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Research paper

# Normalization criteria determine the interpretation of nitrogen effects on the root hydraulics of pine seedlings

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**Plant hydraulics is key for plant survival and growth because it is linked to gas exchange and drought resistance. Although the environment influences plant hydraulics, there is no clear consensus on the effect of nitrogen (N) supply, which may be, in part, due to different hydraulic conductance normalization criteria and studied species. The objective of this study was to compare the variation of root hydraulic properties using several normalization criteria in four pine species in response to three contrasting N fertilization regimes. We studied four closely related, yet ecologically distinct species: *Pinus nigra* J.F. Arnold, *Pinus pinaster* Ait., *Pinus pinea* L. and *Pinus halepensis* Mill. Root hydraulic conductance ($K_h$) was measured with a high-pressure flow meter, and values were normalized by total leaf area (leaf specific conductance, $K_l$), xylem cross-section area (xylem specific conductance, $K_s$), total root area (root specific conductance, $K_r$) and the area of fine roots (fine root specific conductance, $K_{fr}$). Controlling for organ size differences allowed comparison of the hydraulic efficiency of roots to supply or absorb water among fertilization treatments and species. The effect of N on the root hydraulic efficiency depended on the normalization criteria. Increasing N availability reduced $K_l$ and $K_s$, but increased $K_h$, $K_r$ and especially $K_{fr}$. The positive effect of N on $K_r$ and $K_{fr}$ was positively related to seedling relative growth rate and was also consistent with published results at the interspecific level, whereby plant hydraulics is positively linked to photosynthesis and transpiration rate and fast growth. In contrast, normalization by leaf area and xylem cross-sectional area ($K_l$ and $K_s$) reflected opposite responses to $K_r$ and $K_{fr}$. This indicates that the normalization criteria determine the interpretation of the effect of N on plant hydraulics, which can limit species and treatment comparisons.**

***Keywords***: ecophysiology, high-pressure flow meter, nitrogen fertilization, *Pinus* spp., plant hydraulics, root hydraulic conductance, specific hydraulic conductance, water relations.

## Introduction

The absorption and transport of water are key physiological processes for plant growth and survival, especially under drought stress (Lambers et al. 2008). Plant hydraulics has evolved concurrently with foliar physiology by placing a limit to the gas exchange capacity of plants (Brodribb 2009). Accordingly, photosynthesis rate and stomatal conductance are frequently positively correlated with plant hydraulics across plant species in several biomes such as rain forests (Brodribb and Feild 2000, Santiago et al. 2004), dry tropical forests (Brodribb et al. 2002), temperate forests (Choat et al. 2011) and in grasslands (Maherali et al. 2008). Such a positive relationship was also found among ferns, conifers and angiosperms (Brodribb et al. 2004). Hydraulic traits thus form part of a unified plant economics spectrum, where high hydraulic conductance is positively linked to a set of attributes associated with high carbon assimilation rate and fast growth (Zhang and Cao 2009, Reich 2014, Mencuccini et al. 2019).

The plant hydraulic system includes roots (water uptake), xylem (water transport) and leaves (main evaporative surfaces) (Cruiziat et al. 2002). The structure and physiology of these organs varies widely, resulting in large differences in water transport capacity among plant species (Mencuccini 2003, Wright et al. 2006, Hernández et al. 2009). Hydraulic conductance ($K_h$), the flow of water through the plant or a specific organ per unit of pressure, scales up with the foliage and root absorption area and the stem xylem water transport capacity of plants (Cruiziat et al. 2002). Therefore, several normalization criteria have been used to compare $K_h$ among plants. Most studies have used the leaf specific conductance ($K_l$), which normalizes $K_h$ by the total leaf area supported by the plant or a specific organ. $K_h$ can also be normalized by xylem cross-section area (xylem specific conductance, $K_s$) or by root surface area (root specific conductance, $K_r$) (Fichot et al. 2011, Nardini and Tyree 1999). These specific conductance assessments are methods for comparing the efficiency of the plant hydraulic system, i.e., the capacity to transport or supply water for meeting the plant water demand.

Plants adjust their water absorption and transport capacity with changes in the environment, such as in response to drought (Cochard et al. 1996) or low temperature (Fennell and Markhart 1998). Soil fertility, especially that of nitrogen (N), can also affect plant hydraulics, particularly during early life stages (Mencuccini 2003, Lambers et al. 2008, Wang et al. 2017). Increase in soil N generally promotes aboveground over belowground growth, resulting in reduced root-to-shoot-mass ratio that potentially creates an imbalance in water uptake relative to plant demand (Birk and Vitousek 1986, Villar-Salvador et al. 2012). However, high soil N availability during growth can increase the diameter and density of xylem conduits, the xylem cross-section area and the expression of aquaporins (Hacke et al. 2010, Borghetti et al. 2016). These changes at the within-species scale in the hydraulic system can be interpreted as anatomical and physiological adjustments to match the water transport capacity with increasing plant water demand due to N availability, which also tends to augment gas exchange, particularly photosynthesis due to increased leaf area and intrinsic carboxylation rate (Evans 1989, Ripullone et al. 2003, Oliet et al. 2013, Zhang et al. 2018). Thus, published literature suggests that plants have acclimation responses to coordinate the hydraulic system, gas-exchange rate and growth with N supply (Hajek et al. 2014). Additionally, root growth undergoes significant adjustments with increasing soil N availability, but changes are not unidirectional. While most studies show that an increase in N increases root growth (Hernández et al. 2009), others have reported a decrease (Wang et al. 2017) or no change (Trubat et al. 2006). In addition, N can reduce the ratio of fine to coarse roots and specific root length (Li et al. 2015). Fine roots are more efficient in the uptake and transport of water than coarse roots (Sands et al. 1982), and they account for a high portion of root surface area and consequently of the water and nutrient absorption capacity of the root system (Green and Clothier 1999, Wells and Eissenstat 2002, Rewald et al. 2011). Changes in fine root growth, therefore, can have important consequences for plant hydraulics at the within-species scale (Brissette and Chambers 1992, Steudle and Meshcheryakov 1996).

The different $K_h$ normalization criteria for comparing the efficiency of the hydraulic system among plants should integrate effects of potential sources of changes on the plant attributes listed above, including soil N. However, published studies that analyzed plant hydraulics in response to soil N availability have used different normalization criteria (Lovelock et al. 2004, Samuelson et al. 2007, Wang et al. 2017), and the results are frequently discordant. Most studies indicate that $K_l$ and $K_s$ (measured in roots or shoots) decrease with N (Bucci et al. 2006, Hernández et al. 2009, Luis et al. 2010, Wang et al. 2016), while fewer studies have shown a positive or no effect of N availability (Clearwater and Meinzer 2001, Hacke et al. 2010). Similarly, the effect of N on $K_r$ is in most cases neutral or positive (Steudle and Meshcheryakov 1996, Trubat et al. 2006, 2012, Wang et al. 2017), although some negative effects have been reported (Hernández et al. 2009, Luis et al. 2010). In addition to differences in normalization criteria and the experimental conditions, it is possible that variation among studies may be attributed to differences in species ecology, as species might differ in their response of hydraulics and biomass allocation patterns to N (Hernández et al. 2009).

The objective of this study was to compare the variation of root hydraulic properties using several normalization criteria in seedlings of four ecologically distinct pine species in response to N supply. These species (*Pinus halepensis* Mill., *Pinus pinea* L., *Pinus pinaster* Ait., *Pinus nigra* Arnold) are widespread across southern Europe, are distributed along an aridity and temperature gradient, and show contrasting ecological and growth patterns (Climent et al. 2011, Fernández-Pérez et al. 2018). *Pinus halepensis* and *P. pinea* are found in low- and mid-altitude locations in a typical Mediterranean climate, with mild to cool and humid winters and hot, dry summers (Navarro Cerrillo et al. 2013, Puértolas et al. 2013). At these sites, summer water stress is the main limiting factor for plant life (Mitrakos 1980). At the other extreme, the high mountain species *P. nigra* thrives in environments where low winter temperature is the main limiting factor for plant life (Barbero et al. 1998, Peñuelas et al. 2013). Finally, *P. pinaster* is usually found in habitats with intermediate drought and temperature conditions compared with the previous species (Lafuente et al. 2013). These pine species differ in their response of frost tolerance (Toca et al. 2018) and dynamics of root growth and architecture (Toca et al. 2019) to N supply. Due to their ecological variation, these pine species have different biomass allocation pattern (Climent et al. 2011), which may also differ in response to increasing soil N that can differentially affect their hydraulic architecture. In addition, using closely related species reduces phylogenetic bias in comparative studies. Because fine roots are directly related to the rate of water and nutrient absorption (Green and Clothier 1999, Wells and Eissenstat 2002, Rewald et al. 2011), we proposed to normalize root $K_h$ using fine root ($\leq$1 mm in diameter) surface area (fine root specific hydraulic conductance, $K_{fr}$). We hypothesized that the efficiency of the root hydraulic system, measured as $K_l$, $K_s$, $K_r$ and $K_{fr}$, would increase in response to N availability in order to match the water transport capacity to the increase in assimilation and transpiration capacity of the plant. To test this hypothesis, we performed an experiment where seedlings were cultivated under several N fertilization regimes and their root hydraulic conductance measured and compared using several normalization criteria. We used seedlings as this life stage is an important bottleneck for pine recruitment (Herrero et al. 2013).

## Materials and methods

### Plant material and experimental design

Seeds of the four pine species were collected in the southern part of the Iberian range (eastern Iberian Peninsula, Alía Miranda et al. 2009). Climate of these provenances, geographical coordinates of seed sources and mean climatic characteristics in the species range in Spain are described in Table S1 available as Supplementary Data at *Tree Physiology* Online. To obtain the climatic characteristics, we used distribution data for continental Spain from the third Spanish Forest Inventory, which analyzed adults and saplings of all woody species in plots distributed over forest ecosystems according to a 1-km$^2$ grid. For each studied species, we selected those plots classified as natural pine forests based on the Spanish Regions of Provenance (Ruiz-Benito et al. 2012) that had presence of seedlings. Mean values of climatic variables were calculated overlapping the selected plots on a climate map with 1-km$^2$ spatial resolution (Gonzalo 2008).

Seeds were sown in February 2012 at the Centro Nacional de Recursos Genéticos Forestales 'El Serranillo' (Central Spain, 40°40′N, 3°10′W, 650 m above sea level (a.s.l.)) into plastic trays (Plasnor® 190/300-45, Legazpi, Spain), which have 45,300-ml cells, at a cultivation density of 283 plants m$^{-2}$. Growing medium was *Sphagnum* peat moss pH = 4.7, enriched with a 16-10-20 NPK slow release fertilizer at a rate of 0.9 kg m$^{-3}$ (Kekkila® White 420 F6, Finland). Trays were kept in an unheated greenhouse during germination and emergence phases to avoid late spring frost damage. On 17 May, 2012, all trays were transferred to the School of Forestry at the Technological University of Madrid (40°27′N; 3°43′W, 664 m a.s.l.) where the plants remained outdoors for the rest of the experiment. On the same date, 10 seedlings per species were randomly harvested to assess seedling mass for future relative growth rate (RGR) measurements (see RGR description below). Samples were then dried at 60 °C for 48 h and weighed to assess their mass. The initial mass of the seedlings for *P. halepensis*, *P. pinea*, *P. pinaster* and *P. nigra* was 0.37 ± 0.02, 1.49 ± 0.09, 0.73 ± 0.06 and 0.58 ± 0.06 g, respectively. Plants were then assigned to one of three fertilization treatments: (i) high and (ii) low fertilization, where each plant was supplied with 150 and 20 mg N, respectively, from 22 May to 19 September, 2012 (pre-hardening period), and (iii) fall fertilization, where each plant was supplied with 60 mg N during the pre-hardening period + 40 mg N plant$^{-1}$ during the fall, from 26 September to 8 November, 2012. Fertilizer was applied by hand at a weekly constant rate using a water-soluble fertilizer. The fertilizer was 20 N–20$P_2O_5$–20$K_2O$ plus proportional amounts of other macro- and micronutrients (Peters Professional, Scotts Co., Marysville, OH, USA) with N sources being ammonium nitrate (10%) and urea (10%). The extra supply for fall fertilization was applied as ammonium nitrate. At each fertilization, all seedlings received 55 ml of the fertilization solution. After fertilization, seedlings were watered for 5 min to remove the fertilizer remaining on the needles. Supplemental irrigation was applied every day with a broadcast system for an average of 70 ml plant$^{-1}$ (excepting fertilization days), which kept the media at field capacity almost permanently, according to predetermined weight of trays at field capacity and periodical weight controls after irrigation. Each fertilization treatment had three trays, resulting in nine trays per species. Trays were completely randomized in space, and their position was rotated every 15 days to minimize edge effects. The experimental design was a two factorial, with species (four levels) and fertilization treatments (three levels) as main effects. Seedlings were grown under full sun except from 20 June to 15 September, where plants grew under shading with a 20% light transmission to reduce evapotranspiration.

### Hydraulic conductance and morphology measurements

On 15 April, 2013, six seedlings per species and treatment (two per tray) were randomly selected for maximum root hydraulic conductance measurements. The shoot was excised 5 mm under the cotyledon insertion scar with the entire seedling, including the root plug, immersed in water to avoid entrance of air into the tracheids of the root system. The remaining stem of the root system was then connected to a high-pressure flow meter (HPFM, Dynamax Inc., Houston, TX, USA) after debarking 2 cm. The method for measuring root hydraulic conductance using the HPFM apparatus was described in detail by Tyree et al. (1995). Briefly, the HPFM perfuses degasified water into the base of the root system under an increasing pressure of 3–7 kPa s$^{-1}$ while measuring the corresponding flow every 3 s. The pressure was increased up to 0.5 MPa. The hydraulic conductance ($K_h$) was measured three times per plant and was calculated from the slope of the linear region of the regression of water flow against pressure. Later, shoots and roots were frozen to −20 °C until processing. Once defrosted, plants were washed with tap water and rinsed in distilled water for 3 min. Then, shoots were separated into needles and stem. The diameter of the stem without bark in contact with the HPFM was measured to calculate the xylem cross-section area, assuming the stem to be a cylinder. Leaves were scanned (Lide 120, Canon, Tokyo, Japan), and the surface area was calculated with ImageJ (version 1.46r, Madison, WI, USA). Roots were scanned with a scanner (EpsonScan v.304S 10000XL 3.4, Epson, Tokyo, Japan) and the images analyzed with the software WinRhizo Pro 2007.d® (Regent Instruments Inc., Québec, Canada) to obtain the total length, diameter and surface area of roots. Later, roots were differentiated into two categories: fine (≤1 mm in diameter) and coarse (>1 mm in diameter) (King et al. 2002). Samples were then dried at 60 °C for 48 h and weighed to assess their mass. The leaves, stem and roots of two seedlings were composited and ground in a ball mill (PM 100, Retsch, Haan, Germany) to make three composite samples per treatment (one per tray). Nitrogen concentration of these samples was done by the standard Kjeldahl method using an auto-analyzer (CFA SAN++, Skalar, Breda, The Netherlands). Nitrogen content of organs was calculated as the product of organ mass and its respective N concentration. Plant N content was calculated as the sum of the root, stem and foliage N content.

RGR was calculated as the difference between the natural logarithms of final mass and the mass of seedling of the same batch 43 weeks before divided by time between the two measurements. The root maximum $K_h$ values were used to calculate leaf ($K_l$), xylem ($K_s$), root ($K_r$) and fine root ($K_{fr}$) specific hydraulic conductance by dividing $K_h$ value by total leaf area, xylem cross-section area, root system and fine root surface, respectively.

### Statistical analysis

We assessed the effect of plant species and fertilization treatment on plant morphology and hydraulic traits. For all analyses, $P$-values were computed using $F$-tests as well as a randomization protocol that generated null distributions of variables and interactions by randomly reshuffling these data across species 10,000 times (Monte Carlo method, Crowley 1992). For each of these subsamples, we obtained the $F$-value of a two-way ANOVA analysis with species and treatments as main factors including interactions. The resulting 1000 $F$-values were compared with the $F$-value (ANOVA) from the observed data. The $P$-value is the proportion of all data arrangements resulting in a test statistic at least as extreme in magnitude as the $F$-value from the observed data. $P$-values thus generated are expected to be more robust against potential biases in data (Crowley 1992). All analyses were performed in R software (R Foundation for Statistical Computing, Vienna, Austria).

## Results

### Seedling morphology and N content

Nitrogen supply significantly affected seedling growth, root-to-shoot ratio and N content; however, for most traits the magnitude of this effect depended on the species (species × fertilization interaction, Table 1). Nitrogen fertilization consistently increased the N content, mass, RGR, total leaf area, root surface area and xylem cross-section area of seedlings (Table 2). Overall, across species, high fertilization increased the root, leaf and xylem cross-section area by 53, 148 and 123%, respectively, with respect to low-fertilized plants. *Pinus halepensis* showed the largest differences between high and low fertilization for all traits, while *P. pinea* showed the lowest difference. For instance, total leaf area of high-fertilized plants was 3.8 times greater than for low-fertilized plants in *P. halepensis*. This difference was 1.7, 1.9 and 2.3 times in *P. pinea*, *P. pinaster* and *P. nigra*, respectively. The relative differences in root surface area between the high and low fertilization treatments were smaller than for total leaf area and xylem cross-section area and seedling mass in all species except for *P. halepensis*. In contrast to previous traits, fertilization reduced the root-to-shoot ratio and the fraction of fine root area in all species (Table 2). Overall, across species, fall fertilization increased the root, leaf and xylem cross-section area by 22, 63 and 76%, respectively, with respect to low-fertilized plants. The effect of fall fertilization on growth, morphology and N content varied among species. While for *P. pinaster*, trait values of fall-fertilized plants were closer to low-fertilized plants, for other species such as *P. pinea*, values were closer to high-fertilized seedlings. For the latter species, traits such as root surface area were maximum with fall fertilization (Table 2).

*Pinus pinea* and *P. pinaster* had the highest values for all growth and morphological variables except for the root–shoot ratio and RGR, while *P. nigra* and *P. halepensis* showed the lowest morphological values and the highest root–shoot ratio. *Pinus halepensis* had the highest RGR of all species and had higher leaf and root surface area than *P. nigra*, but lower than *P. pinea* and *P. pinaster* (Table 2). *Pinus pinea* had higher N content compared with other species, which did not differ in their N content (Figure 1).

### Hydraulic conductance

$K_h$ significantly increased with N fertilization and N content in all species (Table 1, Figure 1). On average, across species, high fertilization and fall fertilization increased $K_h$ by 79 and 57%, respectively, with respect to low-fertilized plants. Low-fertilized seedlings showed the lowest $K_h$ values in all species, while, with the exception of *P. pinaster*, fall- and high-fertilized seedlings showed similar values (fertilization × species interaction, Table 1, Figure 1). In *P. pinaster*, high-fertilized plants had the highest $K_h$, low-fertilized plants had the lowest value,

Table 1. Statistical results of the effect of species and fertilization treatments on plant variables.

| | Species | Treatment | Species × Treatment |
|---|---|---|---|
| Seedling mass | **39.4 (<0.001)** | **105.5 (<0.001)** | **2.8 (0.020)** |
| N content | **26.9 (<0.001)** | **90.9 (<0.001)** | **2.76 (0.036)** |
| Root surface area | **7.1 (<0.001)** | **21.1 (<0.001)** | **4.4 (<0.001)** |
| Leaf surface area | **25.4 (<0.001)** | **63.7 (<0.001)** | **3.2 (<0.001)** |
| Xylem cross-section area | **44.2 (<0.001)** | **79.0 (<0.001)** | **4.1 (0.002)** |
| Relative growth rate (RGR) | **28.2 (<0.001)** | **122.8 (<0.001)** | **3.0 (0.012)** |
| Root-to-shoot-mass ratio | **61.7 (<0.001)** | **40.5 (<0.001)** | 1.6 (0.163) |
| Fraction of fine root surface % | **8.1 (<0.001)** | **21.2 (<0.001)** | 1.4 (0.226) |
| $K_h$ | 2.6 (0.060) | **44.7 (<0.001)** | **2.4 (0.038)** |
| $K_l$ | **22.7 (<0.001)** | **8.3 (<0.001)** | 2.0 (0.083) |
| $K_s$ | **30.8 (<0.001)** | **12.2 (<0.001)** | 1.4 (0.246) |
| $K_r$ | **7.3 (<0.001)** | **6.0 (<0.004)** | 2.1 (0.064) |
| $K_{fr}$ | 1.6 (0.197) | **15.4 (<0.001)** | **3.3 (0.008)** |

Data are *F*-values (*P*-values in brackets). $K_h$, Root hydraulic conductance; $K_l$, root hydraulic conductance per leaf area; $K_s$, root hydraulic conductance per xylem cross-section area; $K_r$, root hydraulic conductance per root area; $K_{fr}$, root hydraulic conductance per fine root area. Bold values are statistically significant effects ($P < 0.05$)

and fall-fertilized plants had slightly higher $K_h$ than low-fertilized plants (Table 2). Specifically, $K_h$ of high-fertilized plants was 2.0, 1.6, 1.8 and 1.8 times greater than low-fertilized seedlings in *P. halepensis, P. pinea*, *P. pinaster* and *P. nigra*, respectively.

$K_l$ and $K_s$ significantly differed among species (Table 1), which overall were in the following order: *P. nigra* ≥ *P. halepensis* ≥ *P. pinaster* > *P. pinea* (Figure 2a and c). Fertilization had the same effect on $K_l$ and $K_s$. Specifically, low- and fall-fertilized seedlings had the highest $K_l$ and $K_s$ values, while high-fertilized seedlings consistently showed the lowest values (Table 1 and Figure 2a and c).

$K_r$ also varied among species (Table 1). *Pinus nigra* had the highest $K_r$ followed by *P. pinaster*, while *P. halepensis* and *P. pinea* showed the lowest $K_r$ values (Figure 2b). In contrast to $K_l$ and $K_s$, fertilization increased $K_r$. Fertilization had a similar effect on $K_{fr}$ as $K_r$ in all species, but the differences among fertilization treatments within species were higher for $K_{fr}$, resulting in a significant species × fertilization interaction (Table 1). Specifically, fall-fertilized plants had the highest $K_{fr}$ values in *P. halepensis*, while in *P. pinaster* fall- and low-fertilized seedlings had similar values. High- and fall-fertilized plants had significantly higher $K_{fr}$ in *P. pinea* and *P. nigra* than low-fertilized plants.

## Discussion

Root hydraulic efficiency in pine seedlings in response to N fertilization showed different results depending on the normalization criteria. Increasing N availability had a negative effect on $K_l$ and $K_s$, but a positive effect on $K_r$ and especially on $K_{fr}$. This indicates that the interpretation of the effects of N on root hydraulic efficiency depends on whether root or shoot traits are used to normalize $K_h$. Thus, if the purpose of the specific hydraulic conductance ($K_l$, $K_s$, $K_r$, $K_{fr}$) is to compare the efficiency of the hydraulic system (i.e., the capacity of the system to transport or supply water to meet the water demand of the plant), it must be recognized that the criteria for normalizing variables results in different meanings and therefore different interpretations. We discuss the effects of N on the organs and tissues involved in hydraulic conductance and their consequence for interpreting the results of different normalization criteria below.

According to Ohm's law analogy for plant hydraulics, transpiration rate in unstressed plants and under steady-state conditions is directly related to the water potential gradient from the soil to leaf and especially to the plant hydraulic conductance (Cruiziat et al. 2002, Brodribb 2009). Accordingly, plant gas exchange rate has been found to be positively linked to hydraulic conductance across species (Brodribb and Feild 2000, Brodribb et al. 2004, Santiago et al. 2004, Maherali et al. 2008, Brodribb 2009, Choat et al. 2011, Liu et al. 2019), which may explain in part RGR differences among species (Reich 2014). Because increase in N availability often enhances stomatal conductance, photosynthesis and transpiration rate (Evans 1989, Ripullone et al. 2003, Domec et al. 2009, Oliet et al. 2013, Villar-Salvador et al. 2013, Zhang et al. 2018), RGR and field survival (Kontunen-Soppela et al. 2000, Oliet et al. 2009, Villar-Salvador et al. 2012), it can be expected that normalized $K_h$ will increase or keep constant with N availability to keep pace with the increasing water demand of the plant. In our study, N fertilization increased root $K_h$ and RGR and modified the biomass allocation pattern to the main components of plant hydraulics: roots, stems and leaves (Table 2). However, root $K_l$ and $K_s$ were consistently reduced with increasing N availability in pine species.

Nitrogen fertilization increased total leaf area and xylem cross-sectional area proportionally more than root $K_h$, which explains the overall reduction of $K_l$ and $K_s$ with N availability.

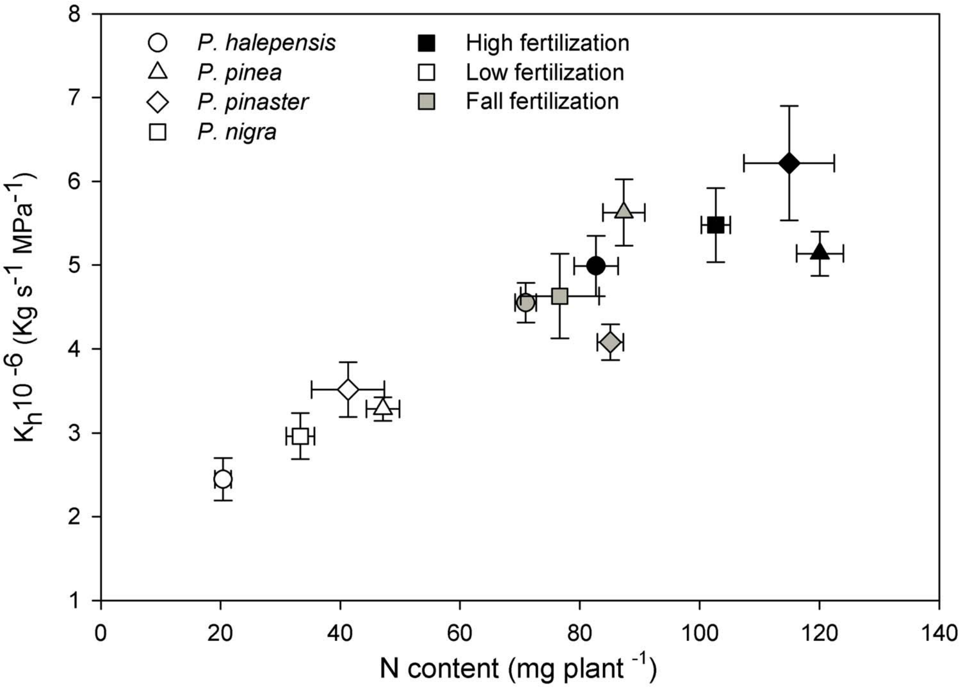

Figure 1. Relationship between seedling N content and root hydraulic conductance in four pine species. Seedlings were cultivated under three N fertilization regimes (low, high and fall fertilization). Data are means ±1 SE.

Similar to our findings, other studies have shown a negative effect of N on root $K_l$ (Mencuccini 2003, Hernández et al. 2009, Luis et al. 2010, Wang et al. 2016) and shoot $K_l$ (Bucci et al. 2006). Nonetheless, a few studies have reported positive or no effects of N fertilization on shoot $K_l$ (Clearwater and Meinzer 2001, Samuelson et al. 2007, Hacke et al. 2010). However, Hacke et al. (2010) and Samuelson et al. (2007) used different methods to measure $K_h$, which also might limit the comparability of these results. Fewer studies have analyzed the effect of N on $K_s$, and the results do not show clear trends (Lovelock et al. 2004, Samuelson et al. 2007, Hacke et al. 2010). It is also possible that the reduction of $K_l$ and $K_s$ in our study, and discordance among studies, occurs because normalization by leaf or xylem cross-section area (i.e., $K_l$ and $K_s$) does not consider other leaf physiological processes and xylem anatomy features that are intimately linked to water transport and demand. These physiological processes and anatomical features also covary with leaf and xylem cross-section area in response to N availability. For instance, plants increase stomatal conductance and photosynthesis rate with N availability (Evans 1989, Ripullone et al. 2003, Oliet et al. 2013, Villar-Salvador et al. 2013), increasing water demand per leaf area. N fertilization also increases water-use efficiency (Hernández et al. 2009, Zhang et al. 2018) at an intraspecific level because stomatal conductance, and consequently transpiration rate, increases at a lower rate with N supply than does photosynthesis rate (Zhang et al. 2018). Therefore, at leaf level, maximum stomatal conductance or transpiration and photosynthesis rate might be more important than leaf area *per se* for driving the coordination of plant hydraulic capacity with plant water demand during plant ontogeny. For example, Liu et al. (2019) found that stomatal conductance was positively correlated with the hydraulic conductance of leaf venation. However, according to a meta-analysis by Zhang et al. (2018), stomatal conductance and transpiration rate vary little with N availability, while the production of leaves is strongly increased. Consequently, the maximum plant transpiration, calculated as the product of maximum stomatal conductance and total leaf area, might increase at a similar or a lower rate than $K_h$ and at a lower rate than leaf area in response to an increase in N availability. Hence the normalization of $K_h$ by plant maximum transpiration, rather than by leaf area, might provide a more precise picture of plant hydraulic efficiency because it represents the plant's specific demand of water. At the xylem level, high N availability can increase the density of conduits per sapwood area (Borghetti et al. 2016) and the diameter of the conduits (Hacke et al. 2010, Zhang et al. 2018). The conducting potential of a conduit, according to the Hagen–Poiseuille law, is proportional to the fourth power of its radius (Zimmermann 1983, Castro-Díez et al. 1998). In addition, N can increase the expression of water transporters such as aquaporins (Hacke et al. 2010). Therefore, the relationship between the xylem cross-section area and hydraulic conductance can be variable (see Cruiziat et al. 2002); small changes in xylem anatomy and aquaporins with N addition could have a disproportionate high impact on its conductance, augmenting the capacity of shoot

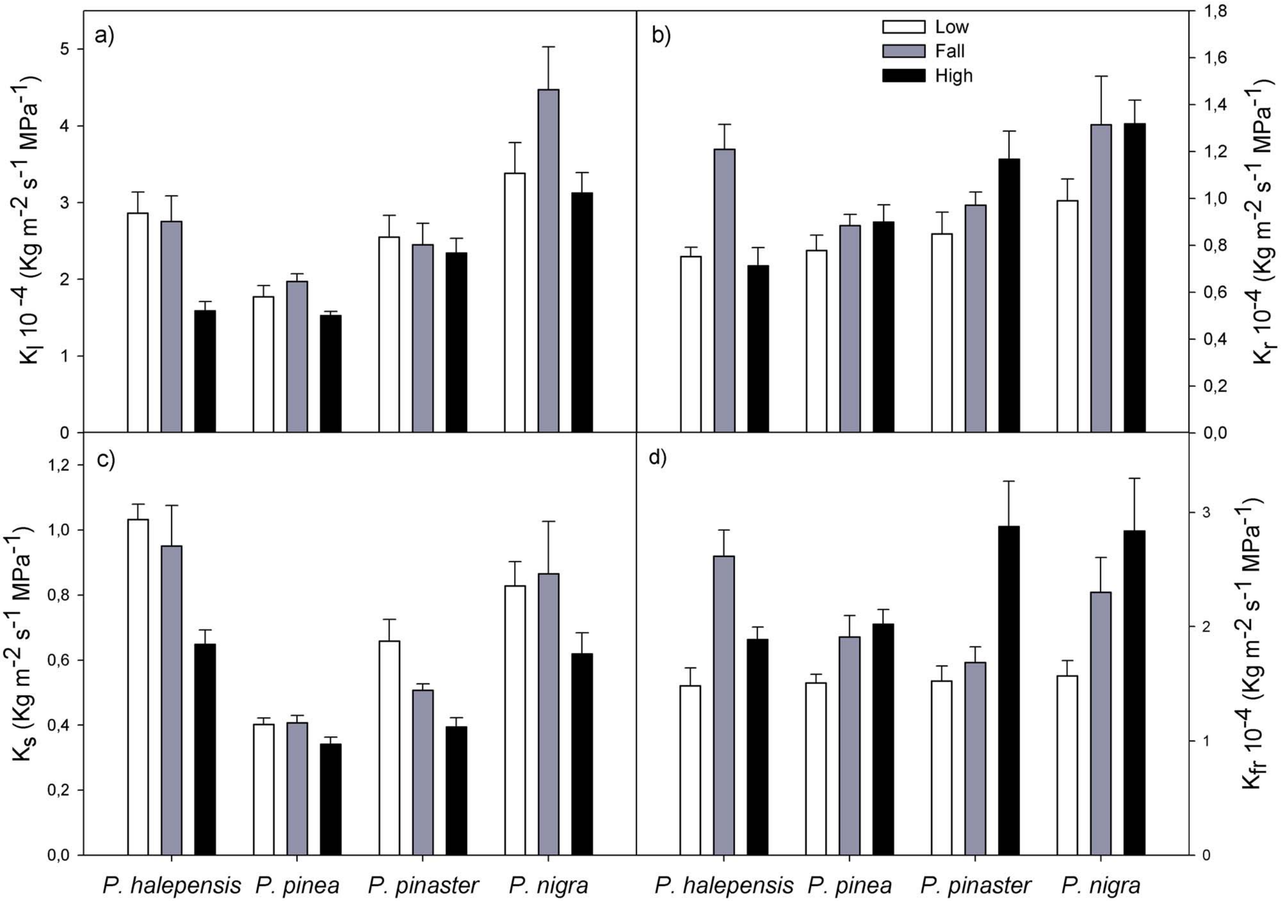

Figure 2. Root hydraulic conductance per leaf surface area ($K_l$) (a), root surface area ($K_r$) (b), xylem cross-section area ($K_s$) (c) and fine root surface ($K_{fr}$) (d) in 1-year-old seedlings of four pine species that were cultivated with three nitrogen fertilization treatments (low, high and fall fertilization). Data are means ±1 SE.

xylem to supply water to the high demanding leaves of high N fertilized seedlings.

Nitrogen fertilization increased the root surface in all species (Table 2) but contrary to results for leaf area, total and fine root surface increased at a lower rate than $K_h$. This explains the positive effect of N fertilization on $K_r$ and $K_{fr}$ and suggests an increase in the hydraulic efficiency of root system with N. The effect of N on root growth varies among studies; most studies report an increase in root growth in response to N fertilization (Hernández et al. 2009), while less frequently others have reported a decrease (Wang et al. 2017) or no effect (Trubat et al. 2006). Response of $K_r$ to N supply also varies among studies, showing mostly a slightly positive or no effect in response to N (Steudle and Meshcheryakov 1996, Trubat et al. 2006, 2012, Wang et al. 2017) and a few negative reports (Hernández et al. 2009, Luis et al. 2010). Total root surface includes roots with different roles in plant hydraulics: fine roots, which determine water uptake, and coarse roots, which primarily serve as anchorage and water transport (Wells and Eissenstat 2002, Kalliokoski et al. 2010, Rewald et al. 2011). Similar to $K_l$ and $K_s$, $K_r$ may also account for some other root physiological and structural processes affected by N availability. On one hand, most published studies that measured $K_r$ used plants that were grown in pots of small volume that might limit root growth (Poorter et al. 2012). However, for Iberian pine species, a reduction in container volume along a gradient from 7 to 0.2 l pots increased the relative biomass allocated to roots (Climent et al. 2011). On the other hand, N reduces the fine-to-coarse-root ratio (Table 2) (Li et al. 2015) but increases the genetic expression of aquaporins (Hacke et al. 2010), potentially increasing the water absorptive capacity and hydraulic conductance of the root system per unit of root area. Consequently, differences in the effect of N on $K_r$ observed among most studies seem to stem from variation in how root morphology and physiology is affected by N availability.

We suggest that the normalization of root $K_h$ by fine root surface ($K_{fr}$), which account for most of the water and nutrient absorption of the root system (Green and Clothier 1999, Wells and Eissenstat 2002, Rewald et al. 2011) instead of total root surface, gives a more precise picture of functional changes of root hydraulics in response to N fertilization. Similar to $K_r$, however, some of the effects of N on root anatomy and

Table 2. Plant morphological and nutrient traits of 1-year-old seedlings of four pine species that were cultivated with contrasting N fertilization regimes.

| Species | Treatment | Seedling mass (g) | Leaf area (cm$^2$) | Xylem surface (mm$^2$) | Root surface (cm$^2$) | Root-to-shoot-mass ratio | Fraction of fine root surface (%) | RGR $10^2$ (week$^{-1}$) | Root hydraulic conductance $K_h$ $10^{-6}$ (Kg s$^{-1}$ MPa$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| *Pinus halepensis* | Low | 2.53 ± 0.15 | 86 ± 8 | 2.4 ± 0.2 | 327 ± 32 | 0.73 ± 0.02 | 52.4 ± 3.4 | 4.44 ± 0.15 | 2.45 ± 0.25 |
| | Fall | 5.49 ± 0.52 | 173 ± 12 | 5.3 ± 0.8 | 385 ± 19 | 0.57 ± 0.03 | 46.4 ± 1.9 | 6.05 ± 0.16 | 4.55 ± 0.24 |
| | High | 9.25 ± 1.09 | 324 ± 37 | 8.0 ± 1.0 | 735 ± 91 | 0.48 ± 0.02 | 41.9 ± 3.6 | 7.41 ± 0.26 | 4.99 ± 0.36 |
| *Pinus pinea* | Low | 6.62 ± 0.23 | 192 ± 18 | 8.2 ± 0.2 | 434 ± 36 | 0.42 ± 0.03 | 51.6 ± 3.1 | 3.46 ± 0.08 | 3.28 ± 0.14 |
| | Fall | 11.89 ± 0.94 | 288 ± 24 | 14.1 ± 1.3 | 635 ± 23 | 0.35 ± 0.02 | 47.4 ± 2.7 | 4.80 ± 0.19 | 5.63 ± 0.40 |
| | High | 14.32 ± 0.64 | 337 ± 17 | 15.1 ± 0.4 | 585 ± 46 | 0.30 ± 0.02 | 44.8 ± 3.4 | 5.26 ± 0.10 | 5.14 ± 0.26 |
| *Pinus pinaster* | Low | 5.02 ± 0.43 | 142 ± 13 | 5.5 ± 0.5 | 422 ± 31 | 0.54 ± 0.02 | 55.2 ± 2.4 | 4.45 ± 0.19 | 3.52 ± 0.33 |
| | Fall | 6.53 ± 0.24 | 172 ± 12 | 8.2 ± 0.7 | 426 ± 31 | 0.47 ± 0.04 | 58.3 ± 2.1 | 5.09 ± 0.08 | 4.08 ± 0.21 |
| | High | 12.89 ± 1.16 | 268 ± 24 | 15.9 ± 1.4 | 542 ± 46 | 0.41 ± 0.02 | 41.9 ± 2.0 | 6.63 ± 0.21 | 6.22 ± 0.68 |
| *Pinus nigra* | Low | 3.62 ± 0.25 | 92 ± 11 | 3.7 ± 0.4 | 306 ± 28 | 0.75 ± 0.03 | 63.1 ± 1.8 | 4.21 ± 0.15 | 2.96 ± 0.27 |
| | Fall | 5.22 ± 0.90 | 108 ± 15 | 6.1 ± 1.2 | 384 ± 64 | 0.69 ± 0.05 | 56.8 ± 3.2 | 4.92 ± 0.00 | 4.63 ± 0.51 |
| | High | 8.43 ± 0.50 | 178 ± 19 | 8.9 ± 0.4 | 420 ± 39 | 0.54 ± 0.02 | 48.9 ± 5.2 | 6.19 ± 0.14 | 5.48 ± 0.44 |

Data are means ±1 SE.

physiology, such as the increase in the expression of aquaporins (Hacke et al. 2010), persist if $K_h$ is normalized by fine root surface. The positive effect of N on $K_{fr}$ concurs with the higher RGR in our plants (Table 2) and photosynthesis rate of high-fertilized seedlings reported in the literature (see references above) in accordance with the root system role of plant water provider. This link between plant functional attributes and $K_{fr}$ at the intraspecific level agree with those at the interspecific level, whereby hydraulic conductance is positively related to RGR (Reich 2014) and transpiration and photosynthesis rate (Brodribb 2009). The increase in $K_{fr}$ with N fertilization could compensate for the high potential vulnerability to water stress of high N fertilized plants due to low root-to-shoot ratio (Grossnickle 2012) and could also help explain the higher survival rate of high N fertilized plants over low N fertilized plants under outplanting conditions (Kontunen-Soppela et al. 2000, Oliet et al. 2009, Villar-Salvador et al. 2012).

The four pine species showed differences in root hydraulic efficiency in response to N (Figure 2). However, these differences in root hydraulic efficiency were not related to traits associated to species ecology (see Introduction). This contrasts with results on frost tolerance, growth dynamics and root architecture in response to N supply in these same species, which were related to species ecology (Toca et al. 2018, 2019). The normalization criteria used in this study to assess the effect of N supply on root hydraulics within species potentially may have the same limitations for comparing the hydraulic system across species. Species strongly differ in gas exchange rate (Maherali et al. 2006) and water-use efficiency (Ponton et al. 2006), xylem conduit diameter and density (Cruiziat et al. 2002, Fernández-Pérez et al. 2018). However, at the interspecific scale, evidence shows that $K_l$ is related to gas exchange and growth rates (Brodribb and Feild 2000, Brodribb et al. 2004, Santiago et al. 2004, Maherali et al. 2008, Choat et al. 2011, Reich 2014, Liu et al. 2019), likely because the differences among species in $K_h$ override the differences in physiological attributes that covary with leaf area.

## Conclusions

This study shows that N affects the root hydraulics of pine species, but the different normalization criteria of Root hydraulic conductance $K_h$ provided radically different pictures of the efficiency of the plant hydraulic system: high N availability had a negative effect on leaf specific conductance $K_l$ and xylem specific conductance $K_s$, yet a positive effect on root specific conductance $K_r$ and especially on fine root specific conductance $K_{fr}$. The effect of N on plant hydraulics described by $K_{fr}$ and $K_r$ seems to be consistent with the evidence at intraspecific and interspecific levels, where plant hydraulics is linked to other physiological processes such as growth rate and photosynthesis. Discrepancies among the various normalization criteria were associated with different biomass allocation patterns of plant fractions with N fertilization: while foliage and xylem transversal area increased faster than $K_h$, root area increased with N fertilization at a lower rate than that of $K_h$. Normalization of $K_h$ by leaf or xylem transversal area (i.e., $K_l$ and $K_s$) probably does not account for leaf physiological processes and xylem anatomy features that are intimately linked to water transport capacity, which also covaries with leaf and xylem cross-section area in response to N availability. Future research is needed to develop normalization methods that more accurately reflect the coordination between the water transport capacity of the hydraulic system and water demand of the plant.

## Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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## Conflict of interest

None declared.

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