Uscola, M. et al., 2014. Nitrogen form and concentration interact to affect the performance of two ecologically distinct Mediterranean forest trees. *European Journal of Forest Research*, 133(2), pp.235–246.

Available at http://dx.doi.org/10.1007/s10342-013-0749-3

Nitrogen form and concentration interact to affect the performance of two ecologically distinct Mediterranean forest trees

Mercedes Uscola[1*], Juan A. Oliet[2], Pedro Villar-Salvador[1], Eugenio Díaz-Pinés[2, 3], Douglass F. Jacobs[4]

[1] Forest Ecology and Restoration Group, Departamento de Ciencias de la Vida, Universidad de Alcalá, E-28871, Alcalá de Henares, Madrid, Spain

[2] Departamento de Silvopascicultura, Escuela de Ingeniería Forestal y del Medio Natural, Universidad Politécnica de Madrid, E-28040 Madrid, Spain

[3] Present address: Division of Bio-Geo-Chemical Processes, Institute of Meteorology and Climate Research, Karlsruhe Institute of Technology, 82467, Garmisch-Partenkirchen, Germany

[4] Department of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center, Purdue University, West Lafayette, IN 47907-2061, USA

*Author for correspondence [email: mercedes.uscola@uah.es; phone: +34 (91) 8856401; fax: +34(91) 8854929]

Abstract

Most studies examining inorganic N form effects on growth and nutrition of forest trees have been conducted on single species from boreal or temperate environments, while comparative studies with species from other biomes are scarce. We evaluated the response of two Mediterranean trees of contrasting ecology, *Quercus ilex* L. and *Pinus halepensis* Mill., to cultivation with distinct inorganic N forms. Seedlings were fertilized with different NH_4^+ / NO_3^- proportion at either 1 or 10 mM N. In both species N forms had small effects at low N concentration, but at high N concentration they markedly affected plant performance. A greater proportion of NH_4^+ in the fertilizer at high N caused toxicity as it reduced growth and caused seedling death, with the effect being greater in *Q. ilex* than in *P. halepensis*. An increase in the proportion of NO_3^- at high N strongly enhanced growth relative to low N plants in *P. halepensis* but had minor effects in *Q. ilex.* Relatively more NH_4^+ in the fertilizer enhanced plant P concentration but reduced K concentration in both species, while the opposite effect occurred with NO_3^-, and these effects were enhanced under high N concentration. We conclude that species responses to inorganic N forms were related to their ecology. *P. halepensis*, a pioneer tree, had improved performance with NO_3^- at high N concentration and showed strong plasticity to changes in N supply. *Q. ilex*, a late successional tree, had low responsiveness to N form or concentration.

Key Words: Ammonium; ecophysiology; growth; nitrate; *Pinus halepensis*; *Quercus ilex.*

Introduction

Nitrogen (N) is a macronutrient usually limiting primary productivity in natural and managed terrestrial ecosystems (LeBauer and Treseder 2008). N is present in soils as inorganic forms, such as ammonium (NH_4^+) and nitrate (NO_3^-), and organic forms, such as amino acids

(Marschner 1995). Plants have the potential to acquire all types of N forms (Paungfoo-Lonhienne et al. 2008) but they frequently show preferential uptake for the most abundant N form in their habitat or successional stage (Kronzucker et al. 2003; Weigelt et al. 2005). Thus, early successional species tend to exhibit high NO_3^- but low NH_4^+ and amino acid uptake rates, whereas late successional species preferentially use NH_4^+ and amino acids and also show low responsiveness to changes in N availability (Kronzucker et al. 2003; Weigelt et al. 2005).

Plants differ not only in N-form uptake preference but also in their functional response to N-forms. For instance, the proportion of NO_3^- and NH_4^+ in soil can affect plant growth and biomass allocation (Guo et al. 2002). While some species perform better when fertilized with NH_4^+ (Cruz et al. 1993; Britto and Kronzucker 2002), others show improved performance when grown with NO_3^- (Atkin and Cummins 1994) or mixtures of NH_4^+ and NO_3^- (Öhlund and Näsholm 2001; Nicodemus et al. 2008). Most studies on the response of plants to N forms have been conducted in crop plants and in boreal and wet temperate forest species (Gigon and Rorison 1972; Falkengren-Grerup 1995; Horchani et al. 2010). Moreover, these studies have generally been carried out with a single species and have used different ranges of N concentration, making it difficult to draw general patterns across experiments. In a comparative study, van den Driessche (1971) demonstrated that boreal conifers grow faster when supplied with NH_4^+ than with NO_3^-. Similarly, Falkengren-Grerup (1995) found that forest herbaceous species that performed well when cultivated with NH_4^+ usually had reduced performance when grown with equimolar mixtures of NH_4^+ and NO_3^- and vice versa. Metcalfe et al. (2011) also found that two temperate conifers grew better when supplied with NH_4^+ yet reported that the biomass of two shrub species was independent of the proportion of NH_4^+ and NO_3^- in fertilizer.

As a cation, NH_4^+ is adsorbed in the substrate, thereby reducing N leachate losses after fertilization compared with NO_3^-. This has important environmental benefits (Raven et al. 1992) and often increases crop N use efficiency. However, NH_4^+ fertilization tends to acidify the substrate because it induces proton efflux in the rhizosphere, reduces concentrations of other cations in plants and causes toxicity when applied at high rates, hindering plant growth, especially of roots (Öhlund and Näsholm 2001; Britto and Kronzucker 2002). In contrast to NH_4^+, NO_3^- can be commonly supplied at higher concentration without harmful effects on plants but easily leaches from the substrate causing potential environmental impacts (Landis et al. 1989; Cruz et al. 1993).

Forest plantations in Mediterranean regions frequently exhibit poor performance (Vallejo et al. 2012). Seedling outplanting performance strongly depends on seedling morphology and physiology (Grossnickle 2012; Villar-Salvador et al. 2012). Nitrogen fertilization greatly influences seedling N reserves, morphology, photosynthesis rate and stress tolerance and, therefore, it frequently enhances outplanting performance (Oliet et al. 2006; Islam et al. 2009). However, inadequate N fertilization can result in nutritional and morphological imbalances and delay stress acclimation in plants (Islam et al. 2009; Andivia et al. 2011). Conventional fertilization programs in forest container nurseries provide between 100 and 150 ppm (7-10 mM) N to cultivated plants (Landis et al. 1989). However, when the objective is to ensure high N concentration in seedlings, fertilizer N concentration can be greater than 150-200 ppm (10-14 mM) (Hawkins et al. 2005; Metcalfe et al. 2011). Fertilization in forest nurseries is usually accomplished by applying fertilizers with similar proportions of NH_4^+ and NO_3^- (Landis et al. 1989), which is a practice that mainly stems from nutrient studies on boreal conifers (van den Driessche 1971; Ingestad 1979). However, because the effect of N form proportions on the functional attributes of plants seems to be species-dependent (see references cited previously), the optimal proportion of NH_4^+ and NO_3^- must be determined to maximize fertilization efficiency and plant quality of non-conifer species or conifers from other biomes. Mineral nutrition of Mediterranean species is comparatively less well understood (Oliet et al. 2004) and as far as we know, knowledge of N-forms response in Mediterranean tree species has been limited to only two species, *Ceratonia siliqua* L. and *Pinus pinaster* Ait. (Cruz et al. 1993, 1997; Warren and Adams 2002).

We investigated whether two ecologically distinct Mediterranean trees have different functional responses to inorganic N forms (NH_4^+ and NO_3^-). We addressed this question by using seedlings of *Quercus ilex* L. *ballota* (Desf) Samp (holm oak) and *Pinus halepensis* Mill. (Aleppo pine), which are widely distributed in the Mediterranean basin and commonly used in afforestation. *P. halepensis* is a fast growing shade-intolerant pioneer tree (Zavala et al. 2000) that mostly thrives on limestone soils where NO_3^- is usually the dominant N form (Gimeno-García et al. 2001). *Q. ilex* is a slow growing, shade-tolerant late successional tree (Zavala et al. 2000) that is indifferent to the soil chemical composition (Ruiz de la Torre 2006). NH_4^+ is often the main N form in *Q. ilex* forest soils (Serrasoles et al. 1999). According to previous literature and given the predominant N-form in *P. halepensis* and *Q. ilex* forests soils, we hypothesized that these species would show variable responses to N forms and concentration. Thus, we predicted that *P. halepensis* will perform better when fertilized with NO_3^-, while *Q. ilex* will have improved performance when fertilized with NH_4^+ or equimolar mixtures of NH_4^+ and NO_3^-. To fulfill our objective, we evaluated the morpho-physiological performance of seedlings grown for six months with three proportions of NO_3^- and NH_4^+ at two N levels.

Material and Methods

Plant material, growing conditions and experimental design

The experiment was conducted in the greenhouse facilities of the Botanical Garden Juan Carlos I at the Universidad de Alcalá (Madrid, Spain). Seeds of *Q. ilex* ssp. *ballota* (Desf)

Samp (holm oak) and *P. halepensis* Mill. (Aleppo pine) from inland Spain provenances were planted as germinants into Super-Leach™ containers (Bardi S.A.L., Navarra, Spain). This container has 35 cavities of 305 ml. Growing media was unfertilized peat moss, pH 5.5±0.1 (Kekkilä B0, Kekkilä Oy, Finland). A 2×3 full factorial experimental design was used for each species: factor N concentration (low, 1 mM N; and high, 10 mM N) and factor NH_4^+/NO_3^- proportion (only NH_4^+ or NO_3^- and equimolar amounts of both N forms, hereafter [N+A]). Each treatment was applied to one container. Containers were re-arranged twice per week throughout the experiment duration.

Nutrient solutions were prepared according to Ingestad (1979) and Landis et al. (1989). The low N solution (1 mM) composition varied according to N form (Table 1). Phosphorous (P) and potassium (K) concentration in the low N solution were 0.33 and 1 mM, respectively, and they were ten times more concentrated in the high N solution. A 10 mM N concentration was chosen because it represents a typical concentration used in nursery culture, especially for N loading (Hawkins et al. 2005). These concentrations were previously found to support conifer growth at deficient and sufficiency levels, respectively (Hawkins et al. 1999; Metcalfe et al. 2011). The low N treatment had similar inorganic N concentration as *Q. ilex* forest soils (Serrasoles et al. 1999). Both nutrient solutions were supplemented with 0.1 g l^{-1} of a commercial micronutrient mixture (Hortrilon, Compo, Barcelona, Spain). Electrical conductivity (EC) and pH of nutrient solutions were measured periodically. pH ranged between 6.8 and 7.1, with no significant differences among treatments. EC of 1 mM N solutions ranged from 317±25 to 416±34 µS cm^{-1} for the NO_3^- and NH_4^+ solutions, respectively, while EC of 10 mM N solutions ranged between 2048±235 and 2940±236 µS cm^{-1}, respectively. Finally, EC of 1 and 10 mM [N+A] solutions were 391±29 and 2850±122 µS cm^{-1}, respectively.

The experiment was conducted from 18 February to 23 July 2009. Light transmission of the greenhouse was 60 %, and daily mean temperature varied from 16 to 29 ºC. Fertilization started on 11 March. To ensure accurate fertilizer delivery, fertilizer was applied individually to each seedling twice a week with a syringe, 20 mL of fertilizer solution during the first two months and 40 mL during the rest of the culture. The volume of fertilizer solution was increased as seedling transpiration increased. We chose these volumes because they did not cause fertilizer leaching and plants were kept well hydrated. Additionally, seedlings were watered once a month with at least 60 mL until leachate was observed in order to flush out accumulated salts. At the end of the experiment approximately 1440 ml was added to each seedling.

Morphology and nutrient concentration

On 23 July, 15 alive seedlings per treatment and species were randomly sampled 24 h after the last fertilization and immediately frozen at -30 ºC until analysis. After thawing, shoots were cut at the point of insertion of the cotyledon and separated into leaves, stems, and root plug. Root plugs were carefully washed to eliminate growing media and roots were separated into fine (< 2 mm diameter) and coarse roots. All seedling fractions were gently washed with tap water, rinsed in deionized water, oven-dried at 60 °C for 48 h and weighed to determine their mass. Root fibrosity was calculated as fine root mass relative to total root mass ratio.

The same 15 seedlings per species used for morphological determinations were used for N, P and K concentration analysis. Three seedlings were randomly pooled to form a total of five composite samples. Samples were ground in a planetary ball mill (PM100, Retsch Haan, Germany). Determination of N concentration was done by the standard Kjeldahl method and that of P concentration followed the methodology described in Allison et al. (1962) using an auto-analyzer (CFA SAN++, Skalar, Breda, The Netherlands). K concentration was determined from perchloric acid extracts in an auto-analyzer (SAN ++) by gas segmented continuous flow coupled to a flame photometer (Sherwood Model 410, Cambridge, UK).

Gas exchange and photosynthetic pigments

Net photosynthetic rate (*A*) was measured at the end of the cultivation period in five seedlings per treatment and species. Measurements took place between 7:00 and 10:00 h (solar time), with an infrared gas analyzer (LCA-4, ADC BioScientific Ltd, Herts, UK). The second flush of *Q. ilex* seedlings with fully expanded leaves and the terminal shoot of *P. halepensis* seedlings were used for gas-exchange measurements. Air temperature inside the cuvette was maintained at 24 °C. Photosynthetic photon flux density was set to 1000 and 1500 µmol m^{-2} s^{-1} for *Q. ilex* and *P. halepensis*, respectively, according to the light saturation point of each species (Loreto et al. 1996; Fernández and Martín 2005). Once *A* stabilized, data were recorded. Following gas exchange measurements, leaf area was calculated with a digital image analyzer (Delta-T Image Analysis System, 1.12, Delta-T Devices LTD, Cambridge, UK).

Photosynthetic pigment concentrations were determined on the same samples used for gas exchange measurements. Three fully expanded leaves of each *Q. ilex* seedling and three subsamples of the needles of the shoot apex of each *P. halepensis* seedling were randomly chosen. Samples were frozen at -30 °C until analysis. Fifty mg of fresh samples were extracted for chlorophyll a and b and total carotenoids (xanthophylls + carotenoids) determination following methodology in Barnes et al. (1992). Pigment concentration was calculated according to equations in Wellburn (1994). Total chlorophyll concentration was obtained by addition of chlorophyll a and chlorophyll b concentration. All values were calculated on a leaf area basis.

Chemical composition of growing media extracts

The growing media of each seedling used for morphology and nutrients analysis was collected before washing the roots. Five composite samples were formed by pooling the substrate of three randomly chosen plants per species and treatment. Additionally, nine cavities were filled with growing media; three of these were left without any seedling (control growing media) and the other six contained one seedling per cavity (three with *Q. ilex* and another three with *P. halepensis* seedlings, respectively) (control growing media with seedling). The nine cavities were not fertilized and were irrigated only with deionized water. These cavities were used as reference samples for substrate analysis. Reference samples were analyzed individually. Growing media extracts were obtained by shaking aliquots of growing media composite samples for 2 h with deionized water under saturated conditions, filtered with a 40 µm pore size filter (DP400 130, Albet, Germany) in a Buchner´s funnel (BR-1611, JP Selecta, Spain) and stored at -30 °C until analysis. Electrical conductivity and pH of the saturated extract were measured with an EC-Meter Basic 30+ (CRISON, Spain) and micropH 2000 meters (CRISON, Spain), respectively. An extraction-distillation approach was used to determine NO_3^- and NH_4^+. From the saturated extracts, 25 mL were analyzed for total inorganic N and NH_4^+ concentration by the steam-distillation method of Mulvaney (1996) in a steam distiller (Kjeltec^{TM} 2100, FOSS, Denmark) and for pH in an automatic titrator with potentiometric (702 titrino, Metrohm, Switzerland). NO_3^- concentration was calculated as the difference between total inorganic N and NH_4^+ concentration, as nitrite concentration was considered negligible, due to absence of anoxic conditions in the growing media. All analyses were made in duplicate.

Statistical analysis

The effect of N form and concentration on plant performance was assessed by two-way ANOVA for a fully randomized design. For the chemical composition of growing media extracts, two different analyses were carried out. First a one-way ANOVA, which compared the three different controls (control growing media, control growing media with *P. halepensis* or *Q. ilex* seedlings). Second, remaining data were analyzed by a two-way ANOVA with a hanging control group (Ministry of Forestry and Range 2007). With this ANOVA procedure, F and P values for the control group (control growing media without seedling) corresponds to the contrast comparing the control mean with the average of all other treatments while maintaining the two-way analysis for the factors. Species were analyzed separately because they showed strong heterocedasticity when analyzing both species together, according to Levene´s test. When significant effects of factors were detected, Fisher's Least Significant Difference test (LSD) was used to identify differences between treatment means. Significance level was established at $\alpha = 0.05$. Several variables were heterocedastic and were analyzed by the Kruskal-Wallis non parametric test and the average planned comparisons non parametric test was used for treatment multiple comparisons. Statistical analyses were conducted with STATISTICA 6 software (StatSoft, Inc, Tulsa, USA).

Results

Morphology

The effect of the interaction between N concentration and N form was significant for all morphological parameters in both species, with the exception of root fibrosity of *Q. ilex* seedlings (Table 2 and Figure 1).

In *Q. ilex*, total mass and most plant fractions did not differ among N forms at 1 mM N, except for coarse roots, which were smaller in NO_3^- than in NH_4^+ fertilized seedlings (Figure 1a). At 10 mM N, an increase in the proportion of NH_4^+ in the fertilizer reduced mass of both total seedling and plant fractions. Ten mM NH_4^+ and [N+A] *Q. ilex* seedlings were smaller than seedlings grown with N forms at 1 mM N except for 10 mM NO_3^-, which overall did not differ from the 1 mM N treatments (Figure 1a). Ten mM NH_4^+ showed generalized leaf necrosis and 32% of the plants were dead by the end of cultivation. Mortality or leaf necrosis was not found in the remaining treatments. Root fibrosity was higher for NO_3^- than for NH_4^+ or [N+A] fertilized seedlings (0.22, 0.16, and 0.18, respectively), and was higher at low N concentration than at high N concentration (0.20 and 0.18, respectively), although concentration effect was marginally significant (Table 2).

Nitrogen forms did not affect either total or plant fractions mass at 1 mM N in *P. halepensis*. In contrast, at high N concentration, total mass or mass of plant fractions increased with higher proportion of NO_3^-. Ten mM [N+A] and, especially, NO_3^- seedlings had larger mass than those of their equivalents grown with 1 mM N (Table 2, Figure 1b), while total mass and mass of plant fractions in seedlings grown at 10 mM NH_4^+ was similar to that in 1 mM NH_4^+ plants. Only *P. halepensiss* fertilized with 10 mM NH_4^+ had foliar necrosis and 8% of these seedlings were dead by the end of the study. Root fibrosity was higher for NO_3^- than for NH_4^+ *P. halepensis* seedlings at both N concentrations, however differences between N forms were higher at low N than at high N concentration (interaction N source x fertilizer N concentration; Table 2; 0.69 and 0.65 for NO_3^- at low and high N concentration, respectively, and 0.60 at both N concentrations for NH_4^+ seedlings). Root fibrosity of [N+A] fertilized seedlings was intermediate at low N concentration and lowest at high N concentration (0.66 and 0.49, respectively).

Nutrient concentration

N forms affected plant N, P and K concentration in *Q. ilex*, but the effect depended on N concentration (interaction N source x fertilizer N concentration, Table 2, Figure 2). At 1 mM N, there were no differences in plant N concentration among N forms in *Q. ilex* seedlings.

However, at 10 mM N, NO_3^- fertilized seedlings had lower N concentration than seedlings grown with NH_4^+ or [N+A], which showed no differences between them. No differences in N content were observed among 1 mM N seedlings (mean N content was 42 mg N), while N content at 10 mM varied between N forms (interaction N source x fertilizer N concentration P=0.024) being highest in NO_3^- seedlings (60 mg N in NO_3^- seedlings and 44 mg N in NH_4^+ and [N+A] seedlings).

Although all seedlings received the same proportion of macronutrients, NO_3^- seedlings had lower P concentration than those grown with NH_4^+ and [N+A] at both N concentration levels, although the differences among N forms were greater at high than at low N concentration. In contrast, K concentration was higher in NO_3^- than in [N+A] or NH_4^+ plants and, as with P, the difference among N forms was greater at high than at low N concentration. Variation in root and shoot nutrient concentration in *Q. ilex* followed the same trend that was described at the plant level except for N concentration. Variation in plant N concentration was attributed to changes in root N concentration rather than shoot N concentration, which did not differ among N forms within a given N concentration level (data not shown).

Concentrations of N, P, and K differed among N forms in *P. halepensis* seedlings, but differences among N forms were greater at high N concentration than at low N concentration (N source x fertilizer N concentration interaction, Table 2 and Figure 2b). While plant N and P concentration increased with the proportion of NH_4^+ in the fertilizer, K concentration decreased. Variation in root and shoot nutrient concentration in *P. halepensis* followed the same trend as described for the plant level except for K concentration. Shoot K concentration was lower in NH_4^+ than in [N+A] and NO_3^- ($P<0.001$), while root K concentration increased with greater proportion of NH_4^+ in the fertilizer ($P<0.001$) (data not shown). N form affected plant N content in *P. halepensis* but the effect depended on N concentration (interaction N source x fertilizer N concentration P<0.001). At low N concentration, NH_4^+ seedlings had higher N content than the remaining N forms (17, 13 and 12 mg N for NH_4^+, [N+A] and NO_3^-, respectively), while at high N concentration, NO_3^- seedlings had higher N content than the remaining treatments (17, 22 and 24 mg N for NH_4^+, [N+A] and NO_3^-, respectively).

Gas exchange and photosynthetic pigments

N form effect on *A* was different depending on N concentration (interaction N source x fertilizer N concentration, Table 3). In *Q. ilex*, *A* was higher in NH_4^+ and NO_3^- seedlings than in [N+A] plants at low N fertilization. In *P. halepensis*, *A* did not differ between N forms at low N fertilization. At high N fertilization, *A* was enhanced with the increase in NO_3^- proportion in the fertilizer for both species.

Nitrogen form had no influence on *Q. ilex* photosynthetic pigments at low N fertilization but at high N fertilization NH_4^+ fertilized seedlings had lower photosynthetic pigments than NO_3^- and [N+A] (interaction N source x fertilizer N concentration, Table 3). *Pinus halepensis* seedlings fertilized at 1 mM had lower chlorophyll and carotenoid concentrations than those fertilized at 10 mM N, but N form had no effect (Table 3).

Growing media chemical characteristics

Control growing media had higher pH than the control with seedling (4.8±0.1 for control and 4.1±0.2 and 4.2±0.2 for control with *Q. ilex* and *P. halepensis* seedlings on average, respectively; P=0.019). The growing media containing seedlings that had been fertilized had lower pH than the control growing media (Table 4). The *g*rowing media from *Q. ilex* seedlings fertilized at 1 mM with NO_3^- had higher pH than [N+A] or NH_4^+, but no differences in pH were detected at 10 mM N (N form × Fertilizer N concentration interaction). Growing media from *P. halepensis* seedlings at 1 mM had the same pH, while at high N fertilization NH_4^+ resulted in decreased pH. Only the 1 mM NO_3^- plants had higher growing media pH than that of the 10 mM NH_4^+ plants (Table 4).

Growing media EC and NH_4^+ and NO_3^- concentrations had very similar patterns across treatments in both species. EC in growing media from 10 mM N seedlings was 6 to 10 times higher than the EC in growing media from 1 mM N seedlings (Table 4). NO_3^- had lower EC than NH_4^+, while [N+A] fertilization resulted in intermediate EC values in both species. EC of control growing media was lower than all fertilization treatments in both species. Growing media NH_4^+ and NO_3^- concentrations were greater at 10 mM than at 1 mM N and increased as did the proportion of NH_4^+ and NO_3^- in the fertilizer, respectively. However this effect was greater at 10 mM than at 1 mM N (significant interaction N source x fertilizer N concentration, Table 4). NH_4^+ and NO_3^- concentrations in the control growing media with plants in both species were similar to that in the growing media of seedlings grown at 1 mM.

Discussion

Effects of inorganic N forms

Quercus ilex and *P. halepensis* seedlings had distinct performance in response to supply with different inorganic N forms. The effect of N forms, however, was small at low N concentration, as indicated by the relatively low responses in growth, *A* and photosyntethic pigment concentration for both species at 1 mM N. Similar results were reported by Metcalfe et al. (2011) in several boreal woody species that were fertilized at 1 mM N and by Warren and Adams (2002), who found negligible growth differences between N forms in seedlings of the Mediterranean pine, *P. pinaster*, fertilized at < 2 mM N. As Mediterranean forest soils usually have N concentrations below 2 mM (Bonilla and Rodá 1992), our results suggest that differences in the relative proportion of NO_3^- and NH_4^+ under natural conditions likely have limited effects on the functional attributes of both species. In addition, we cannot discard that the limited response to N forms at low N concentration might also be attributable to the fairly small differences in the proportion of NO_3^- and NH_4^+ in the growing media among N-form treatments (Table 4). The low concentration and similar proportion in N forms in the growing media at 1 mM N is probably the consequence of plant nutrient uptake that depleted supplied N, such that N form concentration in growing media reflected N forms after peat decomposition and microbial N transformation. This idea is supported by similar N form concentrations in the 1 mM N treatments and in the reference growing media.

Species had distinct functional responses to N forms at high concentration. First, high NH_4^+ fertilization caused toxicity in both species as it reduced growth yet increased plant N concentration (Salifu and Timmer 2003). Toxicity of NH_4^+ at high concentration has also been reported in several boreal conifers (Öhlund and Näsholm 2001; Rothstein and Cregg 2005). Although *Quercus* species are generally classified as NH_4^+-tolerant (Britto and Kronzucker 2002), *Q. ilex* was more sensitive than *P. halepensis* to high NH_4^+ fertilization, as growth reduction relative to 1 mM N plants (Figure 1) and mortality were greater in the former than in the latter species. Moreover, *Q. ilex* plants fertilized with high [N+A] also showed growth reduction relative to 1 mM N plants but this effect did not occur in *P. halepensis*. Results, therefore, do not support our hypothesis that *Q. ilex* would perform better with NH_4^+. Second, consistent with our hypothesis, an increase in the proportion of NO_3^- in the fertilizer promoted *P. halepensis* growth compared to plants fertilized with 1 mM N, especially when NO_3^- was the only N source, while it had a negligible effect on *Q. ilex*. Growth stimulation in *P. halepensis* at high NO_3^- can be explained, in part, by an increase in photosynthetic capacity (Table 3) as was similarly reported for *Pinus radiata* D. Don (Bown et al. 2010). Although high NO_3^- fertilization increased *A*, this effect cannot be ascribed to either higher shoot N (Field and Mooney 1983) or higher photosynthetic pigment concentration in high NO_3^- seedlings. This might be explained by the same N content being differentially partitioned to and within the photosynthetic apparatus, a process that varies depending on N form in fertilizer (Warren et al. 2000).

N form not only influenced N concentration but also concentration of P and K, probably reflecting the need of plants to equilibrate the electrochemical balance in cells (Britto and Kronzucker 2002). Specifically, NH_4^+ uptake competes with (and reduces) uptake of other cations, such as K, and enhances uptake of anions, such as P (Sotiropoulos et al. 2005; Rothstein and Cregg 2005); yet, the opposite effect occurs for NO_3^- uptake whereby K uptake is enhanced (Britto and Kronzucker 2002). Our results suggest that this effect is magnified under higher N concentration, explaining the low K^+ concentration in seedlings highly fertilized with NH_4^+. Also, as indicated by low pH in growing media from NH_4^+ fertilized seedlings, NH_4^+ metabolization produces the extrusion of protons (Cruz et al. 1993; Marschner 1995). The efflux of K^+ from the roots increases through the process of K^+-H^+ co-transport and consequently the net uptake of K^+ declines (Marschner 1995). Also, the higher concentration of protons in the media promotes the $H_2PO_4^-$ form, the main P form actively uptaken by plants, at the expense of HPO_4^{-2} (Marschner 1995).

Growing media pH affects growth and nutrition of plants (van den Driessche 1971; Marschner 1995). Although the fertilizer pH was neutral, growing media pH was lower than the pH recommended for nursery cultivation of forest species (5.5 and 6.5 for conifers and hardwoods, respectively, Landis et al. 1989). Optimum growing media pH varies among species. For instance, van den Driessche (1971) observed that growth and needle N concentration in *Pseudotsuga menziesii* (Mirb.) Franco were highest at pH 4.5, while performance in *Picea sitchensis* (Bong.) Carr. was lowest . Moreover, he reported that the effect of different N forms on plant performance was independent of the growing media pH. Rygiewicz et al. (1984a; 1984b) showed that uptake rate of both NH_4^+ and NO_3^- by *P. menziesii* was constant over a pH range from 3.0 to 5.5. Finally, in the Mediterranean pine *P. pinaster*, the greatest root biomass occurred at pH 3.5 (Arduini et al. 1998). As far as we know, no published research has examined pH effects on the functional performance of our studied species. However, as growing media pH differences among treatments were small in our study, we believe that performance differences are attributed to N forms rather than to pH. Specifically, growth reduction in *Q. ilex* under high NH_4^+ fertilization cannot be attributed to changes in substrate pH, given that high NH_4^+ fertilization did not influence this parameter, as similarly reported by Guo et al. (2002).

Substrate salinity was greater in highly N fertilized plants, especially in NH_4^+ and [N+A]. However, while seedlings of many forest tree species suffer damage beyond an EC of 2500 µS cm^{-1} (Jacobs and Timmer 2005), *P. halepensis* exhibits optimal performance up to a salinity level of 6000 µS cm^{-1} (Oliet et al. 2004). *Quercus ilex* has a lower salinity tolerance than *P. halepensis* but it can tolerate salinity levels up to 3000 µS cm^{-1} (Miyamoto et al. 2004), which is slightly lower than the values obtained in this experiment.

NH_4^+ toxicity was probably due to tissue NH_4^+ accumulation, which can be toxic at high concentration (Landis et al. 1989) if not quickly metabolized after absorption (Britto and Kronzucker 2002; Warren and Adams 2002). The high tissue N concentration in the 10 mM NH_4^+ treatment in both species supports this idea. However, reduction in growth at high NH_4^+ fertilization could also have exacerbated high tissue N concentration, thus preventing N dilution (Sanz-Pérez et al. 2007). We suggest that growth reduction associated with high NH_4^+ in our experiment could be explained by the high energy cost involved in NH_4^+ metabolism to minimize its adverse effects (Britto and Kronzucker 2002; Guo et al. 2002). The lower K concentration in plants fertilized only with NH_4^+ may have also contributed to NH_4^+ toxicity because increased plant K and Ca concentrations can help alleviate NH_4^+ toxicity (Roosta et al. 2009).

High doses of NH_4^+ also hindered fine root production and consequently root fibrosity relative to NO_3^- fertilized plants. Similar results have been observed by Cubera et al. (2009) in *Q. ilex* even at low doses (1 mM). Also *Ceratonia siliqua* L. plants showed inhibition of lateral roots when grown with NH_4^+ (Cruz et al. 1997). Two processes might explain the lower fine root growth in NH_4^+ fertilized seedlings. First, as carbon compounds are used for NH_4^+ assimilation, high NH_4^+ concentration might reduce carbon availability for root growth (Horchani et al. 2010). Second, a root system with a low amount of fine roots potentially has less capacity for NH_4^+ uptake, thereby preventing NH_4^+ tissue accumulation relative to a fibrous root system (Cruz et al. 1997). However, root mass reduction in high fertilized plants for both N forms and especially the root fibrosity reduction observed in NH_4^+ plants, can also hinder water and mineral nutrient uptake (Cruz et al. 1993; Rothstein and Cregg 2005) and might limit seedling establishment in forest plantations (Grossnickle 2012). Fine root reduction may also explain the reduction in overall growth observed in NH_4^+ fertilized seedlings.

Results of this study have important implications for better understanding the ecology of these widespread Mediterranean trees as we demonstrate that they have different N response patterns. *P. halepensis*, a pioneer species, is more plastic to changes in N supply and shows improved performance with NO_3^- at high N concentration. Results in *P. halepensis* are consistent with its ecology, as it mostly thrives on degraded limestone soils, where NO_3^- concentration is frequently higher than that of NH_4^+ (Gimeno-García et al. 2001). Similar to *P. halepensis*, pioneer trees of wet temperate areas and calcicole herbs grow better under NO_3^- than NH_4^+, which has been linked to inability to avoid excess tissue NH_4^+ accumulation even when grown at low NH_4^+ concentration (0.1-3 mM N) (Gigon and Rorison 1972; Kronzucker et al. 2003). For instance many boreal or temperate conifers that mainly thrive in NH_4^+ dominated soils shown higher growth with NH_4^+ (van den Driessche 1971; Britto and Kronzucker 2002; Metcalfe et al. 2011). In contrast to *P. halepensis*, *Q. ilex*, a late successional species, has relatively low responsiveness to N form or N concentration. Absence of morphological plasticity to N in *Q. ilex* relative to *P. halepensis* may be due to low intrinsic plasticity to environmental factors in *Q. ilex* (Valladares et al. 2000, Puértolas et al. 2010). Moreover, *Q. ilex* has considerably larger seeds than *P. halepensis,* which allows for a prolonged reliance on seed N reserves during early seedling development stages (Villar-Salvador et al. 2010), rendering *Q. ilex* seedlings relatively independent of soil N abundance or form. In addition, *Q. ilex* may have low morphological plasticity to nutrients due to low sufficiency levels to N supply as reported for *Quercus rubra* L. (Salifu and Jacobs 2006). Finally, *Q. ilex* that thrives in every types of soils (Ruiz de la Torre 2006)., did not show higher performance with either N form, although is more sensitive to high NH_4^+ concentrations.

Seedling size and tissue nutrient concentration affect outplanting performance (Villar-Salvador et al. 2012; Grossnickle 2012). In our study, except for plants grown with 10 mM NH_4^+, which exceeded the optimal N range, all treatments had N concentrations within optimum ranges of N and P for *P. halepensis* (Oliet et al. 2006) and *Q. ilex* (Villar-Salvador et al. 2004). However, the plants grown at low N concentration were K deficient, while those grown at high N concentration had values within the optimal K ranges (Landis et al. 1989). In contrast to tissue nutrient concentration, plant size was smaller than that recommended for 1-year old seedlings for both species (Villar-Salvador et al. 2004; Oliet et al. 2006). However, this is because our experiment was 3-4 months shorter than the typical cultivation length in Mediterranean nurseries.

Conclusions

This study demonstrates that the effect of N availability on *Q. ilex* and *P. halepensis* performance is not straightforward as it depends on N form and species. N form affected the performance of both species, but only at high N concentration. Ten mM NH_4^+ caused toxicity in both species, while 10 mM NO_3^- promoted *P. halepensis* growth and did not increase *Q. ilex* growth relative to N forms at low N concentration. With an increasing proportion of NH_4^+

in the fertilizer at 10 mM, both species showed decreased fine root formation. An increase in NH_4^+ promoted uptake of P and inhibited that of K, while a higher proportion of NO_3^- caused the opposite response. Our experiment provides new insights for nursery cultivation of these species as we demonstrate that N form at high concentration strongly affects growth and nutritional status that are related to their ecological differences. Both species should preferentially be grown with NO_3^- as an N source when fertilized at high N concentration, instead of using mixtures of both N forms. To validate the results of this study, however, further experiments testing intermediate inorganic N concentration values (especially in *Q. ilex*) and field outplanting performance are needed for both species.

Acknowledgements

We are grateful to the Jardín Botánico Juan Carlos I of the Universidad de Alcalá and to Centro Nacional de Recursos Genéticos Forestales "El Serranillo" for their technical support. Agustín Rubio from the UPM is acknowledged for support in substrate analyses. This study was supported by a FPU-MEC grant to M. Uscola, and by projects AGL2006-12609-C02-01/FOR, AGL2011-24296 ECOLPIN (MICIIN), and the network REMEDINAL 2 (S2009/AMB/1783) of the Community of Madrid. We appreciate constructive reviews from anonymous referees and subject editor.

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Table 1. Compounds (mM) contained within the different 1 mM N solutions. Compounds and ratios were identical for 10 mM, just 10 times more concentrated. Solutions were made with deionized water.

Compounds	NH_4^+	$NH_4^+ + NO_3^-$	NO_3^-
KNO_3		0.5	1
$(NH_4)_2SO_4$	0.33	0.168	
NH_4Cl	0.33	0.168	
$CaHPO_4$			0.33
$MgSO_4$ * $7H_2O$	0.25	0.25	0.25
$CaSO_4$ * H_2O			0.25
K_2HPO_4	0.33		
KH_2PO_4		0.33	
$CaCl_2.2H_2O$	0.58	0.58	
KCl	0.33	0.17	

Table 2. Summary of two-way ANOVA (p-values) for N forms (NH_4^+, NO_3^-+NH_4^+ equimolar concentration, and NO_3^-), and N concentration ([N]: high- 10 mM and low- 1 mM) on dry weight of plant fractions and plant nutritional status of *Quercus ilex* and *Pinus halepensis* seedlings.

	N form	[N]	N form × [N]
Quercus ilex			
Plant mass	<0.001	<0.001	<0.001
Shoot mass	<0.001	0.04	<0.001
Fine roots mass	0.004	<0.001	<0.001
Coarse roots mass	0.44	<0.001	<0.001
Plant N concentration	0.17	<0.001	0.008
Plant P concentration	<0.001	<0.001	<0.001
Plant K concentration	<0.001	<0.001	0.049
Root fibrosity	0.007	0.06	0.28
Pinus halepensis			
Plant mass	0.014	<0.001	<0.001
Shoot mass	0.07	<0.001	<0.001
Fine roots mass	<0.001	0.031	<0.001
Coarse roots mass	0.009	0.012	<0.001
Plant N concentration	<0.001	<0.001	<0.001
Plant P concentration	<0.001	<0.001	0.038
Plant K concentration	<0.001	<0.001	0.002
Root fibrosity	<0.001	<0.001	<0.001

Table 3. Net photosynthesis rate (*A*), total chlorophylls and total carotenoids concentrations of *Quercus ilex* and *Pinus halepensis* seedlings cultivated with different N forms (NH_4^+, equimolar amount of NO_3^- and NH_4^+ [N+A], and NO_3^-) at two N concentration ([N]): high (10 mM) and low (1 mM). The three last columns are the p-values of two-way ANOVA. Means followed by different letters denote significant differences at α=0.05 using Fisher's LSD.

	Low N concentration (1 mM)			High N concentration (10 mM)			*P* > F		
	NH_4^+	[N+A]	NO_3^-	NH_4^+	[N+A]	NO_3^-	N form	[N]	N form × [N]
Quercus ilex									
A (µmol m^{-2} s^{-1})	13.2±1.3b	8.8±1.1c	12.1±0.9b	11.6±1.4b	13.6±1.3b	17.5±1.3a	0.04	0.001	0.006
Chlorophylls (mg m^{-2})	1227±52b	1114±41c	1106±30c	1114±81c	1310±58a	1385±59a	0.29	0.68	0.03
Carotenoids (mg m^{-2})	172±7bc	163±9c	159±4c	180±6b	203±8a	200±5a	0.34	0.001	0.05
Pinus halepensis									
A (µmol m^{-2} s^{-1})	24.4±0.9cb	23.2±0.3c	23.2±0.6c	23.5±0.7c	27.2±0.4b	31.6±2.2a	0.15	0.035	0.04
Chlorophylls (mg m^{-2})	982±28b	1078±24b	1002±37b	1227±20a	1189±37a	1208±41a	0.48	<0.001	0.14
Carotenoids (mg m^{-2})	144±3d	160±6c	148±3d	174±4b	172±5b	183±6a	0.46	<0.001	0.21

Table 4. pH, Electric conductivity (EC), N concentration in NH_4^+ form (NH_4^+), and N concentration in NO_3^- form (NO_3^-) of the growing media saturated extract (mean ± SE, n=5) in *Quercus ilex* and *Pinus halepensis* seedlings cultivated with different N forms (NH_4^+, equimolar amount of NO_3^- and NH_4^+ [N+A], and NO_3^-), at two N concentration ([N]): high (10 mM) and low (1 mM). A reference sample treatment consisting of growing media without seedling and watered distilled water was included. The three last columns are the p-values of two way ANOVA. Separated by specific treatment, treatment means within a row followed by different letters denote significant differences at α=0.05 using Fisher's LSD. ^{1} Non-homocedastic variables were analyzed by Kruskal Wallis test.

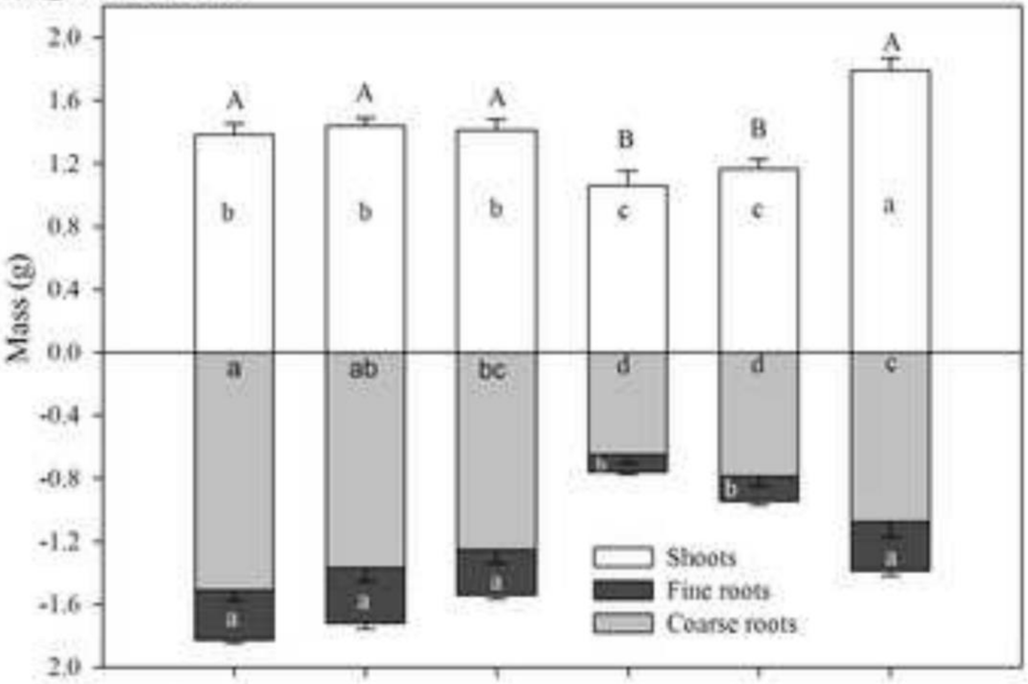
	Reference sample	Low N concentration (1 mM)			High N concentration (10 mM)			*P* > F		
		NH_4^+	[N+A]	NO_3^-	NH_4^+	[N+A]	NO_3^-	N form	[N]	N form × [N]
Quercus ilex										
pH	4.8±0.10a	3.6±0.05d	3.7±0.05cd	4.1±0.06b	3.6±0.02cd	3.7±0.06cd	3.8±0.02c	<0.001	0.06	<0.001
EC (µS cm^{-1})	61±10e	691±40c	708±55c	336±33d	3154±36a	3067±229b	2667±161b	0.002	<0.001	0.85
NH_4^+(µgN ml^{-1})	4±1d	6±1c	4±1d	2±1e	86±2a	59±4b	13±7c		^{1}<0.001	
NO_3^- (µgN ml^{-1})	8.5±0.4c	4±1d	8±1c	10±2c	5±2cd	42±7b	81±4a	<0.001	<0.001	<0.001
Pinus halepensis										
pH	4.8±0.10a	3.8±0.1ab	3.7±0.1ab	4.2±0.4a	3.5±0.1b	3.7±0.1ab	3.8±0.2ab		^{1}<0.001	
EC (µS cm^{-1})	61±10e	536±76c	653±38c	335±21d	4284±322a	3935±96a	2631±227b	<0.001	<0.001	0.26
NH_4^+ (µgN ml^{-1})	4±1c	10±2c	8±1c	4±1c	129±5a	60±1b	6±1c	<0.001	<0.001	<0.001
NO_3^- (µgN ml^{-1})	8.5±0.4d	8±2d	13±1cd	22±3c	11±6d	66±5b	103±10a	<0.001	<0.001	<0.001

Figure captions

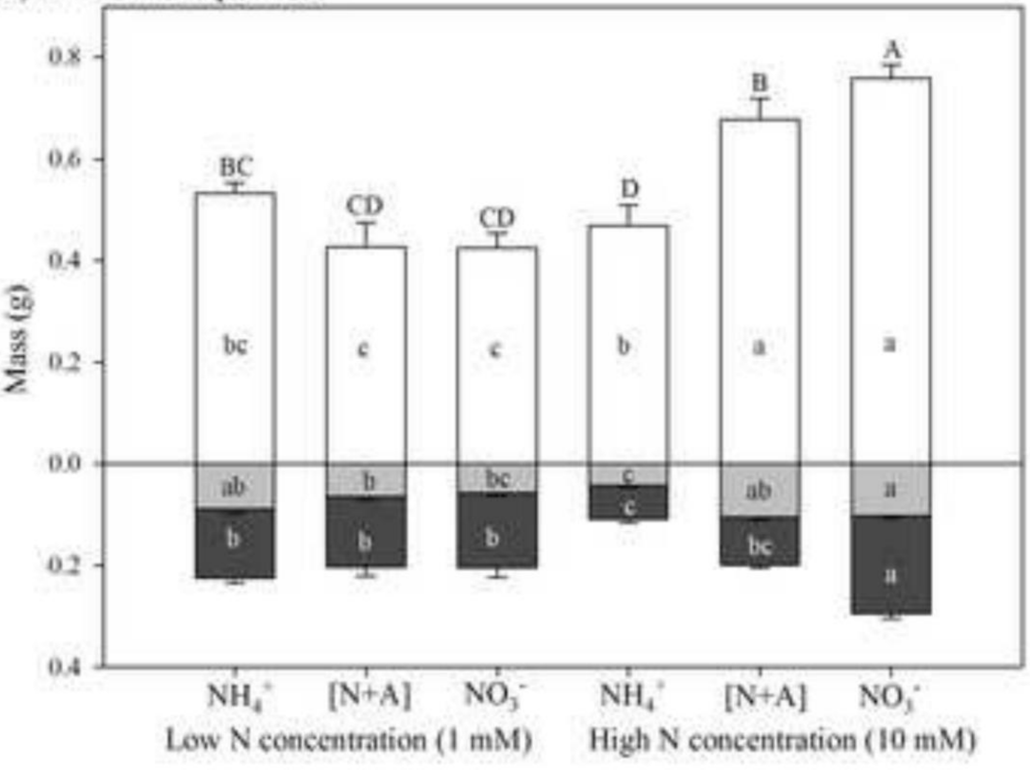
Figure 1. Mass (mean ± SE, n = 15) by fractions of a) *Quercus ilex* and b) *Pinus halepensis* seedlings as affected by fertilizer N form (only NH_4^+ or NO_3^-, and equimolar amounts of NO_3^- and NH_4^+ [N+A]), and fertilizer N concentration (high- 10 mM and low- 1 mM). Within a fraction, different lower-case letters indicate statistical differences among treatments. Different capital letters denote significant differences in total plant mass at $\alpha=0.05$ using Fisher's LSD. To facilitate treatment comparison, mass scale is different for each species.

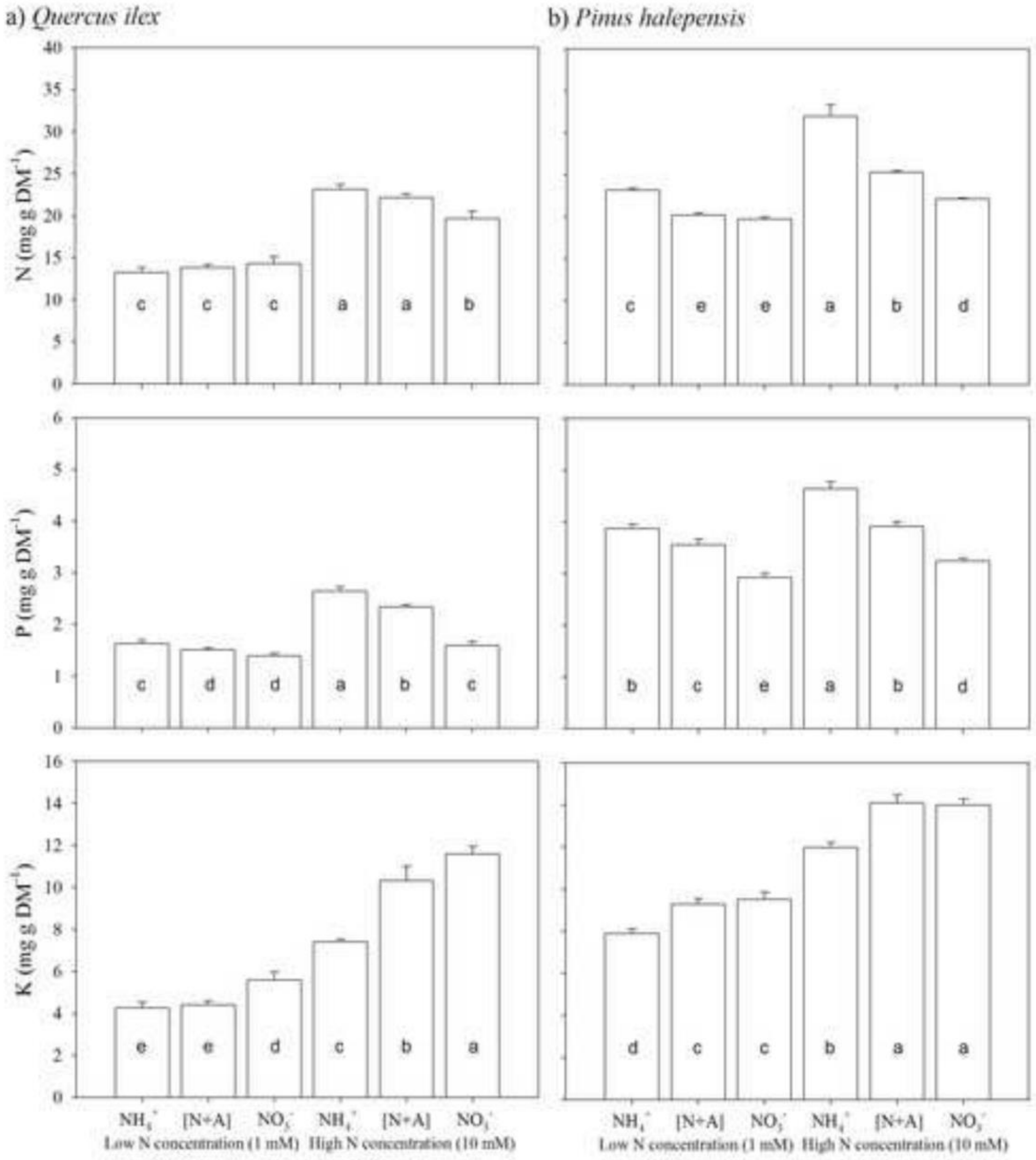
Figure 2. N (upper), P (middle) and K (lower) whole plant concentration (mean ± SE, n=5) of a) *Quercus ilex* and b) *Pinus halepensis* seedlings as affected by fertilizer N form (only NH_4^+ or NO_3^-, and equimolar amounts of NO_3^- and NH_4^+ [N+A]), and fertilizer N concentration (high- 10 mM and low- 1 mM). Means followed by different letters denote significant differences at $\alpha=0.05$ using Fisher's LSD.

a) *Quercus ilex*



b) *Pinus halepensis*




a) *Quercus ilex*
b) *Pinus halepensis*
N (mg g DM^{-1})
P (mg g DM^{-1})
K (mg g DM^{-1})
0
5
10
15
20
25
30
35
40
c c c a a b
c e e a b d
0
1
2
3
4
5
6
c d d a b c
b c e a b d
0
2
4
6
8
10
12
14
16
e e d c b a
d c c b a a
NH$_4^+$ [N+A] NO$_3^-$ NH$_4^+$ [N+A] NO$_3^-$
Low N concentration (1 mM) High N concentration (10 mM)
NH$_4^+$ [N+A] NO$_3^-$ NH$_4^+$ [N+A] NO$_3^-$
Low N concentration (1 mM) High N concentration (10 mM)