 b (1.79)	39.5 abc (1.22)	16
400 N + P	50.1 d (0.89)	4.28 a (0.121)	37.4 a (1.59)	47.4 c (1.42)	35.9 bc (1.62)	19

Different letters indicate a significant difference among treatments ($p < 0.05$) for each species and variable. Standard errors of means are given in parentheses. For mycorrhizal infection, number of replicates for each treatment is $n = 10$, for other variables n is given in the table

nervosa, proportionately more mass was allocated to roots than to shoots (RMR = 57–60 %), and this allocation was maintained irrespective of the rate of N (or P) addition. In *N. obliqua*, the mass allocated to roots was higher than in *N. nervosa*, and decreased from 67 to 50 % in response to N addition (Fig. 1; Table 3); no effect of P was observed at any rate of N supply.

Nitrogen concentrations in green leaves were similar in both species (1.3–1.4 % in the control) and increased significantly as N addition increased (Tables 2 and 4). However, this increment in leaf N was lower in *N. obliqua* than in *N. nervosa* (Table 4), which was most likely due to the dilution effect of higher shoot mass in *N. obliqua*. Consequently, shoot mass and leaf N concentration were

Table 4 Mean values of N and P concentrations in green and senescent leaves and N/P ratio in green leaves with increased N addition with and without P

	N green leaves (%)	P green leaves (%)	N/P green leaves	N senescent leaves (%)	P senescent leaves (%)
Nothofagus nervosa					
Control	1.38 e (0.043)	0.14 a (0.020)	9.9 e (0.24)	0.47 bc (0.007)	0.18 b (0.002)
100 N	1.54 de (0.071)	0.11 b (0.010)	13.6 cd (0.62)	0.45 c (0.019)	0.05 e (0.004)
100 N + P	1.57 de (0.035)	0.16 a (0.003)	10.1 e (0.34)	0.47 bc (0.021)	0.22 a (0.007)
200 N	1.81 bc (0.003)	0.09 c (0.004)	20.8 b (0.99)	1.23 a (0.022)	0.07 d (0.003)
200 N + P	1.60 cd (0.061)	0.15 a (0.004)	11.0 de (0.15)	0.48 bc (0.012)	0.12 c (0.006)
400 N	2.09 a (0.020)	0.07 c (0.003)	29.0 a (1.43)	0.51 bc (0.018)	0.02 g (0.001)
400 N + P	1.90 ab (0.044)	0.11 b (0.002)	16.9 c (0.34)	0.55 b (0.025)	0.03 f (0.002)
Nothofagus obliqua					
Control	1.33 c (0.015)	0.33 a (0.010)	4.0 e (0.18)	0.41 a (0.005)	0.27 a (0.010)
100 N	1.55 a (0.011)	0.19 c (0.005)	8.1 c (0.22)	0.39 a (0.050)	0.14 b (0.010)
100 N + P	1.43 b (0.033)	0.29 ab (0.013)	4.9 e (0.23)	0.44 a (0.018)	0.26 a (0.010)
200 N	1.37 bc (0.020)	0.15 cd (0.004)	9.0 c (0.36)	0.40 a (0.007)	0.06 c (0.003)
200 N + P	1.60 a (0.005)	0.25 b (0.013)	6.3 d (0.30)	0.42 a (0.008)	0.16 b (0.004)
400 N	1.59 a (0.005)	0.13 de (0.003)	12.6 b (0.29)	0.41 a (0.013)	0.03 d (0.001)
400 N + P	1.56 a (0.016)	0.10 e (0.002)	15.3 a (0.21)	0.97 a (0.199)	0.08 c (0.002)

Different letters indicate a significant difference among treatments ($p < 0.05$) for each species and variable. Standard errors of means are given in parentheses; $n = 3$ for all treatments

Table 5 Spearman's rank correlations analyses among several architectural variables and N, P and N/P in green and senescent leaves

	N green leaves (%)	P green leaves (%)	N/P green leaves	N senescent leaves (%)	P senescent leaves (%)	N/P senescent leaves
Nothofagus nervosa						
Shoot mass (g)	0.795 ***	n.s	0.622 **	0.459 *	−0.636 **	0.609 **
Stem length (cm)	0.559 **	n.s	n.s	n.s	n.s	n.s
Basal diameter (mm)	0.817 ***	n.s	0.642 **	0.473 *	−0.643 **	0.639 **
Root mass (g)	0.829 ***	n.s.	0.670 **	0.502 *	−0.683 **	0.648 **
Nothofagus obliqua						
Shoot mass (g)	n.s.	−0.851 ***	0.857 ***	n.s	−0.749 ***	0.875 ***
Stem length (cm)	n.s.	−0.735 **	0.762 **	n.s	−0.691 **	0.791 **
Basal diameter (mm)	n.s	−0.865 ***	0.881 *	n.s	−0.756 ***	0.840 ***
Root mass (g)	0.453 *	−0.871 ***	0.859 ***	n.s	−0.730 **	0.843 ***

n.s. $p > 0.05$; * $p \le 0.05$; ** $p < 0.01$; *** $p < 0.001$

strongly and positively correlated in *N. nervosa* but not correlated in *N. obliqua* (Table 5). Nitrogen concentrations in senescent leaves of both species were on average threefold lower than in green leaves. Furthermore, senescent leaf N concentrations in most treatments did not differ from the control (Table 4), and were below the threshold value of 0.7 % indicative of N-proficiency (Killingbeck 1996). As with green leaves, the correlation between N concentration in senescent leaves and shoot mass was significant and positive for *N. nervosa,* but not for *N. obliqua*.

In contrast to the N results, P concentrations in green leaves of *N. obliqua* were about twice as high as those of *N. nervosa* in all treatments except 400 N + P (Table 4). In both species, P concentrations tended to decrease with N addition probably due to a dilution

effect. This decrease was significant in most treatments of *N. obliqua* associated with higher growth, so correlations between leaf P concentration and several architectural variables (shoot and root masses, stem length and basal diameter) were strong and negative (Table 5). On the contrary, in *N. nervosa*, only treatments without added P had significantly lower leaf P concentrations than the control, and no correlations were observed between leaf P and architectural variables. Phosphorus concentrations in senescent leaves of both species tended also to decrease with increasing N in all treatments, resulting in P proficient plants (<0.05 % sensu Killingbeck 1996) in three treatments of *N. nervosa* and only one treatment of *N. obliqua*. In both species, P in senescent leaves and architectural variables were significantly and negatively correlated (Table 5).

The N/P ratios in green leaves indicated that growth was primarily limited by N (sensu Koerselman and Meuleman 1996) in *N. obliqua* since the values were <14 in six out of seven treatments, and even <10 in five of them (Table 4). On the other hand, the N/P ratios in *N. nervosa* ranged between 10 and 29, indicating that growth was not only limited by N, but also by P in the highest N addition treatments (200 N and 400 N). Consequently, *N. obliqua* showed stronger positive correlations between leaf N/P ratio and shoot and root masses, stem length and basal diameter than *N. nervosa* (Table 5). In both species, only at the highest dose of N with P addition (400 N + P), was the N/P ratio near values considered indicative of N and P co-limitation. The N/P ratios of green and senescent leaves showed a strong relationship across all treatments for each species (Fig. 3), indicating that the treatments had similar impacts on the relative proportions of N and P in actively photosynthesizing and senescent leaves. However, all data points except those from the highest N-only treatment in each species fell below the 1:1 line (Fig. 3), indicating that N/P values were generally lower in senescent than in green leaves and therefore that proportional retranslocation of N exceeded that of P. Together, these data strongly suggest that P resorption (N/P in senescent leaves >N/P in green leaves) was enhanced in both species only at the highest N fertilization rate without P addition (400 N) and that N resorption was the dominant process in all other treatments.

We observed a trend toward decreased ectomycorrhizal infection with increased nutrient addition (Table 3). In *N. obliqua*, it decreased significantly from 67 % in the control to 47–57 % in four of the six fertilized treatments (the lowest value corresponding to 400 N + P), whereas in *N. nervosa*, a significant decrease, from 61 to 51 %, was found only in one treatment (400 N) relative to the control.

Discussion

Nothofagus nervosa and *N. obliqua* seedlings exhibited positive growth responses to increased levels of N supply but not to concurrent addition of P. This result confirms that N is the main limiting nutrient for these species, as was previously suggested for mature stands on the basis of foliar N/P and resorption proficiency (Diehl et al. 2003, 2008). In seedlings of both species, increasing additions of N resulted in higher shoot and root masses, stem height, basal and root diameter and number of nodes, clearly indicating N limitation when growing in their native forest soils.

However, the two species differed in their growth responses, especially to the highest rate of N addition (400 N), where *N. obliqua* was more responsive than *N.*
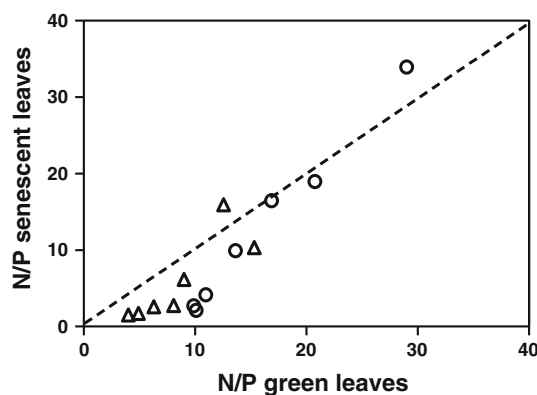

Fig. 3 Relationship between N/P ratio in senescent and green leaves in both *Nothofagus* species. *Symbols* as in Fig. 2

nervosa. A previous study indicated that seedlings of *N. obliqua* have higher photosynthetic rates than *N. nervosa* (Varela et al. 2010), which is consistent with our result of a substantial growth response to N by *N. obliqua*. *N. obliqua* also exhibited higher mass allocation to shoots relative to roots with increased N supply, shifting from 33 to 50 %. One proposed mechanism underlying the response of plants to nutrient addition is the plant's capacity to maximize growth rate by balancing the acquisition of aboveground and belowground resources through a trade-off between shoot and root mass allocation, depending on the most limiting resources (Bloom et al. 1985; Marschner 1995). However, several reviews on the relationship between root mass allocation and nutrient availability suggest that other attributes are more important for the control of nutrient uptake (Wilson 1988; Ericsson 1995; Reynolds and D'Antonio 1996; Aerts and Chapin 2000). Properties such as root length and root area (both in absolute terms and per unit root mass) which increase plant foraging ability, and total plant mass which determines overall nutrient acquisition in terms of the absolute mass of absorbing tissues, may be critically important. In our experiment, *N. obliqua* reduced RMR and specific root area with increased N supply, which implies less ability to explore the soil. However, it also had larger root and total plant mass that may partially compensate for such reductions. On the other hand, nutrient addition negatively affected ectomycorrhizal infection, as has previously been observed for many other tree species (Treseder 2004; Flykt et al. 2008), including a member of the closely related Fagaceae (Oliveira et al. 2010). This reduction in the ectomycorrhizal infection in *N. obliqua* likely decreased the carbon cost of supporting the fungal symbiont and made it possible to increase carbon allocation to shoots.

In the case of *N. nervosa,* the total plant mass of the control seedlings was lower and the proportion of mass allocated to shoots higher than in *N. obliqua*. Nitrogen

addition increased root area and both shoot and root masses, although mass partitioning between both compartments (around 40 and 60 %, respectively) remained unchanged. Another distinguishing feature of *N. nervosa* was that ectomycorrhizal infection was quite similar across most treatments. This species seems to be more sensitive to P limitation than *N. obliqua* since it has half the concentration of foliar P (Table 4) and occupies soils with lower P availability (Table 1; Satti et al. 2007). Maintaining a constant proportion of carbon allocation to roots and the mycorrhizal system implies a cost in terms of shoot growth, but would favor the acquisition of P.

According to the standard indicators of N/P stoichiometry (sensu Koerselman and Meuleman 1996) and nutrient proficiency (sensu Killingbeck 1996), seedling growth of both *Nothofagus* species in our study was primarily limited by N in the control pots (N/P ratio <14, N in senescent leaves <0.7 %, P in senescent leaves >0.05 %). Adding N alone shifted the foliar N/P ratio to higher values than in plants treated with N + P (Table 4), but values indicative of induced secondary P limitation were found only for *N. nervosa*. Apparently, this was not related to growth because no significant differences in plant-size variables were found between N-treated and N + P-treated plants in this species. In contrast, the foliar N/P ratio of *N. obliqua* clearly indicated N limitation in most treatments; only at the highest N rate, were values of N/P within the range indicative of N and P co-limitation, and this corresponded with a large growth increment (Figs. 1 and 2). On the other hand, both species behaved as N proficient in most treatments (<0.7 % N in senescent leaves), with a trend toward P proficiency in some treatments (<0.05 % P in senescent leaves), especially in *N. nervosa*. Therefore, results of both *Nothofagus* species confirmed the pattern of N limitation, but the response of *N. nervosa* also indicated evidence for potential secondary P limitation.

When comparing to adult trees (Diehl et al. 2003, 2008), N/P ratios of green leaves were much lower in seedlings, especially in *N. obliqua* (4 vs. 12 and 10 vs. 13 in *N. nervosa*). The difference is mainly due to lower leaf N concentrations in seedlings of both species (around 1.3 vs. 2.4 % in adult trees). This was associated with high and similar N resorption efficiencies (percentage of reduction between green and senescent leaves) in both control seedlings and adult trees (approx. 70 %), which would allow these plants to increase N storage capacity over time, and consequently, to remobilize higher N amounts to green leaves in adult trees (see review of Millard and Grelet 2010). Contrary to N, little is known about the internal P cycling in trees (Millard and Grelet 2010; Rennenberg and Herschbach 2013). Since in our work P resorption is very low in both control seedlings (0–18 %) and adult trees (35 %), low P storage and remobilization might be

expected. Similarly, in Chilean forests, Hevia et al. (1999) found no P resorption and 72 % N resorption in adult trees of *N. obliqua*, and Gallardo et al. (2012) reported leaf N/P ratios of around 11 in adult trees of *N. obliqua* and *N. nervosa*.

Species adapted to low nutrient conditions may not respond to increased nutrient availability because they have inherently low nutrient requirements, low plasticity and/or nutrient co-limitation with other factors (Drenovsky and Richards 2004; Soudzilovskaia et al. 2005; Subedi et al. 2012). For the species in our study, adaptation to the low P availability of volcanic soils through high ectomycorrhizal infection and mass allocation to roots might have contributed to their null or marginal responses to P addition. The association of ectomycorrhizal infection with P acquisition by tree species has been highlighted in several previous studies (see review of Plassard and Dell 2010). Among the different mechanisms involved, P release from soil organic forms through root phosphatases and dissolution of apatite through root oxalate excretion could be playing an important role in plant P acquisition from the young volcanic soils characteristic of our region. In both species, the addition of P resulted in a reduction of specific root area, confirming also the importance of extended roots in the uptake of immobile phosphates (Aerts and Chapin 2000; Plassard and Dell 2010).

Although these *Nothofagus* species are close taxonomic relatives and occur in habitats with similar abiotic conditions (parent material, climate and duration of soil development), each species has a distinctive imprint on soil properties, especially those related to N and P availability. Slower cycling of base cations and higher concentrations of active Al (higher "andosolization" rates) in *N. nervosa* soils result in higher P retention and lower nitrification rates than in soils of *N. obliqua* (Satti et al. 2007). Lower P availability in *N. nervosa* soils is consistent with the secondary P limitation with increased N supply that we observed in seedlings of this species. Similarly, in an experiment with N addition to five tree species of a northeastern USA forest, species-specific influences on N and P cycling have been observed, resulting in two species being more sensitive to N-induced P limitation than the others (Weand et al. 2010). Determining the nature and characteristics of nutrient limitation is important to understanding the competitive and complementary interactions between plants (Olde Venterink et al. 2003), as well as to predicting the consequences of increased soil N availability resulting from atmospheric N deposition or global warming (Rustad et al. 2001; Weand et al. 2010; Dijkstra et al. 2012).

Results from this study also provide insights into how nutrient availability affects developmental cues in *N. obliqua* and *N. nervosa*. Nutrient additions to these species

did not affect their branching patterns compared with those of either control plants in this experiment or plants growing under natural conditions (e.g., Raffaele et al. 1998). Nonetheless, quantitative responses to increasing levels of nutrient addition, such as longer and thicker stems and more nodes, were recorded for both species. Stem length and slenderness (i.e., stem length/basal diameter) of *N. obliqua* showed sharp increments in the highest N and N + P treatments (Fig. 2), whereas these treatments resulted in decreased slenderness in *N. nervosa*.

This contrast in architectural responses may be explained by inter-specific differences in the extent of neoformation at different levels of N supply. Annual shoots making up the aerial axes in many tree species can include preformed organs and neoformed organs (Barthélémy and Caraglio 2007). In the case of preformed organs, differentiation and extension are separated in time by a period of dormancy, whereas in neoformed organs, differentiation and extension are simultaneous and take place after preformed organs have completed their growth. Neoformation has been interpreted as part of a plastic response of plants to unexpectedly favorable conditions at the time of shoot extension (Guédon et al. 2006). Neoformation has been observed in *Nothofagus* species after the extension of those organs that were preformed in the winter bud (Puntieri et al. 2002). Since the plants in this study were grown under similar conditions for a year before nutrient addition, it is likely that all plants had similar numbers of preformed organs at the beginning of their second growing season when nutrients were added. The variations in stem length, slenderness and number of nodes with progressively higher rates of N addition might imply that neoformed organs were more frequent as N availability increased. Our results suggest that neoformation increased sharply in *N. obliqua* plants under the highest level of N compared with plants in the other treatments. Thus, although N availability may enhance the competitive abilities of young plants of both species (King 1990), the larger growth response of *N. obliqua* may provide seedlings of this species with a competitive advantage over *N. nervosa* at high N availability when growing in mixed stands. Nevertheless, longer studies would be needed to provide more insights about the effects of N and P in preformation versus neoformation of growth units.

Conclusions

Growth of *N. obliqua* and *N. nervosa* seedlings in their native forest soils is primarily limited by soil N availability. In both species, the addition of N increased shoot and root masses, stem height, basal and root diameter and number of nodes, more notably so in *N. obliqua*. Adding N

without P revealed that P can become a secondary-limiting nutrient for *N. nervosa* when N availability is increased. This is consistent with the relatively high soil P retention, the low P concentration in green leaves, and the maintenance of high ectomycorrhizal infection and the RMR in *N. nervosa* compared to *N. obliqua*.

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