Root uptake of inorganic and organic N chemical forms in two coexisting Mediterranean forest trees

Uscola, M.[1,2*], Villar-Salvador, P.[1], Oliet, J.[3] & Warren, C.R. [4]

[1] *Forest Ecology and Restoration Group, Departamento de Ciencias de la Vida, U.D. Ecología, Universidad de Alcalá, Apdo. 20, E-28805, Alcalá de Henares, Madrid, Spain.*

[2] current address: *Hardwood Tree Improvement and Regeneration Center, Department of Forestry and Natural Resources, Purdue University, West Lafayette, IN 47907-2061, USA.*

* author for contact: muscolaf@purdue.edu

[3] *E.T.S. Ingenieros de Montes, Departamento de Sistemas y Recursos Naturales, Universidad Politécnica de Madrid, 28040 Madrid, Spain*

[4] *School of Life and Environmental Sciences, Heydon-Laurence Building A08, The University of Sydney, 2006, Sydney, New South Wales, Australia*

Submitted to Plant and Soil on 17 June 2016

10 Text pages and 2 Figures

Abstract

Background and aims: Plants differ in their ability to use different nitrogen (N) forms and these differences can be related to their ecology and drive community structure. The capacity to uptake intact organic N has been observed in plants of several ecosystems. However, soil organic N uptake by Mediterranean plants is unknown despite organic N being abundant in Mediterranean ecosystems. We compare the uptake of different N forms in two widespread coexisting Mediterranean forest trees with contrasting ecophysiological characteristics: *Quercus ilex* and *Pinus halepensis.*

Methods: To estimate root uptake rate of each N form we used equimolar solutions (1 mM N) of $^{15}NO_3^-$, $^{15}NH_4^+$ and ^{15}N-^{13}C glycine.

Results: NH_4^+ and glycine were taken up at a similar rate, but faster than NO_3^- in both species. Intact dual labeled glycine was found in both species, demonstrating that both species can absorb intact organic N.

Conclusions: Despite their ecological differences, both species had similar preference for N forms suggesting no niche complementarity for N uptake. The higher preference for NH_4^+ and glycine over NO_3^- possibly reflects adaptation to the differing proportions of N forms in Mediterranean soils.

Key words: Amino acid; ammonium; nitrate; *Pinus halepensis*; *Quercus ilex*; root uptake preferences

Introduction

Nitrogen (N) is the most limiting nutrient in many terrestrial ecosystems (LeBauer and Treseder 2008). The capacity of plants to use organic N as a source of N has been demonstrated for cold-climate (Kielland 1994; Näsholm et al. 1998; McKane et al. 2002) and wet temperate ecosystems (Warren 2006; Schulz et al. 2011), and among some crop plants (Näsholm et al. 2000). In cold-climate ecosystems, where soil inorganic N is low, uptake of organic N leads to complementarity in soil N use permitting the coexistence of a higher number of plant species (Kielland 1994; Näsholm et al. 1998; McKane et al. 2002). Soils in Mediterranean ecosystems are poor in N but have large amounts of organic N relative to inorganic N (Delgado-Baquerizo et al. 2011). Uptake of organic N may be significant in Mediterranean ecosystems, despite the absolute concentration of organic N often being lower than in other ecosystems (Delgado-Baquerizo et al. 2011). Recently, Uscola et al. (2014b) showed that two Mediterranean forest trees are able to absorb organic N through their leaves. However, it is still unknown if Mediterranean plants are also able to take up intact organic N through the roots.

Species vary in their ability to take up different N forms (Kronzucker et al. 1997; Aidar et al. 2003; Kielland et al. 2006). Preference differences for N forms among plants could reflect adaptation and/or acclimation to the most abundant N forms in their habitat (Kielland et al. 2006; Song et al. 2015). The proportion of soil NH_4^+ relative to NO_3^- increases through ecological succession (Kronzucker et al. 1997; Aidar et al. 2003). Thus, pioneer species frequently have higher preference for NO_3^- than late successional species, which prefer NH_4^+ and amino acids (Kronzucker et al. 2003; Aidar et al. 2003; Metcalfe et al. 2011). Coexisting species may also vary in their preference for N forms (Miller and Bowman 2003; Song et al. 2015), which allows for ecological niche differentiation that can in turn affect community structure (McKane et al. 2002; Boudsocq et al. 2012; Li et al. 2015). Water stress is considered the main driver of plant community structure in

Mediterranean ecosystems (Zavala et al. 2000; Sánchez-Gómez et al. 2006). However, Mediterranean ecosystems are not dry year-round and for many months water is not a limiting resource. Other soil resources such as N and their different forms may also play a significant role in plant performance and community structure (Kahmen et al. 2006; Sardans et al. 2006; Delgado-Baquerizo et al. 2011). However, knowledge of the preferences for different N forms in Mediterranean forest trees is very limited (Cruz et al. 1993; Warren and Adams 2002; Dias et al. 2014).

The objectives of this study are 1) to assess whether the seedlings of two major Mediterranean forest trees, *Pinus halepensis* Mill. (Aleppo pine) and *Quercus ilex* L. (holm oak), are able to uptake intact organic N from soil, and 2) to compare the capacity of both species to take up organic and inorganic N forms. Both species are widely distributed in the Mediterranean basin, coexist in the mid-successional stages but they have contrasting ecological and morpho-physiological characteristics. *Pinus halepensis* is a fast growing shade-intolerant pioneer tree, while *Q. ilex* is a slow growing shade-tolerant late-successional species (Zavala et al. 2000; Baquedano and Castillo 2006).

Material and methods

Plant material and ^{15}N pulse into the soil

One-year-old container-grown seedlings of *P. halepensis* and *Q. ilex* from an inland Spain provenance were used. Detailed information on seedling cultivation can be found in Supplementary material. Trace of N from previous fertilization were removed from roots before labeling experiments began by immersing roots in a 0.5 mM KCl solution for 15 s and then repeatedly washing in deionized water. Seedlings were transplanted into 2.5 L containers filled with vermiculite (pH 7.2) and placed in a greenhouse for 3.5 months until new roots protruded the plug and colonized most of the transplanting substrate. To allow acclimation to the different N sources, 28 days prior to labeling

seedlings were watered three times per week with 200 ml of a fertilizer solution (pH=6.8) that included 1 mM N as equimolar amounts of nitrate, ammonium and glycine (i.e. 0.33 mM KNO_3, 0.165 mM $(NH_4)_2SO_4$ and 0.33 mM glycine). Inorganic N concentration was similar to that reported for *Q. ilex* forest soils (Bonilla and Rodá 1992) and soils in other Mediterranean ecosystems (Delgado-Baquerizo et al. 2011).

To determine uptake of N-forms, plants were supplied with 200 ml of one of four isotope-labeled solutions. The four fertilizer solutions all contained 1 mM of N with the three N sources in equimolar proportions, but differed from one another in which N source was labeled (either $^{15}NH_4^+$, $^{15}NO_3^-$, ^{15}N-^{13}C glycine or no N source labeled). Full details of labeling compounds and procedure, and the ^{15}N and ^{13}C abundance of unlabeled samples can be found in the Supplementary material and Table S1. Labeled solutions were applied individually to each of eight or six replicate seedlings per species, for glycine or NO_3^- and NH_4^+ respectively. The different N forms of unlabeled solutions were applied individually to four replicate seedlings per species. After 6 h seedlings were harvested. New roots that protruded out of the root plug were cut and washed in 0.5 mM KCl to remove traces of fertilizer, twice with tap water and once with distilled water (Figure S1, supplementary material). The roots remaining in the plug were washed to eliminate peat. Finally, all fractions including the shoot were oven-dried at 60 °C for 48 h and weighed. New roots were ground in a ball mill (PM100, Retsch, Haan, Germany) and the concentration of N and C and the abundance of ^{15}N and ^{13}C were determined by isotope ratio mass spectrometry (EF-IRMS Isochrom, Micromass, UK) at the UC Davis Stable Isotopes Laboratory (Sharp 2005).

Intact glycine absorption estimation

We followed two methods to assess whether glycine was taken up intact in new roots. The first method calculates the proportion of glycine absorbed intact by comparing how much the slope of the regression line between $C_{absorbed}$ against $N_{absorbed}$ determined by

IRMS in new roots deviates from the regression line of slope = 1 predicted from the stoichiometry of intact dual labeled glycine uptake (i.e., 1 mol of ^{13}C per mol of ^{15}N) (Näsholm et al. 1998; Warren 2012). The second method analyzed the amount of 2-$^{13}C^{15}N$-glycine in root samples by gas chromatography-mass spectrometry (see methodological and calculation details in the Supplementary material).

Statistical analysis

Uptake of N by roots was assessed by two-way ANOVA, with species and N forms as fixed factors. Differences between species in N absorption rate of intact glycine, dual labeled glycine and organ mass were assessed by a t-test. Differences between species in the slope of the linear regressions between $C_{absorbed}$ *vs*. $N_{absorbed}$ were conducted by checking the significance of covariate ($C_{absorbed}$) × Species interaction on $N_{absorbed}$ as a dependent variable (Sokal and Rohlf 2012, page 494). Statistical analyses were conducted with R version 3.1.0 (Spring Dance).

Results

Quercus ilex seedlings had higher total plant mass than *P. halepensis* (5.34±0.09 and 2.12±0.04g respectively, t_{38}=-32.17; *P*=0.002) but *P. halepensis* had 2.7 times more new roots than *Q. ilex* (108±9 and 39±5mg, respectively; t_{38}=-7.73; *P*=0.029). Both species had similar total N uptake rate and glycine and NH_4^+ were taken up the fastest, while uptake of NO_3^- was three to four times slower (Figure 1).The slope of the $C_{absorbed}$ *vs*. $N_{absorbed}$ regression line was steeper in *P. halepensis* than in *Q. ilex* ($F_{1,14}$=5.48; *P*=0.034). Based on the slope of fitted lines, estimation of intact glycine absorption was 92% in *P. halepensis* and 86% in *Q. ilex* (Figure 2a). Dual labeled glycine was detected by GC–MS in roots of both species. Dual labeled glycine absorption rate in roots was higher in *Q. ilex* than in *P. halepensis* (t_{14}=2.31; *P*=0.037; Figure 2b). The absorption rate of intact glycine through roots as estimated by GC-MS in *Q. ilex* and *P. halepensis* seedlings was

145 1.37 and 0.67%, respectively of the absorption rate estimated from the regression slopes
146 method.

147 Discussion

148 *Uptake of intact glycine by roots.*

149 Intact dual labeled glycine was detected by GC-MS indicating that both species were able
150 to absorb intact glycine (Näsholm et al. 1998). This is the first study demonstrating that
151 Mediterranean trees can take up intact amino acids from soil. The small amount of intact
152 dual labeled glycine detected within roots can be explained by the quick metabolization
153 of the glycine and/or transport to other parts of the plant (Warren 2012).

154 According to the ^{13}C-^{15}N molar ratio method, the estimated proportion of glycine
155 taken up intact was high for both species. Notably, this result is similar to those reported
156 for species from other ecosystems (Nasholm and Persson 2001; Persson et al. 2003;
157 Metcalfe et al. 2011). However, intact uptake may be overestimated if labeled amino acids
158 are mineralized in the soil and ^{13}C and ^{15}N are taken up independently (Persson and
159 Näsholm 2001; Warren 2012). The ^{13}C-^{15}N molar ratio of labeled roots can also be
160 affected by post uptake losses of $^{13}CO_2$ via respiration and transfer of ^{15}N and/or ^{13}C
161 throughout the plant (Persson and Näsholm 2001; Warren 2012). As these metabolic
162 changes can be species-specific, this could explain the apparent disparities in intact
163 glycine uptake from both methods. Results of this study, taken together with the fact that
164 transporters in root epidermis have broad affinity for many amino acids (Svennerstam et
165 al. 2011) and that organic N accounts for a high proportion of soil N in Mediterranean
166 ecosystems (Delgado-Baquerizo et al. 2011) suggest that amino acids may be a major N
167 source for the studied trees.

168 *Differences in N acquisition and N forms preferences between species.*

169 Fast growing species usually have higher inherent N uptake capacity than slow growing
170 species (Osone and Tateno 2005; Schulz et al. 2011). In our study both species had
171 similar rates of N uptake, despite *P. halepensis* being faster growing than *Q. ilex* (Uscola
172 et al. 2015). However, because *P. halepensis* had higher new root growth than *Q. ilex* it
173 can be expected that *P. halepensis* will have higher N uptake per plant than *Q. ilex*.

174 Despite their ecological and functional differences, *P. halepensis* and *Q. ilex* did
175 not differ in relative uptake rates of N forms. Preference differences for N forms among
176 plants could reflect adaptation to the most abundant N forms in their habitat (Kielland et
177 al. 2006; Song et al. 2015). Mature forest soils often contain more NH_4^+ than NO_3^-, thus
178 forest species might preferentially take up NH_4^+ and grow better when NH_4^+ is the main
179 N form (Metcalfe et al. 2011). The dominant N forms in Mediterranean forest soils are
180 organic N and NH_4^+ (Bonilla and Rodá 1992; Delgado-Baquerizo et al. 2011). Plant
181 species that coexist can also show different N-form preferences, with dominant species
182 having higher NH_4^+ preference than subordinate ones (McKane et al. 2002; Boudsocq et
183 al. 2012). Consistent with these ideas, both studied trees are structural species in many
184 forests across the Mediterranean basin and present a strong preference for NH_4^+ and
185 glycine.

186 We believe that relative differences in N-form uptake results were not biased by
187 soil pH in our experiment. Soil pH can shift preferences for N forms, which could be
188 important for competitive relationships because both species coexist and thrive in a broad
189 range of soil pH values (Pemán García et al. 2014). The pH of the peat and vermiculite
190 mixtures such as the used in this study usually ranges from moderately acid to neutral
191 (Pemán García et al. 2014), which is in the range of soil pH values in which these species
192 live (Pemán García et al. 2014).

193 The potential benefit of greater preference for NH_4^+ is that it has lower metabolic
194 costs than NO_3^- metabolism, which might increase plant growth. Boudsocq et al. (2012)

suggested that greater preference for NH_4^+ may maximize primary productivity of plant communities. Consistent with the model of Boudsocq et al. (2012), *Q. ilex* and especially *P. halepensis* seedlings have slightly higher growth, photosynthesis and leaf nutrient concentration when cultivated with NH_4^+ than with NO_3^- at the same N concentration used in this experiment (Uscola et al. 2014a).

Plants show plasticity in N forms preference in response to changes in N form availability and the presence of competitors (Houlton et al. 2007; Ashton et al. 2010). Consequently, assessment of the ecological implications of N forms preferences for both species needs to be addressed under different plant-plant interaction scenarios. Additionally, mycorrhiza can modify plant N uptake and N form preferences (Chalot and Brun 1998). We did not inoculate our plants with any mycorrhiza or measure mycorrhization in our seedlings. Therefore, future experiments should address the importance of mycorrhiza on the uptake of N forms in both species.

Acknowledgments

We are grateful to C.N. R.G. F. "El Serranillo" (MAGRAMA) for cultivating the plants. This study was supported by a FPU-MEC grant to MU, and by projects AGL2006-12609-C02-01/FOR (ENCINUT), AGL2011-24296 ECOLPIN (MICIIN), CGL2014-53308-P SERAVI (MINECO) and Remedinal 3 (S2013/MAE-2719, CAM). CW was supported by a Future Fellowship from the Australian Research Council (FT100100024).

References

Aidar MPM, Schmidt S, Moss G, et al (2003) Nitrogen use strategies of neotropical rainforest trees in threatened Atlantic Forest. Plant, Cell Environ 26:389–399.

Ashton IW, Miller AE, Bowman WD, Suding KN (2010) Niche complementarity due to plasticity in resource use: plant partitioning of chemical N forms. Ecology 91:3252–3260.

Baquedano FJ, Castillo FJ (2006) Comparative ecophysiological effects of drought on seedling of the Mediterranean water-saver *Pinus halepensis* and water-spenders *Quercus coccifera* and *Quercus ilex*. Trees 20:689–700.

Bonilla D, Rodá F (1992) Soil nitrogen dynamics in a holm oak forest. Vegetatio 99:247–

257.

Boudsocq S, Niboyet A, Lata JC, et al (2012) Plant preference for ammonium versus nitrate: a neglected determinant of ecosystem functioning? Am Nat 180:60–9.

Chalot M, Brun A (1998) Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. FEMS Microbiol Rev 22:21–44.Cruz C, Lips SH, Martins-Loução MA (1993) Interactions between nitrate and ammonium during uptake by carob seedlings and the effect of the form of earlier nitrogen nutrition. Physiol Plant 89:544–551.

Delgado-Baquerizo M, Covelo F, Gallardo A (2011) Dissolved organic nitrogen in Mediterranean ecosystems. Pedosphere 21:309–318.

Dias T, Clemente A, Martins-Loução MA, et al (2014) Ammonium as a driving force of plant diversity and ecosystem functioning: observations based on 5years´ manipulation of N dose and form in a Mediterranean ecosystem. PLoS One 9:e92517.

Houlton BZ, Sigman DM, Schuur EAG, Hedin LO (2007) A climate-driven switch in plant nitrogen acquisition within tropical forest communities. PNAS 104:8902–6.

Kahmen A, Renker C, Unsicker SB, Buchmann N (2006) Niche complementarity for nitrogen: an explanation for the biodiversity and ecosystem functioning relationship. Ecology 87:1244–1255. Kielland K (1994) Amino acid absorption by arctic plants: implications for plant nutrition and nitrogen cycling. Ecology 75:2373–2383.

Kielland K, McFarland J, Olson K (2006) Amino acid uptake in deciduous and coniferous taiga ecosystems. Plant Soil 288:297–307.

Kronzucker HJ, Siddiqi MY, Glass ADM (1997) Conifer root discrimination against soil nitrate and the ecology of forest succession. Nature 385:59–61.

Kronzucker HJ, Siddiqi MY, Glass ADM, Britto DT (2003) Root ammonium transport efficiency as a determinant in forest colonization patterns: an hypothesis. Physiol Plant 117:164–170.

LeBauer DS, Treseder KK (2008) Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 89:371–379.

Li X, Rennenberg H, Simon J (2015) Competition for nitrogen between *Fagus sylvatica* and *Acer pseudoplatanus* seedlings depends on soil nitrogen availability. Front Plant Sci 6:1–11.

McKane RB, Johnson LC, Shaver GR, et al (2002) Resource-based niches provide a basis for plant species diversity and dominance in arctic tundra. Nature 415:68–71.

Metcalfe RJ, Nault J, Hawkins BJ (2011) Adaptations to nitrogen form: comparing inorganic nitrogen and amino acid availability and uptake by four temperate forest plants. Can J For Res 1637:1626–1637.

Miller AE, Bowman WD (2003) Alpine plants show species-level differences in the uptake of organic and inorganic nitrogen. Plant Soil 283–292.

Näsholm T, Ekblad A, Nordin A, et al (1998) Boreal forest plants take up organic nitrogen. Nature 392:914–916.

Näsholm T, Huss-Danell K, Högberg P, Hogberg P (2000) Uptake of organic nitrogen in the field by four agriculturally important plant species. Ecology 81:1155–1161.

Nasholm T, Persson J (2001) Plant acquisition of organic nitrogen in boreal forests.

Physiol Plant 111:419–426.

Osone Y, Tateno M (2005) Nitrogen absorption by roots as a cause of interspecific variations in leaf nitrogen concentration and photosynthetic capacity. Funct Ecol 19:460–470.

Pemán J, Navarro RM, Nicolás JL, et al (2014) Producción y manejo de semillas y plantas forestales. OAPN. Ministerio de Agricultura, Alimentación y Medio Ambiente. Madrid.

Persson J, Högberg P, Ekblad A, et al (2003) Nitrogen acquisition from inorganic and organic sources by boreal forest plants in the field. Oecologia 137:252–257.

Persson J, Näsholm T (2001) A GC-MS method for determination of amino acid uptake by plants. Physiol Plant 113:352–358.

Sánchez-Gómez D, Valladares F, Zavala MA (2006) Performance of seedlings of Mediterranean woody species under experimental gradients of irradiance and water availability: trade-offs and evidence for niche differentiation. New Phytol 170:795–806.

Sardans J, Rodà F, Peñuelas J (2006) Effects of a nutrient pulse supply on nutrient status of the Mediterranean trees *Quercus ilex* subsp. *ballota* and *Pinus halepensis* on different soils and under different competitive pressure. Trees 20:619–632.

Schulz H, Härtling S, Stange CF (2011) Species-specific differences in nitrogen uptake and utilization by six European tree species. J Plant Nutr Soil Sci 174:28–37.

Sharp Z (2005) Principles of Stable Isotope Geochemistry. Pearson/Prentice Hall, 2007

Sokal RR, Rohlf FJ (2012) Biometry: The principles and practice of statistics in biological research, 4th edn. W. H. Freeman, New York

Song M, Zheng L, Suding KN, et al (2015) Plasticity in nitrogen form uptake and preference in response to long-term nitrogen fertilization. Plant Soil 394:215–224.

Svennerstam H, Jämtgård S, Ahmad I, et al (2011) Transporters in Arabidopsis roots mediating uptake of amino acids at naturally occurring concentrations. New Phytol 191:459–67.

Uscola M, Oliet JA, Villar-Salvador P, et al (2014a) Nitrogen form and concentration interactively affect the performance of two ecologically distinct Mediterranean forest trees. Eur J For Res 133:235–246.

Uscola M, Villar-Salvador P, Oliet J, Warren CR (2014b) Foliar absorption and root translocation of nitrogen from different chemical forms in seedlings of two Mediterranean trees. Environ Exp Bot 104:34–43.

Uscola M, Villar-Salvador P, Gross P, Maillard P (2015) Fast growth involves high dependence on stored resources in seedlings of Mediterranean evergreen trees. Ann Bot 115:1001–1013.

Warren CR (2006) Potential organic and inorganic N uptake by six Eucalyptus species. Funct Plant Biol 33:653.

Warren CR (2012) Post-uptake metabolism affects quantification of amino acid uptake. New Phytol 193:522–31.

Warren CR, Adams MA (2002) Possible causes of slow growth of nitrate-supplied *Pinus pinaster*. Can J For Res 32:569–580.

Zavala MA, Espelta JM, Retana J (2000) Constraints and trade-Offs in Mediterranean

312 plant communities: the case of holm oak-Aleppo pine forests. Bot Rev 66:119–149.

Figure captions.

Figure 1. Root N uptake rate of three N chemical forms (glycine, NO_3^- and NH_4^+) by intact new roots in one-year-old seedlings of *Quercus ilex* and *Pinus halepensis*. Data are means ± 1 SE. Means followed by different capital letters denote significant differences at α=0.05. Estimated percentages of the N from fertilizers that were taken up by the roots during the uptake experiment were: for NH_4^+ and glycine about 2.3% in *P. halpensis* and about 0.6% in *Q. ilex*; for NO_3^- it was about 0.6% in *P. halpensis* and 0.2% in *Q. ilex*.

Figure 2. a) Regressions between $C_{absorbed}$ and $N_{absorbed}$ in intact new roots of *Quercus ilex* (r^2=0.85; $F_{1,8}$=725; P<0.0001; y=0.8593x) and *Pinus halepensis* (r^2=0.84; $F_{1,8}$=496; P<0.0001; y=0.9213x) seedlings after fertilizing with dual ^{13}C and ^{15}N labeled glycine. Each point represents an individual. b) N taken up from dual labeled glycine detected by GC-MS in roots in both species and total N taken up from glycine estimated by IRMS after correcting for natural abundance of ^{15}N in samples and the enrichment labeling by fertilizers (see Isotope analyses and calculations in Supplementary material). N from intact glycine by IRMS was estimated by multiplying root N content by the line slope in the regression analysis (2a). The remaining N taken up was assumed to be glycine absorption after de-amination. Means followed by different capital letters denote significant differences for total N taken up from glycine at α=0.05. Within a fraction, different lower-case letters indicate statistical differences between species. Numbers indicate statistical differences in dual labeled glycine detected by GC-MS.

Figure 1.

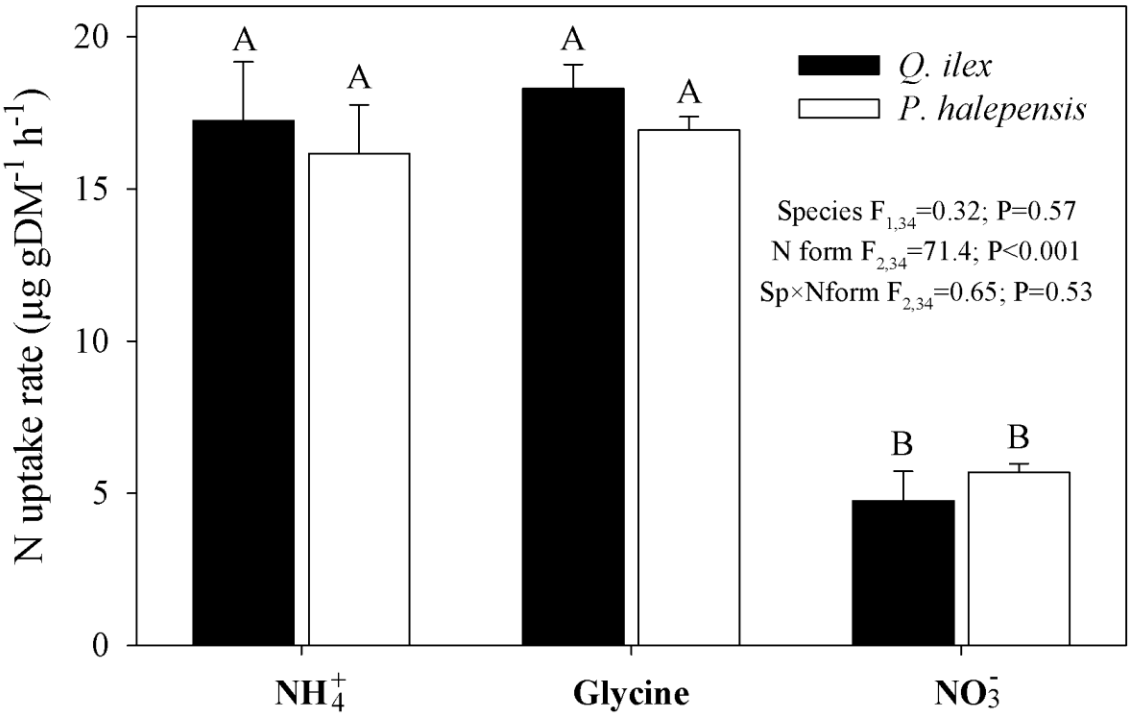
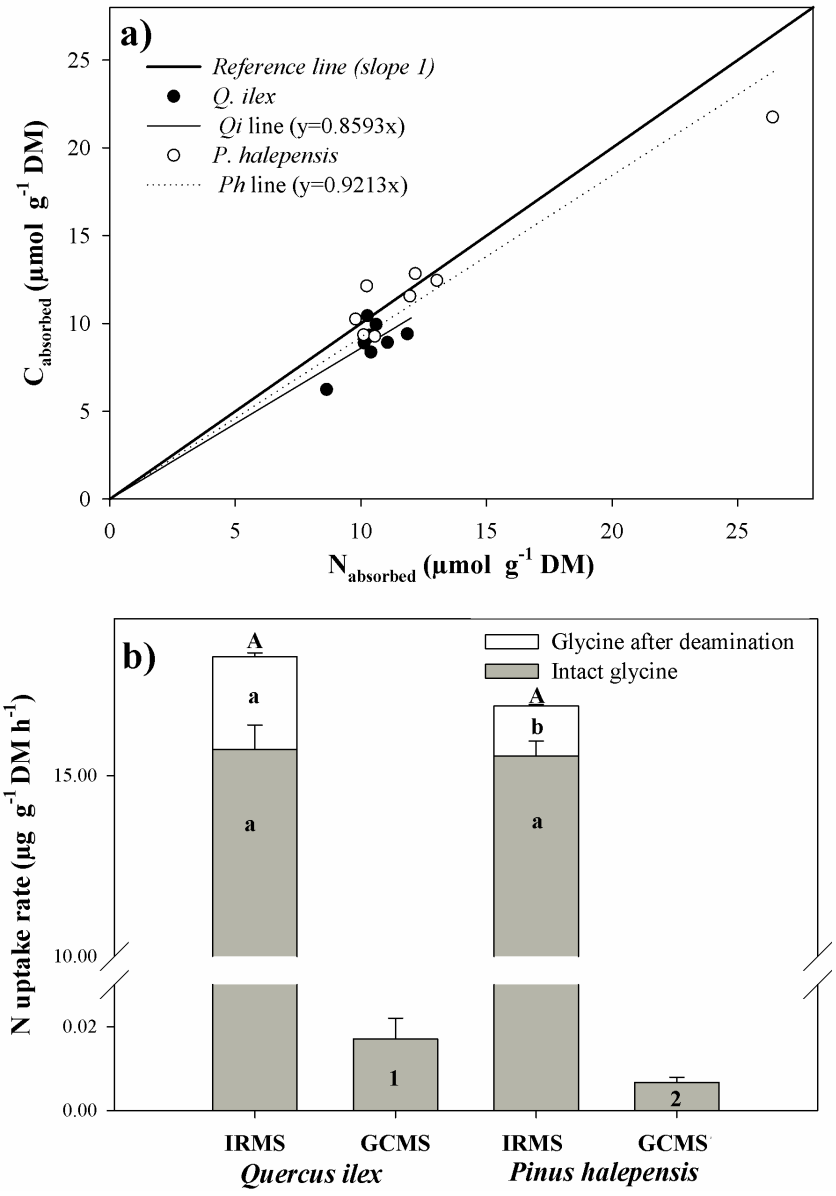

N uptake rate (µg gDM-1 h-1)
20
15
10
5
0
A
A
A
A
B
B
Q. ilex
P. halepensis
Species F1,34=0.32; P=0.57
N form F2,34=71.4; P<0.001
Sp×Nform F2,34=0.65; P=0.53
NH4+
Glycine
NO3-

Figure 2


a)
Reference line (slope 1)
Q. ilex
Qi line (y=0.8593x)
P. halepensis
Ph line (y=0.9213x)
C absorbed (µmol g-1 DM)
N absorbed (µmol g-1 DM)
0
5
10
15
20
25
b)
Glycine after deamination
Intact glycine
N uptake rate (µg g-1 DM h-1)
A
a
a
A
b
a
15.00
10.00
0.02
0.00
1
2
IRMS
GCMS
IRMS
GCMS
Quercus ilex
Pinus halepensis

Supplementary material.

Details on Material and methods.

First year cultivation in the nursery.

One-year-old seedlings of *P. halepensis* and *Q. ilex* from an inland Spain provenance, ES-9 Alcarria for *P. halepensis* and ES-12 La Mancha for *Q. ilex* (Pemán García et al. 2014), were cultivated at the Centro Nacional de Recursos Genéticos Forestales "El Serranillo" (MAGRAMA). One-year-old plants were chosen because seedlings are the most limiting life-stage for tree recruitment (Pulido and Díaz 2005). Additionally, functional traits measured at the seedling stage usually corresponds with functional characteristics of adults (Cornelissen et al. 2003). Plants were grown in Forest Pot 300 ® trays (Nuevos Sistemas de Cultivo S.L., Girona, Spain) that has 50 cavities of 300 ml, which were filled with fertilized (1kg m^{-3} of 16:4:17 N-P-K fertilizer) peat moss, pH 4.5 (Kekkilä F6, Kekkilä Oyi, Finland). Seeding was done in January 2007 and until mid-May, seedlings were cultivated in a greenhouse to avoid spring frosts. Greenhouse temperature ranged from 4 to 25 °C and radiation was approximately 50% of that outside. Seedlings were moved outdoors in mid-May under full sun conditions, fertilized weekly with a 17:5:19, N-P-K + micronutrients water-soluble fertilizer (Hakaphos yellow, BASF, Germany) and irrigated every 2–4 days. Fertilization was accomplished through the overhead sprinkling irrigation system, from the beginning of June to mid-October (20 fertilization events). By the end of nursery culture each seedling had received 129 mg N, 35 mg P, and 134 mg K.

Seedling cultivation during the experiment in the greenhouse

The experiment was carried out in a greenhouse of the Royal Botanic Garden Juan Carlos I at the University of Alcalá. Air temperature varied from 15.3 to 32.0°C and seedlings were watered with tap water twice a week. In addition to N, the acclimation fertilizer solution also contained 0.33 mM KH_2PO_4, 0.33 mM $MgSO_4$, 0.58 mM $CaCl_2*2H_2O$ and 0.34 mM KCl following recommendation by Ingestad (1979) and Landis et al (1989). Micronutrients were supplied using a commercial fertilizer (Hortrilon, Compo, Barcelona, Spain) at a 0.1g l^{-1} concentration.

Soil ^{15}N labeling pulse and calculation of N absorption

Uptake rates of different N source are different when the N forms are applied individually or in mixtures (Näsholm et al. 2009). Thus, "preferences" among N sources should be measured applying all N forms in equimolar amounts. We used the amino acid glycine because it has been

widely used in studies on organic N uptake (Harrison et al. 2008; Warren 2009; Kahmen et al. 2009; Ashton et al. 2010), furthermore it is an abundant amino acid in forest soils (Yu et al. 2002; Andresen et al. 2008) including the Mediterranean soils (Uscola unpublished data). $^{15}NO_3^-$ and $^{15}NH_4^+$ fertilizers were in the form of $K^{15}NO_3$ and $SO_4(^{15}NH_4)_2$, respectively (Sigma Aldrich Co, Milwaukee, USA). The amino acid was in the form of 2-$^{13}C^{15}N$ glycine (Cambridge Isotope Laboratories, London, UK). Abundance of labeled and unlabeled fertilizers is shown in Table S1.

The pots were introduced in plastic bags to avoid liquid losses, and 200 ml of the described solutions were applied. Half of the solution was applied to the soil surface and the other half to the plastic bag to allow the bottom of the pot to be in contact with the solution. Plastic bags were sealed around the pot to minimize the amount of solution that drained out of the pot. Application of the solution took from 07:00 to 08:00 h. Both time of application and time of harvesting were recorded for each seedling and the difference was considered as labeling time.

The amount of N taken up ($N_{absorbed}$) by new roots from a specific labeled N form was calculated for each seedling with isotopic dilution equations (Deléens et al. 1994) as:

$$N_{absorbed} = \frac{X_N \times [N_{root}] \times DM}{N\ atomic\ mass} \times 1000 \qquad (\mu mol) \qquad (3)$$

where $[N_{root}]$ is the N concentration in new roots (mg g DM^{-1}); DM is the mass of the new roots (g); and X_N is the proportion of N in the new roots that came from the specific labeled N form (either NH_4^+, NO_3^- or glycine), which was calculated as:

$$X_N = \frac{(A_{LO}) - (A_{UO})}{(A_{LF}) - (A_{UF})} \qquad (4)$$

where A_{LO} is the ^{15}N abundance (atom%) in new roots in labeled seedling and A_{UO} is the average ^{15}N abundance of new roots in the unlabeled seedlings (Table S1). A_{LF} and A_{UF} are the ^{15}N abundance of the labeled and unlabeled fertilizer, respectively. The amount of C absorbed from the dual labeled ^{15}N-^{13}C glycine ($C_{absorbed}$) was calculated using the same equations but substituting X_N, N_{root} and ^{15}N abundance with X_C, C_{root} and ^{13}C abundance, respectively.

N absorption rate of each N form was calculated as:

$$N_{absorption\ rate} = \frac{N_{absorbed}}{DM \times time} \times 1000 \qquad (\mu g\ g^{-1}\ h^{-1}) \qquad (5)$$

Labeling time and new roots dry mass of each single seedling were used to standardize calculations and avoid differences among seedlings due to small differences in labeling duration and root development, respectively.

GCMS dual labeled glycine methodology

The second method analyzed the amount of 2-$^{13}C^{15}N$-glycine in a new root sample by gas chromatography-mass spectrometry (GC–MS) of *tert*-butyldimethylsilyl derivatives (Warren 2012). 20 mg (±1 mg) of freeze dried and ground root material was extracted with 700 μL of hot methanol by shaking for 30 min at 60 °C. Aqueous and organic phases were separated by addition of 400 μL of chloroform and 800 μL of water. 100 μL of the aqueous phase and 5 μL of internal standard (0.1 mg mL^{-1} norleucine) were dried and taken up in 100 μL of N,N-dimethylformamide. 50 μL of dimethylformamide was added and samples were derivatised by heating at 80 °C for 45 min. Amino acid derivatives were separated by capillary gas chromatography (30 m Long × 0.25 mm ID × 0.25 μm film thickness; Rtx-5SilMS, Restek, Bellfonte, USA). The column eluent was ionised by electron impact (70 eV) and mass spectra were collected from 100 to 600 amu (GCMS-QP2010Plus, Shimadzu, Kyoto, Japan). ^{14}N, ^{12}C glycine was quantified from mass 246. 2-$^{13}C2$, ^{15}N glycine was quantified from mass 248 after subtracting the contribution to mass 248 from the natural isotopes of ^{12}C, ^{14}N glycine.

References

Andresen LC, Jonasson S, Ström L, Michelsen A (2008) Uptake of pulse injected nitrogen by soil microbes and mycorrhizal and non-mycorrhizal plants in a species-diverse subarctic heath ecosystem. Plant Soil 313:283–295.

Ashton IW, Miller AE, Bowman WD, Suding KN (2010) Niche complementarity due to plasticity in resource use: plant partitioning of chemical N forms. Ecology 91:3252–3260.

Cornelissen JHC, Cerabolini B, Castro-Díez P, et al (2003) Functional traits of woody plants : correspondence of species rankings between field adults and laboratory-grown seedlings ? J Veg Sci 14:311–322.

Deléens E, Cliquet JB, Prioul JL. 1994. Use of ^{13}C and ^{15}N plant label near natural abundance for monitoring carbon and nitrogen partitioning. Aust J Plant Physiol 21: 133–46

Harrison KA, Bol R, Bardgett RD (2008) Do plant species with different growth strategies vary in their ability to compete with soil microbes for chemical forms of nitrogen? Soil Biol Biochem 40:228–237.

Ingestad T (1979) Mineral nutrient requirements of *Pinus silvestris* and *Picea abies* seedlings. Physiol Plant 45:373–380.

Kahmen A, Livesley SJ, Arndt SK (2009) High potential, but low actual, glycine uptake of dominant plant species in three Australian land-use types with intermediate N availability. Plant Soil 325:109–121.

Landis T, Tinus R, McDonald AJS, Barnett JP (1989) Mineral nutrients and fertilization. In: Landis TD, Tinus RW, McDonald SE, Barnett JP (eds) The Container Tree Nursery Manual. Vol. 4. Seedling nutrition and irrigation. U.S. Department of Agriculture, Forest Service, Washington, DC, p 67

Näsholm T, Kielland K, Ganeteg U (2009) Uptake of organic nitrogen by plants. New Phytol 182:31–48.

Pemán García J, Navarro Cerrillo RM, Nicolás Peragón JL, et al (2014) Producción y manejo de semillas y plantas forestales. Tomo 2. Organismo Autónomo Parques Nacionales Ministerio de Agricultura, Alimentación y Medio Ambiente

Pulido FJ, Díaz M (2005) Regeneration of a Mediterranean oak: A whole-cycle approach. Ecoscience 12:92–102. doi: 10.2980/i1195-6860-12-1-92.1

Warren CR (2009b) Uptake of inorganic and amino acid nitrogen from soil by *Eucalyptus regnans* and *Eucalyptus pauciflora* seedlings. Tree Physiol 29:401–9.

Warren CR (2012) Post-uptake metabolism affects quantification of amino acid uptake. New Phytol 193:522–31.

Yu Z, Zhang Q, Dahlgren RA, et al (2002) Contribution of amino compounds to dissolved organic nitrogen in forest soils. Biogeochemistry 61:173–198.

Table S1. ^{15}N and 13C abundance (atom%) in unlabeled (A_{UO}) and labeled (A_{LO}) new roots in one-year-old seedlings of *Quercus ilex* and *Pinus halepensis* 6h after of a labeling pulse into the soil. Column in the right indicates fertilizer ^{15}N and ^{13}C abundance. Data are mean± 1 SE.

	Q. ilex	*P. halepensis*	Fertilizer
Abundance in unlabeled samples (A_{UO})			
Control (^{13}C)	1.0808±0.0011	1.0803±0.0006	1.082
Control (^{15}N)	0.3682±0.0004	0.3701±0.0006	0.3664
Abundance in labeled samples (A_{LO})			
$^{15}NO_3^-$	0.5749±0.0631	0.6220±0.0381	60
$^{15}NH_4^+$	1.2018±0.2516	1.2687±0.2395	60
^{15}N-Glycine	1.3829±0.1634	1.6017±0.4316	98
^{13}C-Glycine	1.1072±0.0043	1.1113±0.0107	99

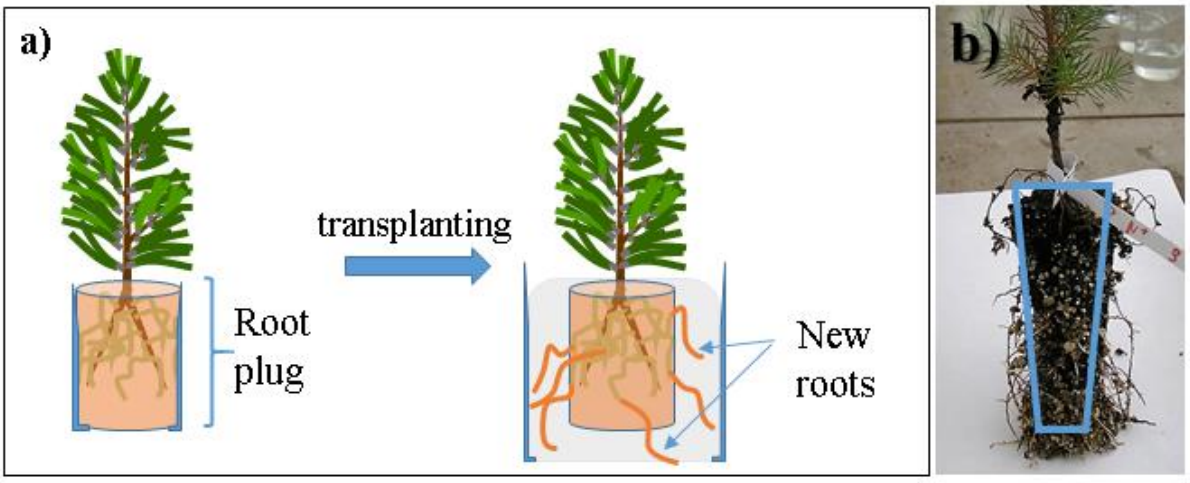


Figure S1 (supplementary material). a) Image showing the root growth of one year-old seedlings, in container-grown plants the plug is a mass build up with the roots that hold the substrate. In the seedlings used in the experiment a plug was formed the first year when cultivated in 0.3L containers. When the seedling were transplanted to a larger pot (2.5 L pots) for the labeling experiment new fine roots protruded out of the plug and colonized the transplanting substrate. b) Seedling at harvesting, the plug is delimited by the blue line.