Universidad de Alcalá
COMISIÓN DE ESTUDIOS OFICIALES
DE POSGRADO Y DOCTORADO

ACTA DE EVALUACIÓN DE LA TESIS DOCTORAL

(FOR EVALUATION OF THE ACT DOCTORAL THESIS)

Año académico (*academic year*): 2018/19

DOCTORANDO (*candidate PHD*): **TOCA, OVIDIU ANDREI**
D.N.I./PASAPORTE (*Id.Passport*): ******5452**
PROGRAMA DE DOCTORADO (*Academic Committee of the Programme*): **D413-ECOLOGÍA. CONSERVACIÓN Y RESTAURACIÓN DE ECOSISTEMAS**
DPTO. COORDINADOR DEL PROGRAMA (*Department*): **CIENCIAS DE LA VIDA**
TITULACIÓN DE DOCTOR EN (*Phd title*): **DOCTOR/A POR LA UNIVERSIDAD DE ALCALÁ**

En el día de hoy 07/03/19, reunido el tribunal de evaluación, constituido por los miembros que suscriben el presente Acta, el aspirante defendió su Tesis Doctoral **con Mención Internacional** (*In today assessment met the court, consisting of the members who signed this Act, the candidate defended his doctoral thesis with mention as International Doctorate*), elaborada bajo la dirección de (*prepared under the direction of*) PEDRO VILLAR SALVADOR // JUAN A. OLIET PALÁ
DOUGLAS F. JACOBS.

Sobre el siguiente tema (*Title of the doctoral thesis*): *THE ROLE OF NITROGEN IN FROST TOLERANCE ROOT GROWTH DYNAMICS AND HYDRAULIC CONDUCTANCE OF ECOLOGICALLY DISTINCT PINE SPECIES*

Finalizada la defensa y discusión de la tesis, el tribunal acordó otorgar la CALIFICACIÓN GLOBAL[1] de (**no apto, aprobado, notable** y **sobresaliente**) (*After the defense and defense of the thesis, the court agreed to grant the GLOBAL RATING (fail, pass, good and excellent)*): SOBRESALIENTE

Alcalá de Henares, a 7 de MARZO de 2019

Fdo. (*Signed*): ENRIQUE ANDIVIA
Fdo. (*Signed*): Alberto Vilagrosa
Fdo. (*Signed*): Guillaume Charrier

FIRMA DEL ALUMNO (candidate's signature),

Fdo. (*Signed*): OVIDIU ANDREI TOCA.

Con fecha 25 de marzo de 2019 la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado, a la vista de los votos emitidos de manera anónima por el tribunal que ha juzgado la tesis, resuelve:

☒ Conceder la Mención de "Cum Laude"
☐ No conceder la Mención de "Cum Laude"

La Secretaria de la Comisión Delegada

Ov

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[1] La calificación podrá ser "**no apto**" "**aprobado**" "**notable**" y "**sobresaliente**". El tribunal podrá otorgar la mención de "cum laude" si la calificación global es de sobresaliente y se emite en tal sentido el voto secreto positivo por unanimidad. (*The grade may be "**fail**" "**pass**" "**good**" or "**excellent**". The panel may confer the distinction of "cum laude" if the overall grade is "Excellent" and has been awarded unanimously as such after secret voting.*).

INCIDENCIAS / OBSERVACIONES:
(Incidents / Comments)

Universidad
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DE POSGRADO Y DOCTORADO**

UNIVERSIDAD DE ALCALÁ, PATRIMONIO DE LA HUMANIDAD

En aplicación del art. 14.7 del RD. 99/2011 y el art. 14 del Reglamento de Elaboración, Autorización y Defensa de la Tesis Doctoral, la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado y Doctorado, en sesión pública de fecha 25 de marzo, procedió al escrutinio de los votos emitidos por los miembros del tribunal de la tesis defendida por *TOCA, OVIDIU ANDREI*, el día 07 de marzo de 2019, titulada *THE ROLE OF NITROGEN IN FROST TOLERANCE ROOT GROWTH DYNAMICS AND HYDRAULIC CONDUCTANCE OF ECOLOGICALLY DISTINCT PINE SPECIES*, para determinar, si a la misma, se le concede la mención "cum laude", arrojando como resultado el voto favorable de todos los miembros del tribunal.

Por lo tanto, la Comisión de Estudios Oficiales de Posgrado **resuelve otorgar** a dicha tesis la

MENCIÓN "CUM LAUDE"

Alcalá de Henares, 25 de marzo de 2019
EL VICERRECTOR DE INVESTIGACIÓN Y TRANSFERENCIA

F. Javier de la Mata

F. Javier de la Mata de la Mata

Copia por e-mail a:
Doctorando: TOCA, OVIDIU ANDREI
Secretario del Tribunal: ENRIQUE ANDIVIA MUÑOZ
Directores de Tesis: PEDRO VILLAR SALVADOR //JUAN A. OLIET PALÁ //DOUGLAS F. JACOBS

Universidad
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ESCUELA DE DOCTORADO.
Servicio de Estudios Oficiales de Posgrado

DILIGENCIA DE DEPÓSITO DE TESIS.

Comprobado que el expediente académico de D./Dª OVIDIU ANDREI TOCA
reúne los requisitos exigidos para la presentación de la Tesis, de acuerdo a la normativa vigente, y habiendo presentado la misma en formato: ☒ soporte electrónico ☐ impreso en papel, para el depósito de la misma, en el Servicio de Estudios Oficiales de Posgrado, con el nº de páginas: 173 se procede, con fecha de hoy a registrar el depósito de la tesis.

Alcalá de Henares a 11 de DICIEMBRE de 2018

Aurora Juárez Abril

Fdo. El Funcionario

RESTAURAR

IMPRIMIR

Universidad de Alcalá

Departamento de Ciencias de la Vida
Unidad Docente Ecología

The role of nitrogen in frost tolerance, root growth dynamics and hydraulic conductance of ecologically distinct pine species

Memoria presentada para optar al grado de
Doctor por la Universidad de Alcalá

Programa de Doctorado:
"Ecología, Conservación y Restauración de Ecosistemas" (D413)

Ovidiu Andrei Toca

Directores:
Juan A. Oliet Palá
Pedro Villar Salvador
Douglass F. Jacobs

Alcalá de Henares, 30 noviembre de 2018

Dedicada a Miriam Villamuelas Revenga.
Gracias por compartir tu pasión por la
naturaleza conmigo

DEPARTAMENTO DE
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hace constar:

que el trabajo descrito en la presente memoria, titulado "The role of nitrogen in frost tolerance, root growth dynamics and hydraulic conductance of ecologically distinct pine species", ha sido realizado bajo su dirección por Ovidiu Andrei Toca en la Unidad Docente de Ecología del Departamento de Ciencias de la Vida de la Universidad de Alcalá, dentro del Programa de Doctorado "Ecología, Conservación y Restauración de Ecosistemas" (D330), reuniendo todos los requisitos necesarios para su aprobación como Tesis Doctoral.

Madrid, 10 de Octubre de 2018.

Dr. Juan A. Oliet Palá

Universidad
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Pedro Villar Salvador, Profesor Titular de Universidad del Departamento de Ciencias de la Vida de la Universidad de Alcalá y director de esta Tesis Doctoral,

hace constar:

que el trabajo descrito en la presente memoria, titulado "The role of nitrogen in frost tolerance, root growth dynamics and hydraulic conductance of ecologically distinct pine species", ha sido realizado bajo su dirección por Ovidiu Andrei Toca en la Unidad Docente de Ecología del Departamento de Ciencias de la Vida de la Universidad de Alcalá, dentro del Programa de Doctorado "Ecología, Conservación y Restauración de Ecosistemas" (D330), reuniendo todos los requisitos necesarios para su aprobación como Tesis Doctoral.

Alcalá de Henares, 10 de Octubre de 2018.

Dr. Pedro Villar Salvador

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Douglass F. Jacobs, Profesor Titular de Universidad del Department of Forestry and Natural Resources, de la Universidad de Purdue y co-director de esta Tesis Doctoral,

hace constar:

que el trabajo descrito en la presente memoria, titulado "The role of nitrogen in frost tolerance, root growth dynamics and hydraulic conductance of ecologically distinct pine species", ha sido realizado bajo su dirección por Ovidiu Andrei Toca en la Unidad Docente de Ecología del Departamento de Ciencias de la Vida de la Universidad de Alcalá, dentro del Programa de Doctorado "Ecología, Conservación y Restauración de Ecosistemas" (D330), reuniendo todos los requisitos necesarios para su aprobación como Tesis Doctoral.

West Lafayette (Indiana, US), 10 de Octubre de 2018.

Dr. Douglass F. Jacobs

Universidad
de Alcalá

MIGUEL ÁNGEL DE ZAVALA GIRONÉS, Coordinador de la Comisión Académica del Programa de Doctorado en ECOLOGÍA, CONSERVACIÓN Y RESTAURACIÓN DE ECOSISTEMAS,

INFORMA que la Tesis Doctoral titulada "**The role of nitrogen in frost tolerance, root growth dynamics and hydraulic conductance of ecologically distinct pine species",** presentada por D/Dª Ovidiu Andrei Toca bajo la dirección del/de la Dr/a. **Pedro Villar Salvador, Juan A. Oliet Palá y Douglass F. Jacobs,** reúne los requisitos científicos de originalidad y rigor metodológicos para ser defendida ante un tribunal. Esta Comisión ha tenido también en cuenta la evaluación positiva anual del doctorado, habiendo obtenido las correspondientes competencias establecidas en el Programa.

Y para que así conste y surta los efectos oportunos, se firma el presente informe en Alcalá de Henares a cinco de Diciembre de dos mil dieciocho.

UNIVERSIDAD DE ALCALÁ - DEPARTAMENTO DE CIENCIAS DE LA VIDA

Fdo.: Miguel Ángel de Zavala Gironés

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Abstract

Background and Aims: Environmental stress, such as frost and drought, determines the evolution and distribution of plants. In Mediterranean biome in which a cold wet season alternates with a dry and warm season, perennial plants undergo a complex process of cold acclimation to overcome the cold season and rapidly expand root systems during the wet season to survive the dry season. Environmental factors, such as temperature and water availability, affect cold acclimation, root development, and hydraulics. However, how nitrogen (N) affects these physiological processes and interacts with species ecology is controversial and poorly understood. The objective of this PhD Thesis is to analyze the effect of N on the frost tolerance, root growth dynamics and architecture, and root hydraulic properties in seedlings of four ecologically different pine species. The general hypothesis of the Thesis is that the adaptation of plant species to abiotic stress drives how N is used for growth and stress resistance.

Methods: We studied closely related, yet ecologically distinct pine species that inhabit southern Europe: *Pinus nigra* J.F. Arnold, *Pinus pinaster* Ait., *Pinus pinea* L. and *Pinus halepensis* Mill. We used seedlings because regeneration is the most vulnerable phase to stress factors and consequently differences in species ecology are noticeable at this ontogenetic stage. We performed three experiments where plants were cultivated under contrasting N fertilization regimes (Low and High pre-hardening: 20 and 150 mg of N, respectively supplied only during the growing season; Fall: 40 mg N during the growing season + 60 mg N supplied in the fall) to promote differences in tissue N concentrations and content. In the first experiment (Chapter 2), root and shoot frost tolerance was measured in mid-fall and in winter by evaluating the percentage of withered needles (visual damage index) after a freezing test. In addition, needle and root N and soluble sugar concentration, and timing of cessation of shoot elongation were measured to assess the physiological mechanisms underlying frost acclimation. In the second experiment (Chapter 3), seedlings were transplanted the following spring into rhizotrons to evaluate root growth dynamics and architecture over 10 weeks. Simultaneously, in the third experiment (Chapter 4) root hydraulic conductance was measured with a high-pressure flowmeter and values were standardized by leaf area (leaf specific conductance, K_l), xylem cross-section area (xylem specific conductance, K_s), total root length (root specific conductance, K_r) and the length of fine roots (fine root specific conductance, K_{fr}).

Key Results: Shoot frost tolerance increased over winter while root frost tolerance did not change through time in any species. Pre-hardening N fertilization affected the frost tolerance of both roots and shoots, although the effect was species-specific: high N reduced the overall root and shoot frost tolerance in *P. pinea* and *P. halepensis* (the thermophilic species inhabiting mild winter areas and with weakest dormancy control), increased the frost tolerance in *P. nigra* (the psychrophilic species living in the coldest areas and with tightest dormancy control), and had no effect in *P. pinaster* (the mesophilic species that lives in intermediate cold sites). N supply in the fall consistently increased frost tolerance of shoots and roots in all species, indicating that plants used N surplus during cold acclimation to enhance frost tolerance. Differences in frost tolerance among species and N treatments were not explained by variations in organ N or soluble carbohydrate concentration, nor by timing of cessation of shoot elongation; although the most frost tolerant species ceased elongation earlier than the least frost tolerant species.

Increased seedling N content enhanced root development, but species showed distinct root growth patterns and architecture in response to differences in N content. *Pinus pinaster*, which inhabits moderate climatic stress environments, showed the greatest root growth plasticity with variation in seedling N content. In contrast, root development was less plastic in the pines adapted to more stressful environments (*P. pinea*, which occurs in sites with strong summer drought and *P. nigra*, which inhabits cold winter areas). Nitrogen use efficiency for root growth increased strongly with increasing N reserves in *P. pinaster*, yet the opposite effect occurred in the other two species. This suggests that *P. pinaster* proportionally relies more on N reserves for root growth than the other pine species. New roots of high N content seedlings had lower specific root length than low N content seedlings in all species, suggesting that internal seedling N status plays a significant role in root architecture.

Increasing N availability reduced K_l and K_s, which conflicts with a higher growth and photosynthesis rate of N fertilized plants. In contrast, high N availability increased K_r and especially K_{fr}, which is consistent with published results at interspecific level, where increase in plant hydraulic efficiency is linked to higher growth and photosynthesis. These contradictory results depending on the standardization method emphasizes the importance and difficulty of developing specific hydraulic comparison criteria that match the hydraulic functionality with water demand and other key ecophysiological processes of plants. In addition, according to the per species results, compared pines showed differences in their hydraulic efficiency, but these differences did not correspond with their ecology.

Conclusions: Despite their close phylogenetic relatedness, species ecology determined the effect of N availability on frost tolerance and root development. Specific differences might reflect a trade-off in the use of N: allocation of N to growth *versus* allocation to stress resistance. The contradictory results of the effect of N supply on root hydraulic efficiency shows the shortfalls of standardization methods to compare plant hydraulics.

Resumen

Antecedentes y objetivos: Los estreses ambientales como las bajas temperaturas y la sequía determinan la evolución y distribución de las plantas. En el bioma mediterráneo, en el que una estación fría y húmeda se alterna con una estación cálida y seca, las plantas perennes experimentan un complejo proceso de aclimatación a la helada para sobrevivir a la estación fría y la rápida expansión del sistema radical durante la estación húmeda para sobrevivir a la estación seca. La temperatura y la disponibilidad de agua pueden afectar la aclimatación al frío y el desarrollo e hidráulica del sistema radical. Sin embargo, el efecto del nitrógeno (N) sobre estos procesos fisiológicos y su relación con la ecología de las especies son controvertidos y poco conocidos.

El objetivo de esta Tesis doctoral es analizar el efecto del N sobre la tolerancia a la helada, la arquitectura y cinética radical y las propiedades de la hidráulica del sistema radical en plántulas de cuatro especies de pino ecológicamente distintas. La hipótesis general de la Tesis es que la adaptación de las plantas a los estreses abióticos condiciona el uso del N para el crecimiento y la resistencia a factores de estrés.

Métodos: Se han estudiado especies de pino filogenéticamente muy cercanas, pero de ecología diferente del sur de Europa: *Pinus nigra* J.F. Arnold, *Pinus pinaster* Ait., *Pinus pinea* L. and *Pinus halepensis* Mill. Para abordar estos los objetivos de la Tesis se han usado plántulas debido a que es la fase de la vida de las plantas más vulnerable al estrés ambiental. Se realizaron tres experimentos en los que las plantas fueron cultivadas bajo tres tratamientos de fertilización (Alta y Baja en primavera-verano: 20 and 150 mg de N, respectivamente, y Otoñal: 40 mg en primavera-verano + 60 mg de N en otoño) diseñados para promover diferencias en el contenido de N en las plantas. En el primer experimento (Capítulo 2) se analizó la tolerancia a la helada de la parte aérea y radical en otoño e invierno mediante la evaluación del porcentaje de acículas muertas (índice de daño visual) después de un test de helada. Para analizar los mecanismos fisiológicos subyacentes a la tolerancia a la helada se ha medido la concentración de N y azucares solubles en hojas y raíces al igual que el momento del cese del crecimiento. En el segundo experimento (Capítulo 3), se analizó la arquitectura y cinética radical de las plántulas mediante rizotrones a lo largo de 10 semanas. En el tercer experimento (Capítulo 4) se midió la conductancia hidráulica del sistema radical con un medidor de flujo de agua a alta presión y los valores fueron estandarizados por el área foliar (conductancia específica foliar, K_l), área transversal del xilema (conductancia específica

del xilema K_s) y la longitud total del sistema radical (conductancia específica radical, K_r) y de las raíces finas (conductancia específica de las raíces finas, K_{fr}).

Resultados clave: La tolerancia a la helada de la parte aérea y aumentó a lo largo del invierno mientras que el de la parte radical no sufrió cambio en el tiempo en ninguna especie. La fertilización alta redujo la tolerancia a la helada de la parte aérea y radical de *P. pinea* y *P. halepensis* (las especies termófilas de regiones con inviernos suaves y un control de la dormancia débil), la incrementó en *P. nigra* (la especie psicrófila de regiones muy frías y con un fuerte control de la dormancia) y no tuvo ningún efecto en *P. pinaster* (la especie mesófila de regiones con un frío moderado). La fertilización otoñal aumento la tolerancia a la helada de todas las especies lo que indica que un aporte extra de N durante la aclimatación a la helada es usado para incrementar la tolerancia a la helada. Las diferencias en la tolerancia a la helada entre especies y tratamientos no se pudieron explicar por la variación en la concentración de N y azucares solubles en los órganos o por el momento del cese del crecimiento, aunque las especies más tolerantes a las heladas cesaron el crecimiento antes que las menos tolerantes.

El aumento en el contenido de N en la planta incrementó el desarrollo del sistema radical en todas las especies. Sin embargo, la respuesta de la arquitectura y cinética radical a variaciones en el contenido de N mostró respuestas muy diferentes entre especies. *Pinus pinaster*, la especie de ambientes con estrés moderado mostró la mayor plasticidad radical en respuesta a variaciones en el contenido de N. En contraste, las especies de hábitats con un alto estrés ambiental (*P. pinea*, que habita regiones con pronunciada sequia estival y *P. nigra*, de regiones con inviernos muy fríos), mostraron una baja plasticidad en respuesta a variaciones en N. La eficiencia en el uso del N para el desarrollo radical aumentó en respuesta al aumento en contenido de N en la planta en *P. pinaster* mientras que las otras especies la eficiencia disminuyó. Las raíces nuevas de plántulas con alto contenido en N tuvieron una longitud radical especifica menor que las plántulas con bajo contenido en N, sugiriendo que el contenido en N de la planta y no la disponibilidad de N en el suelo determina esta característica del sistema radical.

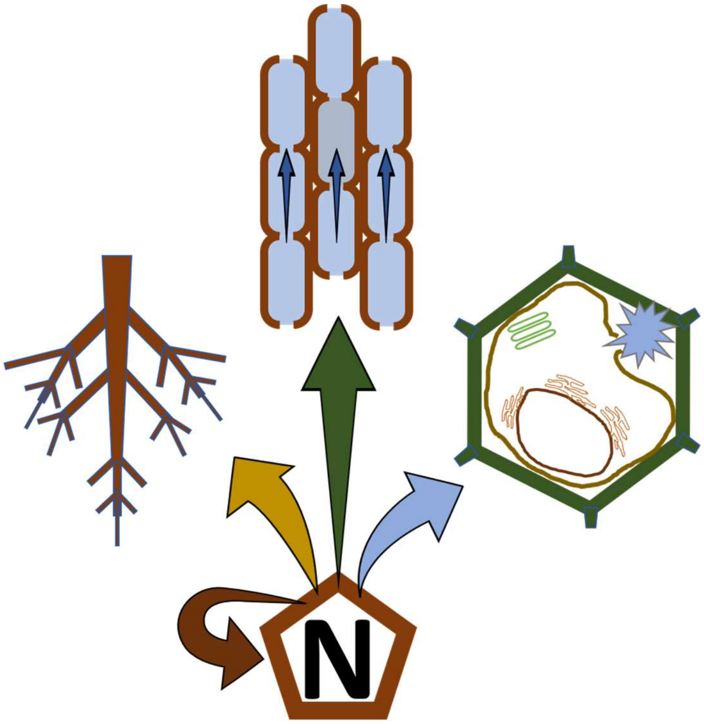
El aumento en la disponibilidad de N redujo K_l y K_s, lo que está en contradicción con el alto crecimiento y tasa fotosintética de las plantas altamente fertilizadas. Por el contrario, K_r y, especialmente K_{fr}, aumentaron en respuesta a una mayor disponibilidad de N, lo que coincide con los resultados publicados a nivel interespecífico que muestran que un incremento en la eficiencia

la hidráulica de las plantas está coordinado con una mayor tasa fotosintética y crecimiento. Estos resultados contradictorios entre los métodos de estandarización de la conductancia radical indican la importancia y dificultad del desarrollo de criterios para comparar la funcionalidad del sistema hidráulico y que reflejen adecuadamente la coordinación de la hidráulica de las plantas con su demanda de agua y otros procesos ecofisiológicos de la planta. Las especies mostraron diferencias en la eficiencia hidráulica de las raíces, pero estas diferencias no se relacionaron con sus diferencias ecológicas.

Conclusiones: A pesar de su proximidad filogenética, la ecología de las especies de pino condicionó el efecto del N sobre la tolerancia a la helada y el desarrollo del sistema radical. Estas diferencias interespecíficas parecen reflejar compromisos en la prioridad de uso del N: uso para apoyar el crecimiento radical o para mecanismos de tolerancia al estrés. Los resultados contradictorios sobre la eficiencia hidráulica del sistema radical indican que los métodos de estandarización tienen limitaciones para comparar el funcionamiento del sistema hidráulico de las plantas.

Chapter 1

Thesis introduction



Thesis introduction

Nature of abiotic stress and resistance mechanisms of seedlings

Temperature and water are main abiotic environmental drivers of plant evolution (Grace 1987, Hawkins et al. 2014). These environmental factors vary in space and time, shaping the distribution of plant species and terrestrial ecosystems (Woodward and Williams 1987, Pockman and Sperry 1997, Schulze et al. 2005). Plants undergo intense acclimation processes (Bigras et al. 2001, Schulze et al. 2005, Brunner et al. 2015) to cope with temporal changes in temperature and water availability. Although the acclimation of plants to stress has traditionally been studied by analyzing environmental factors independently, plants frequently are exposed to multiple stress factors (Mooney et al. 1991) and consequently have to achieve resistance to multiple stresses throughout the year.

Across the life cycle of trees, the seedling phase is the most vulnerable period to environmental stress in most biomes (Bazzaz 1979, Gilbert et al. 2001, Climent et al. 2009). Consequently, the distribution of plant species and population dynamics depends on the performance of seedlings (Lenoir et al. 2009). One of the main reasons that seedlings are highly vulnerable to stress is their small size (Leishman et al. 2000). On one hand, small size hinders the capacity of resource acquisition. For instance, light competition at the seedling phase is critical in tree species due to the small size of shoot (Leishman et al. 2000). Similarly, low rooting depth and volume of soil explored by seedling roots, limits the capacity of seedlings to access stable soil water reserves, which is critical for survival during the dry season and in arid climates (Canadell et al. 1996, Padilla and Pugnaire 2007). On the other hand, roots and foliage of seedlings are more vulnerable to frost damage than the roots and foliage of their adult relatives (Climent et al. 2009) and are closer to the ground where the temperature is lowest in winter and highest in summer (Kolb and Robberecht 1996, Alvarez-Uria and Körner 2007, Charrier et al. 2015).

Mechanisms of stress resistance differ widely among plant species and can be classified into stress avoidance and stress tolerance mechanisms (Schulze et al. 2005). Fast growth is a main component of the stress avoidance strategy. Fast growing plants can avoid seasonal drought by rapidly elongating their roots into deep soil where water reserves are stable (Padilla and Pugnaire 2007) and light competition by escaping the dense shade of forest understory (Leishman et al.

2000). These plants also avoid the extreme temperatures closer to the surface that can damage their roots and leaves (Alvarez-Uria and Körner 2007, Charrier et al. 2015). Tolerance mechanisms allow plants to perform while being exposed to stresses by withstanding certain level of stress in their tissues. For instance, plants accumulate antifreeze proteins that control the osmotic adjustment and inhibit the growth of intracellular ice crystals (Atıcı and Nalbantoğlu 2003, Griffith and Yaish 2004).

Plants demand a high amount of resources to carry out their main functions (growth, reproduction, maintenance and defense *sensu lato*). However, plants live in environments where resources are usually limited, which constrains the amount of resources that can be allocated to the main functions. Since seedlings are highly vulnerable to abiotic and biotic stresses, functional attributes conferring rapid and efficient use of nutrient resources for growth might be important to "escape" the limitations at initial development stages (Cook 1979, Herms and Mattson 1992). However, in many ecosystems a high nutrient use efficiency for growth may conflict with physiological stress tolerance mechanisms that also require significant amounts of resources (Herms and Mattson 1992) establishing a trade-off between these main functions. Hence, differences in plant adaptations to ecological factors could drive the fate of nutrients use: towards growth or stress resistance mechanisms (Figure 1).

The role of nitrogen in plant functioning

The Mediterranean region is characterized by strong limiting environmental conditions for plant growth. Drought and extreme temperatures are the main drivers of plant evolution and the primary productivity in this region (Mitrakos 1980, Lopez et al. 1998, Larcher 2000, Padilla and Pugnaire 2007). However, Mediterranean ecosystems are also limited by low soil fertility, especially of nitrogen (N) (LeBauer and Treseder 2008, Delgado-Baquerizo et al. 2011). Low fertility is due to a combination of harsh climate conditions, which hinders plant productivity and microbe activity, and the chemical composition of Mediterranean woody plants (low nutrient and high lignin and cutin concentration), which slows litter decomposition (Gallardo and Merino 1993).

Many key molecules for plant functioning such as metabolic and structural proteins and nucleic acids have high amount of N. Much of plant N demand is met through root uptake, although some N can also be absorbed via foliage (Uscola et al. 2014). Plant demand for N varies throughout the year, especially in seasonal ecosystems, where growth, reproduction and hardening occur at specific moments of the year. Part of plant N demand can also be met by stored N, which is remobilized depending on plant N demand (Millard 1996, Millard and Grelet 2010). Nitrogen can be stored in different organs that vary among plant species and as different chemical structures, the main forms being amino acids, polypeptides and storage proteins such as the inactive form of the Rubisco (Lambers et al. 2008, Villar-Salvador et al. 2015).

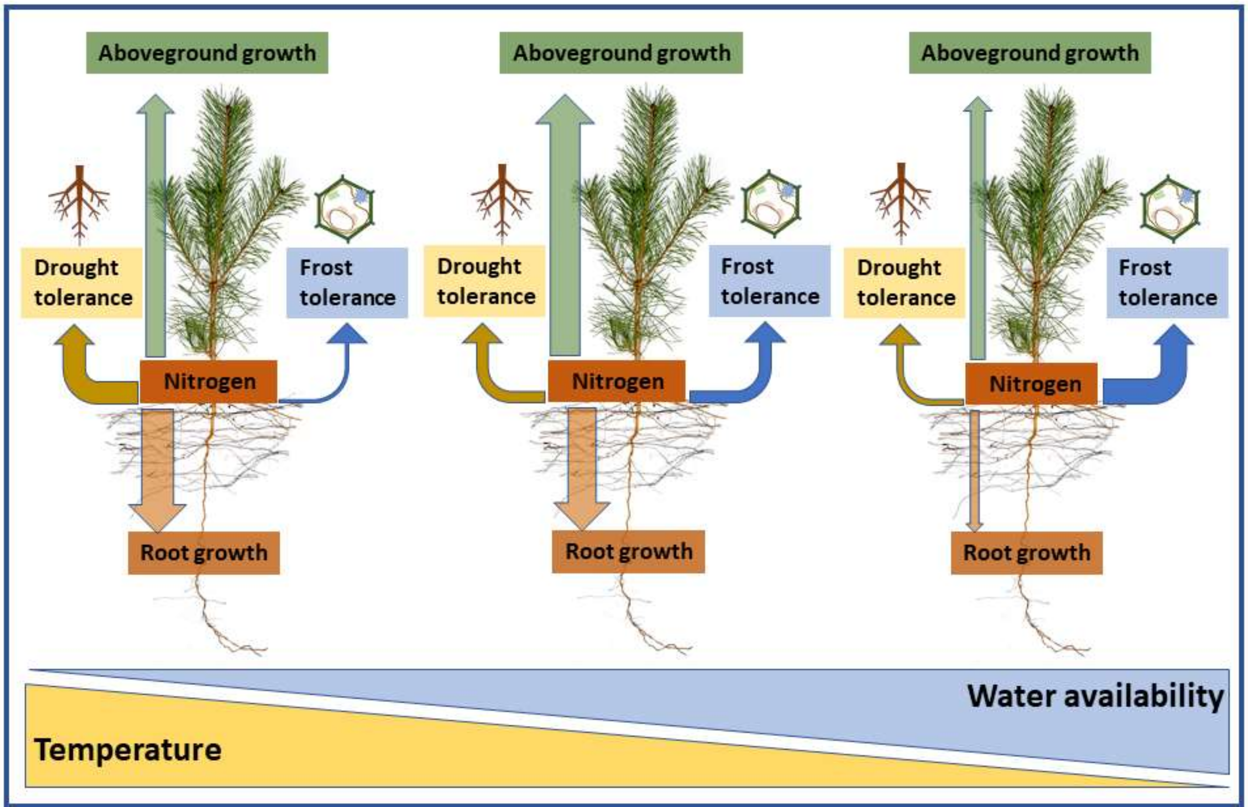


Figure 1: Theorical framework and hypothesis for the relationship between N and frost tolerance and root growth dynamics across a temperature and water availability gradient. Arrows represent the direction of the N investment and the width represents the magnitude of the process.

Symbols: Frost tolerance (chapter one) Root growth dynamics (chapter two)

Growth has a strong positive relationship with N availability over a wide concentration range. This is a consistent pattern that has been observed worldwide in comparative studies among ecosystems, as well as in fertilization experiments (Marschner 1995, Lambers et al. 2008). The positive effect of N on plant growth is partially based on the relationship between N and some of the main physiological processes affecting plant life (Figure 2). For instance, high photosynthetic capacity is frequently positively related to high foliage N concentration (Evans 1989, Reich et al. 1998, Oliet et al. 2013, Zhang et al. 2018). Therefore, photosynthesis links plant N and carbon metabolism. In addition, stored N can be remobilized to support physiological processes acording to their demand and the magnitude of the reserves (see citations above). The efficiency with which plants use N for growth varies widely among species (Chapin 1980). In nutrient-poor ecosystems, high nitrogen use efficiency (NUE) can help plants to maintain their fitness (Aerts 1990). NUE can be quantified in different ways but a standard procedure is to measure the growth per unit of N availability (Berendse and Aerts 1987). Not only growth but also the morphology and architecture of plants can change significantly with N availability, indicating that the pattern of carbon allocation to different organs changes across a gradient of N availability. Generally, an increase in soil N promotes shoot growth at the expense of root growth (Birk and Vitousek 1986, Villar-Salvador et al. 2012).

Water absorption and transport capacity is also tightly linked to the physiological processes underlying plant growth. For example, plant hydraulics has evolved coordinately with the foliage physiology by placing a limit to the photosynthesis and transpiration rates of plants (Brodribb and Feild 2000, Brodribb 2009). The hydraulic system, which is in charge of water transport, is formed by the root surface, and the xylem, as well as leaf intercellular spaces and stomata, which connects the roots with the water evaporation sites (Cruiziat et al. 2002). The structure of all these components of the hydraulic system are highly responsive to changes in N (Marschner 1995, Lambers et al. 2008).

Plant stress resistance has been linked to N nutrition. For instance, plants allocate high amounts of N to physiological mechanisms to resist detrimental effects of environmental stress such as cold and drought (Saneoka et al. 2004, Villar-Salvador et al. 2012, Taulavuori et al. 2014). As N is also a critical nutrient for plant growth, there must be a trade-off between the allocation of N to these two physiological processes (Herms and Mattson 1992). Thus, we hypothesize that species ecology may be among the most important factors determining the direction and magnitude of this N trade-off (Figure 1). For instance, species from highly stressing environments such as dry and cold ecosystems will allocate a significant proportion of N to the development of stress resistance mechanisms, while species from moderately stressing environments will allocate most N to growth (Figure 1). This PhD Thesis focuses on the role of plant ecology as a determinant of the relationship between N availability and three of the main plant ecophysiological processes: frost tolerance, root growth dynamics and architecture, and water transport.

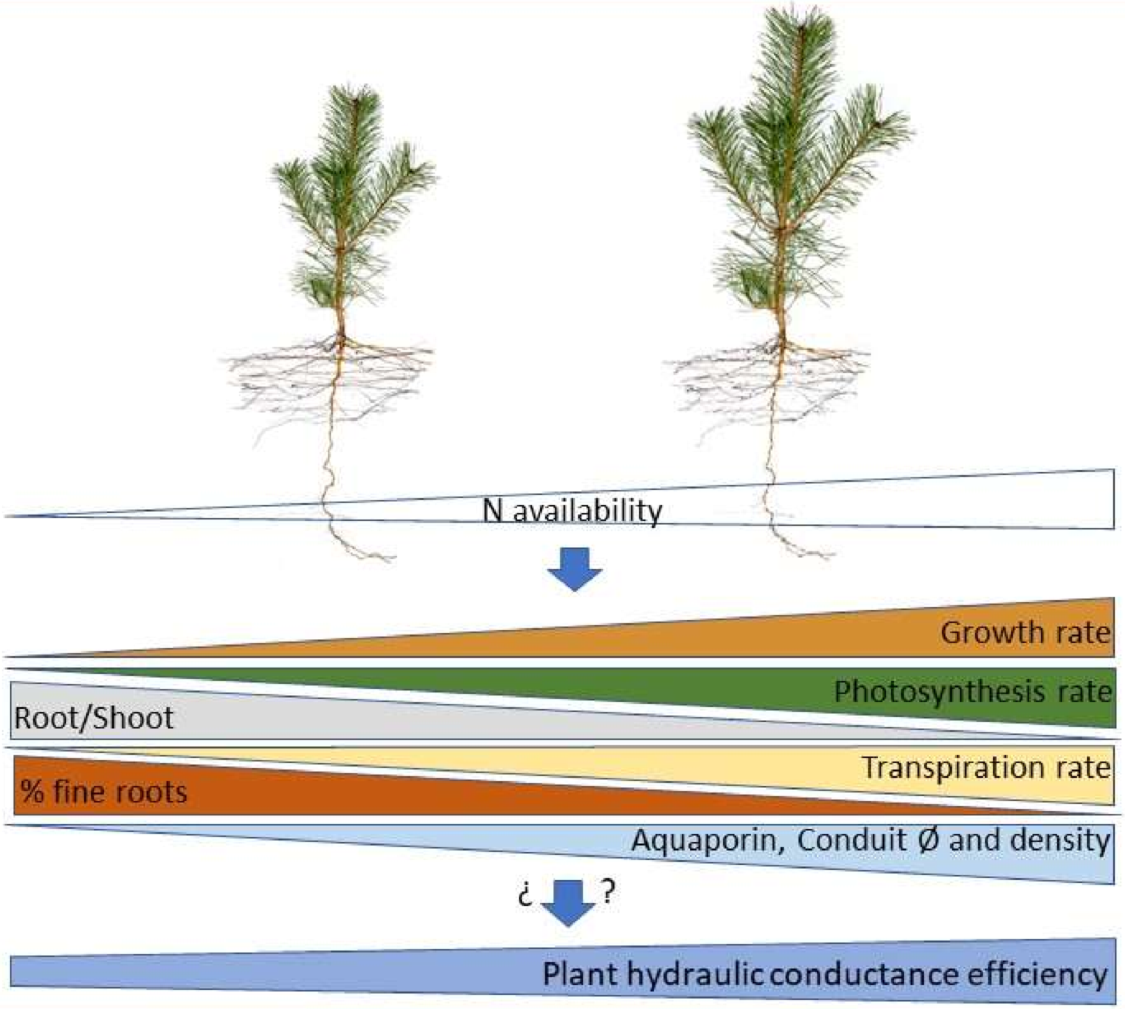


Figure 2: Theorical framework and hypotheses for for the relationship between N and growth, photosynthesis and transpiration rate, root/shoot ratio, percentage of fine roots, xylem morphology and hydraulic conductance in pine seedlings along a N availability gradient.

Influence of nitrogen on the frost tolerance of plants

Perennial plants from cold and temperate biomes experience a complex process of cold acclimation during fall in which they undertake deep reversible physiological changes to overcome frost stress during the cold season (Sakai and Larcher 1987, Bigras et al. 2001, Charrier et al. 2015). Frost hardening starts with the cessation of shoot elongation (Greer et al. 2000, Repo et al. 2000), triggering the accumulation of soluble carbohydrates (Ogren et al. 1997, Tinus et al. 2000, Martínez-Vilalta et al. 2016), amino acids (Lähdesmäki and Pietiläinen 1988), and antifreeze proteins (Atıcı and Nalbantoğlu 2003, Griffith and Yaish 2004). Additionally, perennial plants modify lipid composition of the plasma membrane (Kreyling et al. 2012) to prevent the formation of ice crystals and stabilize the cell membranes (Sakai and Larcher 1987, Lambers et al. 2008) when exposed to freezing temperatures. At a plant scale, organs also differ in frost tolerance, with aboveground vegetative parts having greater frost tolerance than roots (Bigras et al. 2001, Charrier et al. 2015), although there are very few studies that evaluated root frost tolerance. While both temperature and photoperiod control shoot frost tolerance, temperature seems to be the main driver of root frost tolerance (Fernández et al. 2008, Ryyppö et al. 2008).

Nitrogen affects the frost tolerance of plants. However, there is no clear consensus on the relationship between frost tolerance and N availability (see the review by Taulavuori et al., 2014). Several studies have reported that high N availability reduces frost tolerance (Hellergren 1981, Schaberg et al. 2002, Villar-Salvador et al. 2013, Heredia-Guerrero et al. 2014). Others, however, have shown that plant frost tolerance increases with N availability in trees from temperate climates (DeHayes et al. 1989, Andivia et al. 2012, Taulavuori et al. 2014). The negative effects of high N fertilization on frost tolerance are attributed to a reduction of osmolytic soluble carbohydrates that are invested in structures to fed growth (Andivia et al. 2012) and delayed cessation of shoot elongation in fall (Hawkins et al. 1995, Heredia-Guerrero et al. 2014), which affect timing of cold acclimation. The positive effects of N fertilization on frost tolerance are associated with high concentrations of osmolytes such as amino acids and cryoprotection proteins (Lähdesmäki and Pietiläinen 1988, Griffith and Yaish 2004, Berrocal-Lobo et al. 2011). Because frost tolerance of roots and shoots responds differently to environmental stimuli such as temperature and photoperiod (Fernández et al. 2008, Ryyppö et al. 2008), N may also have variable effects on the frost tolerance of plant organs. Additionally, contradictory results of N supply on frost tolerance could be due to differences in N availability and application timing (Oliet et al. 2013), the environmental conditions during the experiment such as temperature and photoperiod (Fernández et al. 2008; Heredia-Guerrero et al. 2014), or more importantly, functional differences among species. Thus, one objective of this PhD Thesis will be identifying the mechanisms linking N status and frost resistance in ecologically different *Pinus* species. In addition, results of this study can shed light on the effects of combined global change drivers such as N deposition (Waldner et al. 2014) and the reduction of snow cover and earlier snow melting on the risk of root frost damage due to lower soil and root insulation (Groffman et al. 2001, Schaberg et al. 2008).

Root growth dynamics and plant nitrogen status relationships

Survival of tree seedlings, especially under arid conditions, depends on the ability to rapidly expand the root system and develop hydrological connectivity with the surrounding soil (Canadell et al. 1996, Grossnickle 2005, Padilla and Pugnaire 2007). Soil fertility, especially of N, greatly affects root development. Increments in soil N can increase, reduce, or have no effect on the belowground biomass and number of growing roots (Mackiedawson et al. 1995, Trubat et al. 2006, Wang et al. 2017). Soil N usually affects root architecture by reducing specific root length (Ostonen et al. 2007). However, internal N can also affect root growth by remobilization of stored N (Millard and Grelet 2010, Villar-Salvador et al. 2015). The magnitude of N remobilization depends mostly on the amount of stored N (Millard and Grelet 2010), therefore high N content plants have a generally greater root development (Grelet et al. 2003, Cuesta et al. 2010b). There is vast literature on the effect of soil N on root growth and architecture (Forde and Lorenzo 2001, López-Bucio et al. 2003, Wang et al. 2013). However, the effects of internal N on root growth dynamics and architecture are poorly known. As far as we know, only a few studies have investigated this relationship in plants; reporting, for example, that plant NO_3^- concentration can modulate root branching in *Arabidopsis thaliana L. (Heyn)* (Zhang et al. 1999). Similarly, Cuesta et al. (2010b) found that high N content *Pinus halepensis* Mill. seedlings developed a greater number of growing fine roots than low N content seedlings.

Because N is involved in plant stress resistance (DeHayes et al. 1989, Saneoka et al. 2004, Villar-Salvador et al. 2013, Taulavuori et al. 2014) and growth (Marschner 1995, Lambers et al. 2008), there may be a trade-off in resource allocation to meet these two main functions (Herms and Mattson 1992). Therefore, the influence of internal N on root growth and architecture might be constrained by the adaptive strategy of a given plant species. As far as we know, no study has compared how differences in N content affect root growth dynamics and architecture in forest tree species with contrasting ecology.

Hydraulic conductance responses to nitrogen availability

Hydraulic architecture affects the capacity of trees to conduct water through the soil-plant-atmosphere continuum. Hydraulic conductance is a key trait for plants to cope with hydric stress, as it affects the ability of plants to uptake water and maintain photosynthesis under low soil moisture conditions (Cruiziat et al. 2002). In this context N, has distinct effects on the main component of the plant hydraulic system, which in turn may affect positively or negatively plant hydraulic conductance. For instance, high N availability reduces the root to shoot ratio (Birk and Vitousek 1986, Villar-Salvador et al. 2012) and the percentage of fine roots (Li et al. 2015), which might potentially cause imbalance in water uptake relative to the water demand of the plant. Similarly, N availability increases the diameter of xylem conduits, conduit number per surface unit and the expression of aquaporins (Hacke et al. 2010, Borghetti et al. 2016), which increases plant water transport capacity.

In most studies, hydraulic conductance (the flow of water through an organ per unit of pressure and time) is expressed as the capacity to supply water per unit of leaf area, which is called the leaf specific conductance (K_l). This standardization allows comparative studies as it controls for size differences, which affect hydraulic conductance. In other studies, the hydraulic conductance is standardized by xylem cross-section surface (called xylem specific conductance, K_s) or the root system surface (called root specific conductance, K_r) (Nardini and Tyree, 1999, Fichot et al. 2011). The effect of N on K_l, K_s, and K_r varies among studies although there are some general trends. Most studies show that an increase in soil N generally results in the development of a less efficient root hydraulic system when standardized by leaf area (Bucci et al. 2006, Hernández et al. 2009, Luis et al. 2010, Wang et al. 2016). When standardized by root surface, the effect of N on hydraulic is generally not significant or positive (Steudle and Meshcheryakov 1996, Trubat et al. 2006, 2012, Wang et al. 2017). Hence, these standardization methods can give a very different functional picture of plant hydraulics, arriving sometimes at contradictory results (Trubat et al. 2006, Hernández et al. 2009). Variation among studies on the effect of N on the efficiency of the hydraulic system could also be attributed to functional differences among ecologically different species (Hernández et al. 2009).

The reduction of K_l with soil N observed at an intraspecific level apparently conflicts with the positive relation between plant hydraulic capacity and other functional attributes involved in the carbon and water economy at an interspecific level such as photosynthesis and transpiration rate (Figure 3) (Brodribb and Feild 2000, Brodribb 2009). N consistently increases photosynthesis, transpiration and the growth rate in plants (Evans 1989, Villar-Salvador et al. 2013, Oliet et al. 2013, Camarero and Carrer 2017). However, it reduces the efficiency of the hydraulic system at an intraspecific level (Bucci et al. 2006, Hernández et al. 2009, Luis et al. 2010, Wang et al. 2016) according to some standardization methods (Figure 2). In this Thesis, we address this apparent contradictory response between N and hydraulic conductance at an intraspecific level and discuss the different methods used to describe the relationship.

Studied species

For this PhD Thesis, we selected four pines species as model species. By selecting species from the same genus, we reduce the potential bias due to phylogeny (Ackerly and Reich 1999, Ackerly 2009). Pines comprise around 110 species that inhabit sites ranging from sea level up to 5000 m a.s.l and from hot semi-arid deserts to rainforests and sub-arctic ecosystems. Therefore, pine species show important differences in their ecology (Richardson 1998, Keeley 2012). Pines are also very important for forest restoration because they are easy to cultivate and most of them are pioneer species that establish very well after planting (Richardson 1998, Puerta-Piñero 2013). In addition, pines are among the most valued tree species for timber and wood pulp throughout the world and they are grown in extensive plantations in relatively dense stands (Palo et al. 2001). Therefore, results of this Thesis are useful for improving pine plantations. Selected four pine species are: *Pinus halepensis* Mill., *Pinus pinea* L., *Pinus pinaster* Ait. and *Pinus nigra* J.F. Arnold. These pines are widespread in southern European regions mostly with Mediterranean climates. Their distribution is segregated along an aridity and altitudinal gradient and exhibit contrasting ecology and growth patterns (Barbero et al. 1998, Climent et al. 2011) (Figure 4).

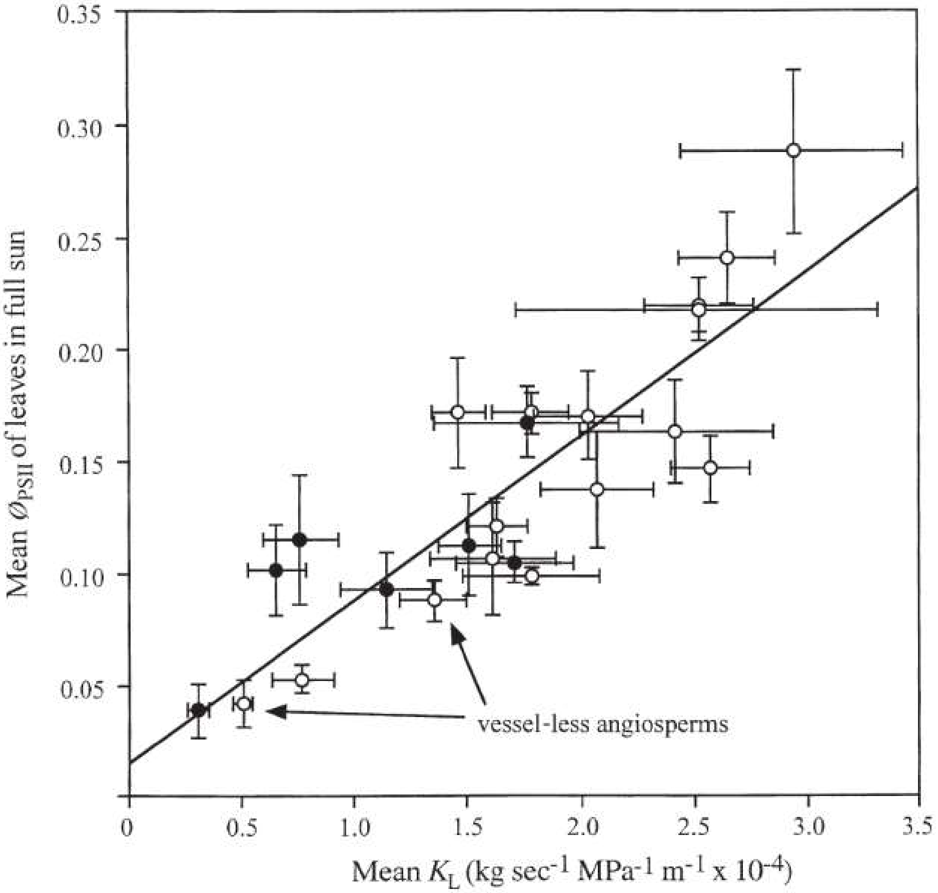


Figure 3: From (Brodribb and Feild 2000). A strong relationship ($r^2 = 0{\cdot}74$) is shown between mean leaf specific conductivity (K_L) of stems (n = 4) from seven conifers (●) and 16 angiosperms (○) and mean quantum yield of PSII electron transport (ØPSII) (a surrogate of photosynthesis rate) measured in full sun (n = 5). The x-intercept of the regression is not significantly different from zero suggesting a proportional relationship. Two vessel-less species from the family *Winteraceae* are arrowed.

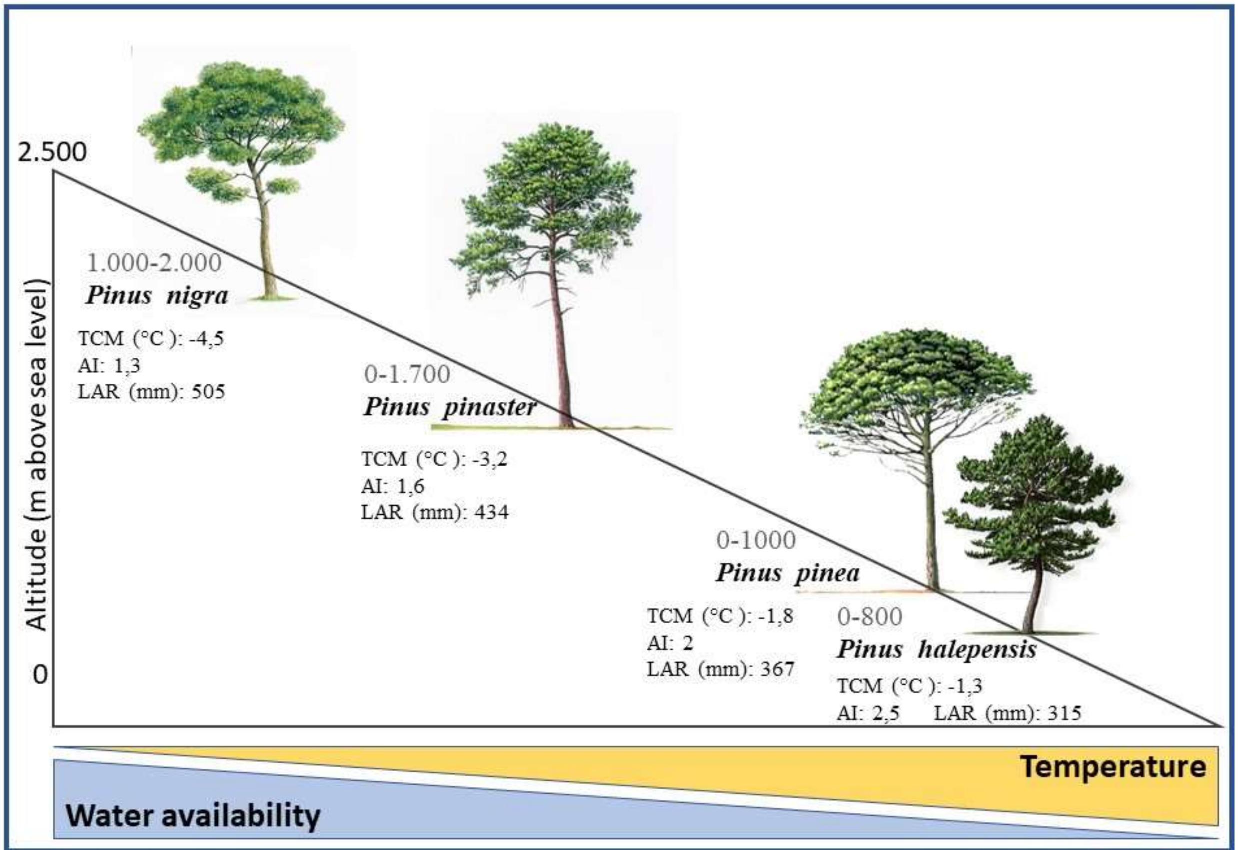
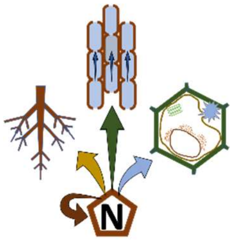


Figure 4: Distribution range of studied pine species along gradients of temperature, water availability and altitude. TCM= mean of the minimum temperatures of the coldest month, AI= highest aridity index in species range, LAR= Lowest annual rainfall in species range

Pinus halepensis Mill. and *P. pinea* L. are thermophilic species that inhabit regions with mild winter conditions and long dry summers (Navarro et al. 2013, Puértolas et al 2013). These species have weak environmental control of dormancy and do not set buds in autumn during the seedling and early sapling stage (Mutke et al. 2012). During winter, these species have high photosynthetic activity and can reactivate growth during mild spells (Puértolas 2005, Climent et al. 2011). The range of *P. halepensis* and *P. pinea* extends widely in the Mediterranean basin, and the largest populations are found in Spain (Navarro et al. 2013, Puértolas et al 2013). *Pinus pinaster* Ait. is a mesophilic species that thrives mostly in mid-elevation mountains with intermediate drought conditions and cool winters. The geographical distribution of *P. pinaster* extends mainly in the western side of the Mediterranean region, with its main populations in Iberian Peninsula. The distribution of this species frequently overlaps with the distribution of the other pine species (Lafuente et al. 2013). *Pinus nigra* is a psychrophilic pine (Barbero et al. 1998) that thrives in high mountain sites, where the main limiting abiotic factor for plant life is low temperature rather than summer drought (Peñuelas et al. 2013). *Pinus nigra* is divided in two subspecies: *Pinus nigra* J.F. Arnold. subsp. *salzmanii* and *Pinus nigra* J.F. Arnold. subsp. *nigra*. For this study, we selected the subspecies *salzmanii* (here after *P. nigra*) that is distributed in the western side of the Mediterranean region, mainly in Spain. *Pinus pinaster* and especially *P. nigra*, are usually exposed to intense and frequent frost events, set buds before dormancy in winter, mediated by a strong endodormancy, and develop secondary needles earlier in their ontogeny than *P. halepensis* and *P. pinea* (Kreyling et al. 2012, Lafuente Laguna et al. 2013, Peñuelas et al. 2013).

General objective and hypothesis of the thesis.



The general objective of the PhD Thesis was to assess if plant ecology determines the effect of N nutrition on plant frost tolerance, root growth dynamics and architecture, and hydraulic conductance. Selected pine specie have distinct ecology (see studied species above). Moreover, we studied these ecophysiological processes in seedlings because it is the most vulnerable stage in the plant's life cycle to environmental stress. Thus, the results of the thesis will be relevant from both an ecophysiological and forest restoration point of view.

We hypothesize that the adaptation of pine species to abiotic stress determines how species use N for growth and stress resistance.

Thesis structure, specific objectives and hypotheses

This PhD Thesis has six chapters. The first chapter corresponds to the general introduction where we present the general framework of the Thesis. To test the hypothesis and address the objectives of the PhD Thesis, we carried out three experiments corresponding to chapters 2-4, which are scientific articles published, submitted or in preparation for international journals. Each of the experimental chapters is structured like a regular article (*i.e.* Abstract, Introduction, Material and Methods, Results Discussion and References), and includes an abstract in Spanish.

In chapter two, we analyzed the role of plant ecology in determining the effect of N availability on the frost tolerance of roots and shoots of four ecologically distinct pine species during cold hardening in mid-fall and in winter. Specific hypothesis are:

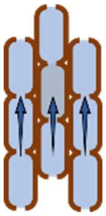
(i) The effect of N availability on frost tolerance during acclimation will be mediated by species ecology: while pine species inhabiting mild winter regions will have reduced frost tolerance under high N availability, those from cold winter regions will be positively influenced.

(ii) The timing of nutrient application (i.e., pre-hardening vs fall hardening) will affect frost tolerance of seedlings, and the magnitude of this effect will depend on species.

(iii) Root frost tolerance will be less affected by N availability and timing of supply than shoots, because roots experience less cold acclimation in response to environmental shifts.

The specific objective of chapter three was to compare the effects of seedling N status on early root growth dynamics and architecture of three ecologically distinct pine species, *P. pinea*, *P. pinaster* and *P. nigra*. We excluded *P. halepensis* from this experiment because there is a previous study on this species with a similar experimental design.

(i) We hypothesized that the effect of N reserves on root growth dynamics and architecture will depend on species ecology. Species from moderate stress environments (*P. pinaster*) will show large differences in root growth dynamics and architecture in response to variations in N reserves. Conversely, species from high stress environments (i.e., drought for *P. pinea* and cold for *P. nigra*) will have lower response to variations in N reserves, as root growth is more constrained according to their ecological acclimation demands and the development of stress resistance mechanisms. Moreover, we hypothesized that root growth would be more rapid for these species adapted to drier habitats.



In chapter four, the specific objective was to compare the variation in root hydraulic properties of four ecologically different pine species in response to N availability. We analyze this relationship through several methods of standardization and discuss their biological significance.

(i) We hypothesize that the efficiency of the hydraulic system will increase in response to N availability to balance the water transport capacity to the increase in transpiration and assimilation.

Finally, chapter five is a general discussion of the main results of chapters 2-4, followed by the conclusions of the Thesis in chapter six and a photographical section in chapter seven. In order to facilitate results description, the supplementary material was introduced after the Results section of the corresponding chapter. This structure leads to some repetition resulting from the publication of chapter 2-4 as independent studies.

In order to create a gradient in seedling N concentration and content, we designed three fertilization treatments where seedlings were subjected to low (20 mg N plant^{-1}) and high (150 mg N plant^{-1}) N fertilization during the active growth period to induce low and high N concentration and content (Villar-Salvador et al. 2013). The third fertilization treatment had 100 mg N per plant of which 60 mg were applied during the seedling active growth period and 40 mg during the fall. The fall fertilization was designed to boost N reserves formation, therefore producing intermediate size seedlings with high N concentration compared to the other treatments (Rikala and Repo 1997, Islam et al. 2009, Andivia et al. 2012) (see picture 2-4, photographical section). This gradient of N availability might also help to understand some of the effects of increasing N deposition (Waldner et al. 2014) on Mediterranean forest ecosystems.

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Chapter 2

Species ecology determines the role of nitrogen nutrition in the frost tolerance of pine seedlings

Resumen

Las heladas determinan la evolución y distribución de las plantas en las regiones templadas y frías del planeta. Varios factores ambientales pueden afectar la aclimatación de las plantas a las heladas, pero el efecto y la magnitud de la disponibilidad de nitrógeno (N) sobre este proceso es controvertido. El objetivo de este estudio es analizar el efecto del N sobre la tolerancia a la congelación de la parte aérea y radical en otoño e invierno de plántulas de cuatro especies de pino de ecología contrastada: *Pinus nigra, P. pinaster, P. pinea* and *P. halepensis*. Para analizar los mecanismos fisiológicos subyacentes a la tolerancia a la helada, se midió la concentración de N y azucares solubles en hojas y raíces, y el momento del cese de la elongación de la parte aérea. Las plantas fueron fertilizadas con una dosis alta y una baja de N durante el periodo de pre-endurecimiento y una dosis moderada de N durante el periodo de endurecimiento. La tolerancia a la helada de la parte aérea aumento durante el invierno. Sin embargo, la tolerancia a la helada de la parte radical permaneció igual a lo largo del tiempo. La fertilización con N durante el periodo de pre-endurecimiento afectó la tolerancia a la helada de la parte aérea y radical, si bien el efecto varió entre las especies: en general la fertilización alta redujo la tolerancia a la helada de la parte aérea y radical en *P. pinea* y *P. halepensis* y tuvo el efecto contrario en *P. nigra;* sin embargo, no tuvo ningún efecto en la tolerancia a la helada de *P. pinaster*. La fertilización otoñal incrementó la tolerancia a la helada de todas las especies. Las diferencias en la tolerancia entre las especies y tratamientos no fueron explicadas por la variación en la concentración de N y azucares solubles en los órganos o por el momento del cese de la elongación de la parte aérea. Sin embargo, las especies más tolerantes a la helada cesaron antes su elongación. A pesar de la proximidad filogenética de las especies, el N tuvo un efecto distinto en las especies, indicando que la ecología de las especies (especialmente la fisiología de aclimatación a la helada y el momento de la aplicación del N) determina el efecto del N sobre la tolerancia a la helada de las especies de pino.

Palabras Clave: Azucares solubles, cese de la elongación del tallo, endurecimiento a la helada, fertilización otoñal, *Pinus*, tolerancia a la helada de las raíces, ecología.

Species ecology determines the role of nitrogen nutrition in the frost tolerance of pine seedlings

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Abstract

Frost determines the evolution and distribution of plants in temperate and cold regions. Several environmental factors can influence frost acclimation of woody plants but the magnitude and direction of the effect of nitrogen (N) availability is controversial. We studied the effect of N availability on root and shoot frost tolerance in mid-fall and in winter in seedlings of four pines of contrasting ecology: *Pinus nigra, P. pinaster, P. pinea* and *P. halepensis*. Needle and root N and soluble sugar concentration, and timing of cessation of shoot elongation were measured to assess the physiological mechanisms underlying frost acclimation. N was supplied at high and low rates only during the pre-hardening period and at a moderate N rate during hardening in the fall. Shoot frost tolerance increased over winter while root frost tolerance did not change in any species. Pre-hardening N availability affected the frost tolerance of both roots and shoots, although the effect was species-specific: high N reduced the overall root and shoot frost tolerance in *P. pinea* and *P. halepensis*, increased the frost tolerance in *P. nigra,* but had no effect in *P. pinaster*. N supply in the fall consistently increased frost tolerance in all species. Differences in frost tolerance among species and N treatments were not explained by variations in organ N or soluble carbohydrate concentration, nor by timing of cessation of shoot elongation; although the most frost tolerant species ceased elongation earlier than the least frost tolerant species. Despite the close phylogenetic relatedness of the studied species, the effect of N availability on seedling frost tolerance differed among species, indicating that species ecology (especially frost acclimation physiology and timing of N supply) drives the effect of N availability on frost tolerance of pine species.

***Keywords*: cessation of shoot elongation, cold hardiness, soluble carbohydrates, fall fertilization, *Pinus*, root frost tolerance, species ecology.**

Introduction

Low temperature is a key environmental factor determining the evolution and distribution of plants (Grace 1987, Hawkins et al. 2014). Frost can damage plants through xylem embolism and the formation of extracellular ice, which causes cell dehydration and disruption of cell membranes (Zwiazek et al. 2001, Willson and Jackson 2006, Charrier et al. 2015). Woody plants have remarkable differences in frost tolerance, which are frequently related to the minimum temperatures within their distribution range (Kreyling et al. 2014). At a plant scale, organs also differ in frost tolerance, with aboveground vegetative parts having greater frost tolerance than roots (Bigras et al. 2001, Charrier et al. 2015). Plants have developed specific adaptations to low temperatures (Levitt 1980, Larcher 2005). Perennial plants from cold and temperate biomes undergo a complex cold acclimation process during fall in which they experience deep reversible physiological changes to survive the cold season (Bigras et al. 2001, Charrier et al. 2015). Consequently, plant frost tolerance increases through the fall to a maximum in mid-winter and releases in spring to a minimum tolerance in early summer. However, decoupling of cold plant acclimation with timing of climate events has increased in the last decades. This phenomenon increases the chance of frost damage to plants due to unusual fall and spring frosts events, which are expected to increase with climate change (Augspurger 2013). At the same time, the reduction of the snow cover and earlier snow melting can increase the risk of root frost damage due to lower soil and root insulation (Groffman et al. 2001, Schaberg et al. 2008).

Cessation of shoot elongation is a requisite for frost acclimation (Greer et al. 2000, Repo et al. 2000, Heredia-Guerrero et al. 2014), triggering the accumulation of nutrients and the synthesis of metabolites involved in frost tolerance (Charrier et el. 2015). Specifically, soluble carbohydrates (SC) progressively accumulate in plant tissues during cold acclimation (Schulze et al. 2005, Martínez-Vilalta et al. 2016). *In vitro* addition of sucrose enhances structural stability of the cell membrane to frost damage and increases cytoplasm osmotic potential, which stabilizes intracellular structures and lowers the freezing point (Wolfe and Bryant 2001, Uemura and Steponkus 2003, Charrier et al. 2015). These cellular changes explain the reported positive relationship between frost tolerance and SC concentrations in plants (Ogren et al. 1997, Tinus et al. 2000, Kreyling et al. 2012). Lipid concentration and composition also varies during frost acclimation, reducing the protein to lipid ratio and increasing the degree of unsaturation of the hydrocarbon chain in membranes. These changes increase membrane fluidity and also reduce their disintegration when plant tissues freeze (Schulze et al. 2005). Finally, antifreeze proteins and several amino acids are involved in the frost tolerance of plants (Griffith and Yaish 2004), inhibiting the growth of ice crystals (Atıcı and Nalbantoğlu 2003) and contributing to osmotic adjustment (Zwiazek et al. 2001).

Photoperiod and temperature trigger and determine the intensity and speed of frost acclimation in woody plants (Welling et al. 2002, Charrier et al. 2015). While both temperature and photoperiod control shoot frost tolerance, temperature seems to be the main control of root frost tolerance (Fernández et al. 2008, Ryyppö et al. 2008). Nutrient availability, especially of nitrogen (N), can also affect plant frost tolerance (Taulavuori et al. 2014, Charrier et al. 2015). However, some studies indicate that roots do not harden in response to environmental variations such as temperature, even in cold climates (Tinus et al. 2000, Schaberg et al. 2008). In contrast to temperature and photoperiod, however, no clear conclusions have been developed regarding the relationship between frost tolerance and N availability (see Taulavuori et al. 2014). Because frost tolerance of roots and shoots responds differently to environmental stimuli such as temperature and photoperiod, N may have variable effects on the frost tolerance of plant organs.

Nitrogen deposition increases soil N and is a major environmental issue associated with global change (Rennenberg and Gessler 1999). Therefore, knowledge of the relationship between N and frost tolerance is important for assessing the potential responses of forest species to N deposition. Some studies have reported that high N availability reduces frost tolerance (Hellergren 1981, Schaberg et al. 2002, Villar-Salvador et al. 2013, Heredia-Guerrero et al. 2014), associated with a lower tissue SC concentration (Andivia et al. 2012) and delayed cessation of shoot elongation in fall (Hawkins et al., 1995, Heredia-Guerrero et al. 2014). In contrast, other evidences suggest that plant frost tolerance increases with N availability in trees from temperate climates (DeHayes et al. 1989, Andivia et al. 2012, Taulavuori et al. 2014). N-rich osmolytes such as amino acids and cryoprotection proteins are expected to increase with soil N availability during frost acclimation (Lähdesmäki and Pietiläinen 1988, Griffith and Yaish 2004, Berrocal-Lobo et al.

2011). This may explain why N supply in fall during cold hardening increases frost tolerance in plants from cold and temperate ecosystems (DeHayes et al. 1989, Andivia et al. 2012).

Contradictory results among studies on the effect of N on tree frost tolerance could be related to differences in N availability and application timing (Oliet et al. 2013) and to environmental conditions during the experiment (Heredia-Guerrero et al. 2014). However, variation among studies could also be attributable to functional differences among species. Particularly, physiology of dormancy as related to species ecology could determine the effects of N on frost tolerance during acclimation. Frost acclimation in trees adapted to mild winters is mainly controlled by temperature, while photoperiod plays a secondary role (Nguyen et al. 1995). These trees maintain growth during fall as long as temperature is not limiting and mild temperature spells in winter can quickly reduce frost tolerance (Charrier et al. 2015). High N availability, especially during the hardening period in fall, may hinder frost tolerance in these tree species (Puertolas et al., 2005). In contrast, frost acclimation in tree species from cold winter climates is usually under tight environmental control. Frost acclimation and cessation of shoot elongation begins early in the fall with photoperiod triggering frost acclimation (Charrier et al. 2015). These species usually maintain high frost tolerance during the cold season irrespective of winter conditions (Repo 1992). We expect that high N availability during the pre-hardening and hardening periods will enhance frost tolerance in these tree species. Therefore, to better understand the relationship between frost tolerance of tree species and N nutrition, and help to disentangle the underlying physiological mechanisms, comparative experiments where species are grown under the same environmental conditions are needed.

The objective of this investigation was to analyze the role of plant ecology in determining the effect of N on frost tolerance. We selected four pine species that have contrasting ecological characteristics and differ in their frost acclimation physiology that were cultivated under the same environmental conditions. We compared frost tolerance of roots and shoots of seedlings during cold hardening in mid-fall and in winter. We also evaluated tissue N and SC concentration in needles and roots, and cessation of shoot elongation to assess the physiological mechanisms underlying frost acclimation. Differences in N and SC concentration in organs and growing cessation were achieved by supplying N at high and low rates during the pre-hardening period (growing season) and N loading during cold hardening in fall.

The selected pine species are widespread in southern European regions characterized by Mediterranean climates. *Pinus halepensis* Mill. and *Pinus pinea* L. are thermophilic species that inhabit mild winter regions and have weak environmental control of dormancy. During winter, these species have high photosynthetic activity, can reactivate growth during mild spells (Puértolas 2005, Climent et al. 2011) and do not set buds in autumn during the seedling and early sapling stage (Mutke et al. 2012). *Pinus pinaster* Ait. is a mesophilic species that thrives mostly in mid-elevation mountains in Mediterranean-climate locations, while *Pinus nigra* J.F. Arnold is a high mountain, psychrophilic pine (Barbero et al. 1998). The last two species, especially *P. nigra* are usually exposed to intense and frequent frost events, set buds before dormancy in winter mediated by a strong endodormancy, and develop secondary needles earlier in their ontogeny than *P. halepensis* and *P. pinea* (Kreyling et al. 2012, Lafuente Laguna et al. 2013, Peñuelas et al. 2013). Some of these species coexist in the limits of their altitudinal range. We hypothesized that 1) the effect of N availability on frost tolerance during acclimation will be mediated by species ecology: while pine species inhabiting mild winter regions will have reduced frost tolerance under high N availability, those from cold winter regions will be positively influenced; 2) while timing of nutrient application (i.e., pre-hardening *versus* fall hardening) will affect frost tolerance of seedlings, the magnitude of this effect will depend on species; and 3) frost tolerance of roots will be less affected by N availability and timing of supply than shoots, because roots experience less cold acclimation in response to environmental shifts.

The findings of this study can shed light on the mechanisms of frost tolerance in response to N availability, which up to date are far from being clarified for forest species (Taulavuori et al. 2014). This could help to better understand the effects of global change drivers such as N deposition on plant functioning, as well as to improve the cultivation of nursery seedlings.

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 (Table S1, in Supplementary Material, Alía et al., 2009). Seeds were sown on February 2012 at the Centro Nacional de Recursos Genéticos Forestales "El Serranillo" (Central Spain, 40º40'N, 3◦10'W, 650 m a.s.l.) into plastic trays (Plasnor® 190/300-45, Legazpi, Spain). These trays have 45 cells of 300 ml, and cultivation density is 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 May 17, 2012 seedlings 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. Plants were assigned to three fertilization treatments: 1) Pre-hardening high and 2) low fertilization, where each plant was supplied with 150 and 20 mg N, respectively, during the seedling active growth period before fall cold hardening, from May 22 to September 19, 2012; 3) Fall fertilization, where each plant was supplied with 60 mg N during the seedling active growth period from May 22 to September 19 2012 plus 40 mg N·plant^{-1} during the fall, from September 26 to November 8, 2012. Fertilizer was applied by hand at a weekly constant rate using a water-soluble fertilizer. Fall fertilization extra supply was applied as ammonium nitrate, while for the rest of treatments the fertilizer was $20N–20P_2O_5–20K_2O$ (Scotts Co., Marysville, OH, USA) with N sources being ammonium nitrate (10%) and urea (10%). On every fertilization date, each seedling 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 to field capacity based on gravimetric methods (Timmer and Armstrong 1987). Each fertilization treatment had three trays, resulting in a total of nine trays per species. Trays were completely randomized in space and their position was rotated every 15 days to minimize edge effects. 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 June 20 to

September 15, where plants grew under a shading with a 20 % light transmission to reduce evapotranspiration.

Frost tolerance measurements

Freezing tests were carried out on two dates: in mid-November 2012, when seedlings were still frost acclimating and in late-January 2013, when plants are fully frost-hardened (Climent et al. 2009, Pardos et al. 2014). The temperature was measured every 10 minutes during the acclimation period using temperature probes placed adjacent to the experiment (Figure S1, in Supplementary Material). Accumulated chilling hours (air temperature ≤ 8 ºC calculated from September 1) in the nursery were 130 h in mid-November and 1367 h in January. Temperature frequently dropped below 0 ºC before the freezing test in January, reaching a minimum temperature of -4.9 ºC, while in November frosts were only registered in three days (Figure S1, in Supplementary Material). The average temperature the 20 days before the freezing tests was 10.0 ºC in autumn and 5.9 ºC in winter (Figure S1, in Supplementary Material). Frost tolerance was assessed on intact plants and freezing separately the roots and the shoots. Six seedlings per species, treatment and plant fraction (two seedlings per tray, 72 seedlings per plant fraction) were randomly selected on each date and subjected to an 8-h frost cycle in freezing chambers with a programmable temperature controller (ASL-Snijders International® CON-550-20, Madrid, Spain and Dycometal® CCK81, Barcelona, Spain, for the November and January tests, respectively). Temperature was reduced from 5 ºC to a target temperature where plants remained for 4 h. Then the temperature was progressively raised until 5 ºC. The target temperatures varied depending on the date and plant fraction (roots and shoots) (Table 1) and were chosen based on previous results obtained in other studies with these species (Puértolas et al. 2005, Villar-Salvador et al. 2013, Kreyling et al. 2012, Climent et al. 2009). Cooling and warming rates in a frost cycle ranged between 2 and 8 ºC h^{-1} in order to simulate natural frost cycle and to keep the freezing test duration to 8 h. The exception was for the target temperature of -23 ºC, where the cooling rate was 13 ºC h^{-1} while warming rates were 18 ºC h^{-1}. Frost tolerance of shoots was assessed by inserting the trays in a polystyrene box that isolated the plugs from the frost (see picture 5, photographical section). A thermocouple was placed inside the insulation cover and attached to the surface of the plug to record its temperature, which was on average 6.5 ºC higher than the air inside the freezing chamber for the -8 ºC freezing test and 12.5 ºC higher for the remaining target temperatures. To assess the frost tolerance of roots, seedlings were placed in a modified cultivation tray in which the wall of the cells was replaced by a 2-mm plastic mesh that allowed the plug to be fully frozen (see picture 6, photographical section). To prevent root desiccation, the plants were watered the day before and the plug was wrapped in aluminum foil during freezing tests. Shoots were insulated from frost by the same polystyrene cover used to isolate the root system.

After freezing tests, seedlings were transferred to a 60% light transmission greenhouse and kept watered at field capacity for two months. The average temperature in the greenhouse was 17 ºC during the two months following November frost tests and 19.5 ºC for the two months after the January frost tests. After the two months, shoot damage was assessed visually by two independent observers as the percentage of withered needles (see picture 10-12, photographical section). This value (visual damage index, VDI) is strongly correlated with other frost tolerance indicators (Andivia et al. 2011) and was considered as an estimator of shoot or root frost tolerance based on previous studies that show that root and shoot damage have an effect on the whole seedling (Carles et al. 2011).

Growth and nitrogen and soluble carbohydrates concentration

From August 21, 2012, prior the hardening stage, to January 3, 2013, seedling height was measured weekly on five randomly chosen seedlings per tray (15 plants per fertilization treatment and species, 180 seedlings in total). Measurements were carried out on the same plants throughout the study period. Shoot height was measured from a set point 5 mm below the cotyledon insertion scar to the apex of the bud in *P. nigra,* and to the tip of the shoot in the remaining species, which do not set a bud during the first years of life. The Julian day of cessation of shoot elongation was determined when weekly shoot elongation was lower than 0.6 cm (Heredia et al. 2014).

To determine seedling mass and N and SC concentration during hardening, 15 seedlings per species and treatment (five seedlings per tray) were randomly harvested at both freezing test dates and frozen to -20 ºC until processing. Once defrosted, shoots were cut 5 mm below the cotyledon insertion point and separated into needles and stems, and roots were carefully washed from the growing medium with tap water. Samples were rinsed in distilled water for 3 min to avoid contamination. Then leaves, stems and roots were dried at 60 ºC for 48 h and weighed to assess their mass. To analyze organ N and SC concentrations, the organs of the five seedlings of the same tray were composited and ground in a ball mill (PM 100, Retsch, Haan, Germany).

SC were analyzed in leaves and roots using a high-performance liquid chromatograph (HPLC). Grounded leaf samples of 50 mg were extracted twice in 80% aqueous ethanol (v/v) at 80 ºC for 90 min. Samples were centrifuged at 13000 g for 5 min and the supernatant was completely evaporated and resolubilized in 5 ml distilled water and boiled for 5 min. The resulting solution was filtered through a 0.45 µm nylon-syringe and 20 µl was injected to HPLC using an Agilent 1100 Series (Agilent Technologies, Palo Alto, CA) equipped with a refractive index detector. A carbohydrate column (Supelcogel^{TM}Ca, 30 × 0.78 cm, Supelco, Bellefonte, PA, US) was used for the analysis. SC peaks were detected by refractive index and were identified and quantified by comparison with retention times of fructose, glucose and sucrose standards (Heredia et al. 2014). Determination of leaf and root N concentration was done by the standard Kjeldahl method using an auto-analyzer (CFA SAN++, Skalar, Breda, The Netherlands).

Data analysis

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 1000 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). The differences between means were identified using Fisher’s least significant difference (LSD) test. All analyses were performed in R software (R Foundation for Statistical Computing, Vienna, AT).

Results

Differences in root frost damage

Species differed in VDI after root freezing at -10 °C both in November and January (Table 1). *Pinus halepensis* and *P. pinea* had the highest VDI values, with no significant differences between them (30.6% ± 6.7 in November and 36.2% ± 9.3 in January for *P. halepensis* and 26.0± 5.7 and 33.5% ± 9.4, respectively for *P. pinea*). In contrast, *P. nigra* had the lowest VDI values (7.6% ± 1.5 and 2.6% ± 0.6 in November and January, respectively), while *P. pinaster* had intermediate VDI values (12.4% ± 1.5 and 20.9% ± 3.4 in November and January, respectively) between *P. nigra* and the other pine species (Figure 1).

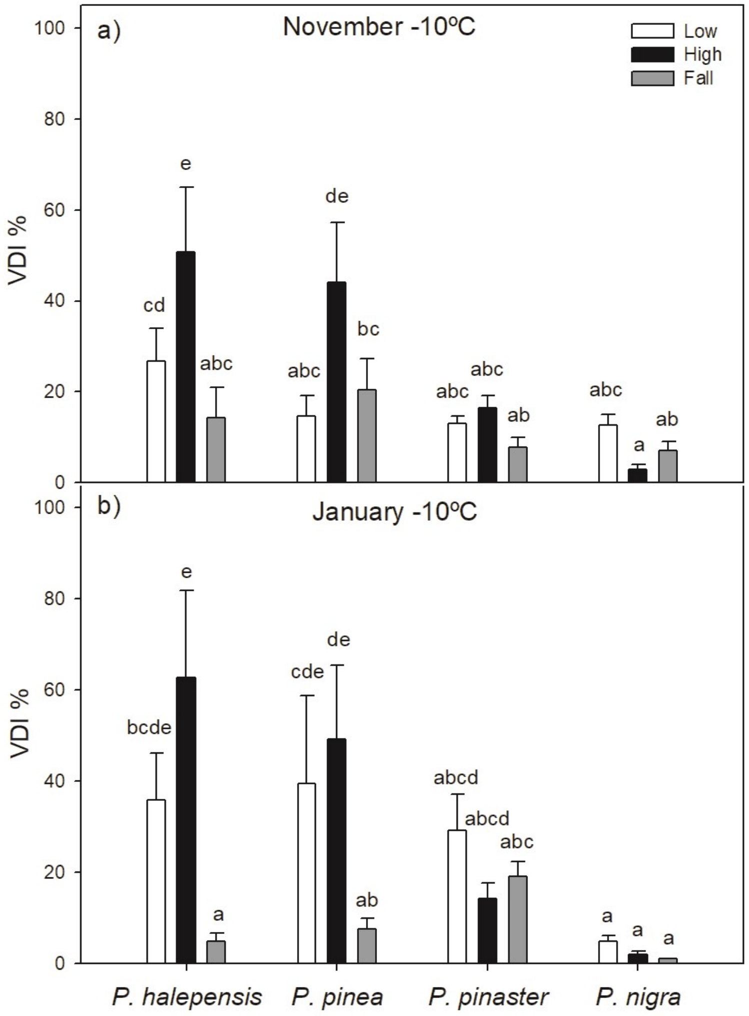

Figure 1: Visual damage index (VDI) after root freezing at -10 °C in November (a) and January (b) in one-year old seedlings of four pine species that were cultivated with three nitrogen fertilization treatments (pre-hardening low and high N, and fall fertilization). Data are means ± 1 SE. Mean values not sharing common letters are significantly different.

Fertilization significantly affected VDI after root freezing, but differences among treatments depended on species in November (interaction Species × Fertilization, Table 1). By this date, the highest VDI occurred for the high pre-hardening fertilized *P. halepensis* and *P. pinea* seedlings, while the opposite was observed for *P. nigra*, with the low pre-hardening fertilized seedlings having the highest VDI. In addition, fall-fertilized seedlings presented similar or slightly lower VDI values than those from low fertilized plants and in most cases, lower VDI values than those from high fertilized plants (Figure 1a).

In January, fall-fertilized seedlings showed the lowest root VDI values (7.0% ± 1.5), while high and low pre-hardening fertilized seedlings showed the highest VDI (Figure 1b). Among species, *P. pinaster* VDI responses to fertilization were the less plastic at both dates. On average, root VDI remained similar between November and January (18.5%± 2.3 and 23.4%± 3.7, respectively).

Differences in shoot frost damage

Species differed in VDI after shoot freezing at -8°C in November, with overall VDI values of 50.0% ± 8.3, 36.0% ± 5.2, 17% ± 2.9 and 4.0% ± 0.7 for *P. halepensis, P. pinea, P. pinaster* and *P. nigra,* respectively. However, a strong Species × Fertilization interaction was observed (Table 1). Low fertilization resulted in the highest VDI values in all species except for *P. pinaster*, which showed the highest VDI values in both high and low fertilized plants. Fall fertilized seedlings had the lowest VDI in *P. halepensis* and *P. pinaster*, while no difference between fall and high fertilization was found in the rest of species. In contrast, high fertilization led to intermediate VDI values between high and fall fertilized plants in *P. halepensis* (Figure 2a).

After the -15 ºC frost in November, VDI differed among species but not among fertilization treatments (Table 1). While *P. halepensis* and *P. pinea* showed almost complete damage (overall mean for both species: 98.7% ± 0.7) irrespective of fertilization treatment, *P. nigra* had the lowest VDI values (17.7% ± 4.2 across fertilization treatments). Finally, *P. pinaster* had intermediate VDI values (61.8% ± 6.1) between *P. nigra* and the other pine species.

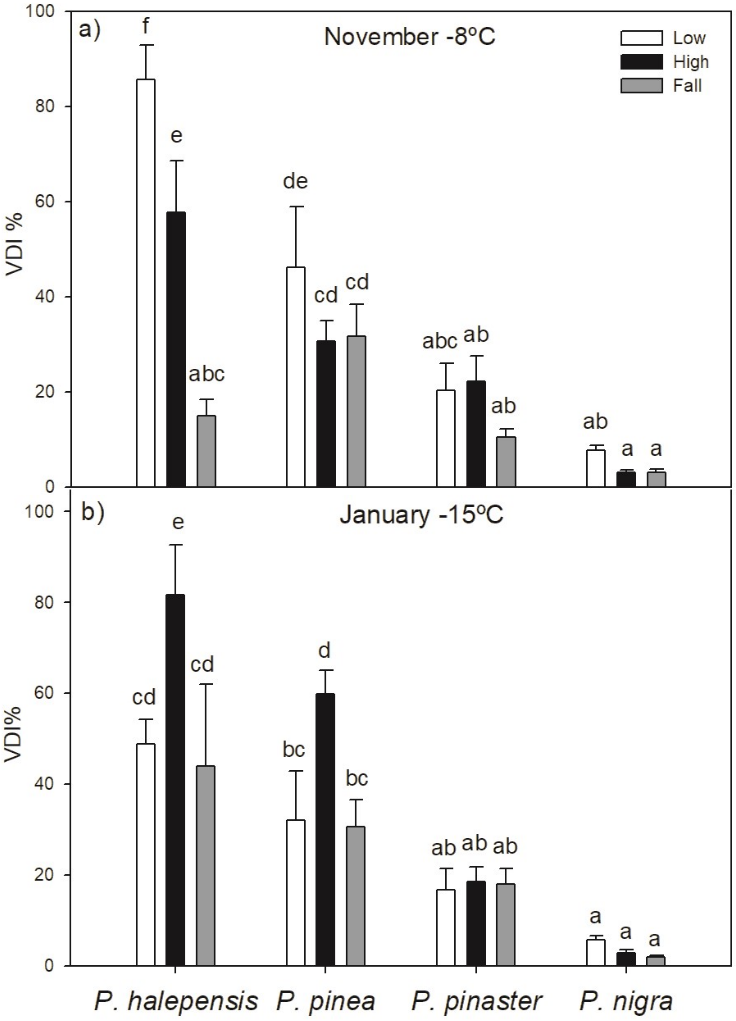

Figure 2: Visual damage index (VDI) after shoot freezing at -8 °C in November (a) and -15°C January (b) in one-year old seedlings of four pine species that were cultivated with three N fertilization treatments (pre-hardening low and high N, and fall fertilization). Data are means ± 1 SE. Mean values not sharing common letters are significantly different.

In January, after a -15 °C frost VDI significantly differed among species (Table 1), with *P. halepensis and P. pinea* showing the highest values (59.3% ± 8.8 and 41.3% ± 5.2, respectively) followed by *P. pinaster* (17.8% ± 2.0) and finally *P. nigra,* which had the lowest VDI value (3.4% ± 0.5). We found a marginally significant Species × Fertilization interaction (P=0.065), explained by a maximum VDI of high fertilized *P. halepensis* and *P. Pinea* seedlings, that did not occur in *P. nigra* and in *P. pinaster* (Figure 2b). On average, shoot VDI after -15°C frost decreased strongly from November to January (69.4 ± 4.3 and 29.7 ± 3.6, respectively).

In January, all seedlings of *P. halepensis*, *P. pinea* and *P. pinaster* died after the -23°C frost test. In contrast, most *P. nigra* seedlings survived with fall fertilized and pre-hardening high fertilized seedlings having the lowest VDI values, without significant differences between them (9.3% ± 2.4 and 16.8% ± 5.5, respectively), while low fertilization the highest (32.5% ± 7.7) (Species × Fertilization interaction, Table 1).

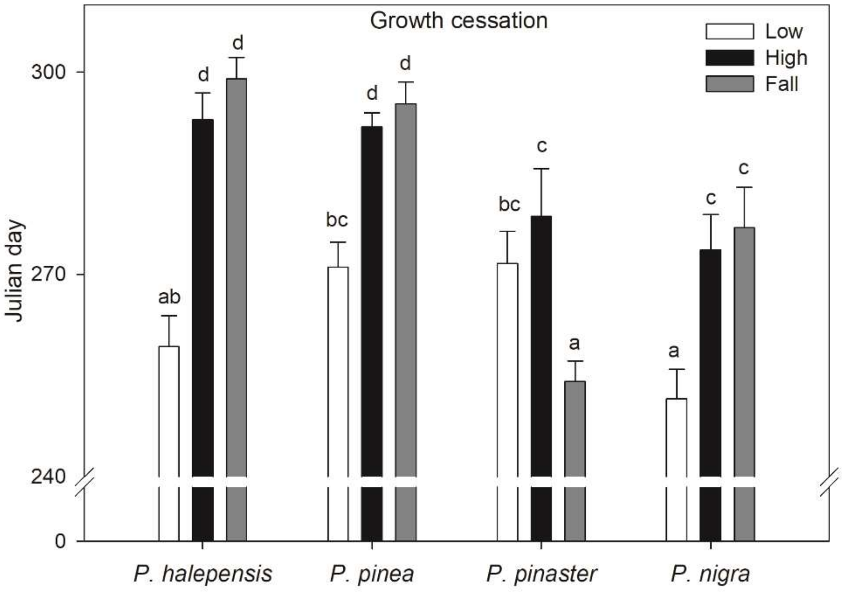

Figure 3: Julian day of cessation of shoot elongation in one-year old seedlings of four pine species that were cultivated with three N fertilization regimes (pre-hardening low and high N, and fall fertilization). Data are means ± 1 SE. Mean values not sharing common letters are significantly different.

Cessation of shoot elongation

On average, *P. nigra* and *P. pinaster* seedlings ceased shoot elongation 17 days earlier than *P. halepensis* and *P. pinea* (F=14.9, P<0.001), while no significant difference existed between the species within the two previous groups (Figure 3). However, a significant Species × Fertilization interaction occurred on cessation of shoot elongation (F=7.8, P<0.01). The effect of N fertilization on the cessation of shoot elongation presented a similar pattern in *P. halepensis, P. pinea and P. nigra*, with high and fall fertilization delaying the cessation of shoot elongation by 20 days on average relative to low fertilized plants (Figure 4). In contrast, fall fertilized *P. pinaster* plants stopped shoot elongation earlier than high and low fertilized plants, which showed no differences between them.

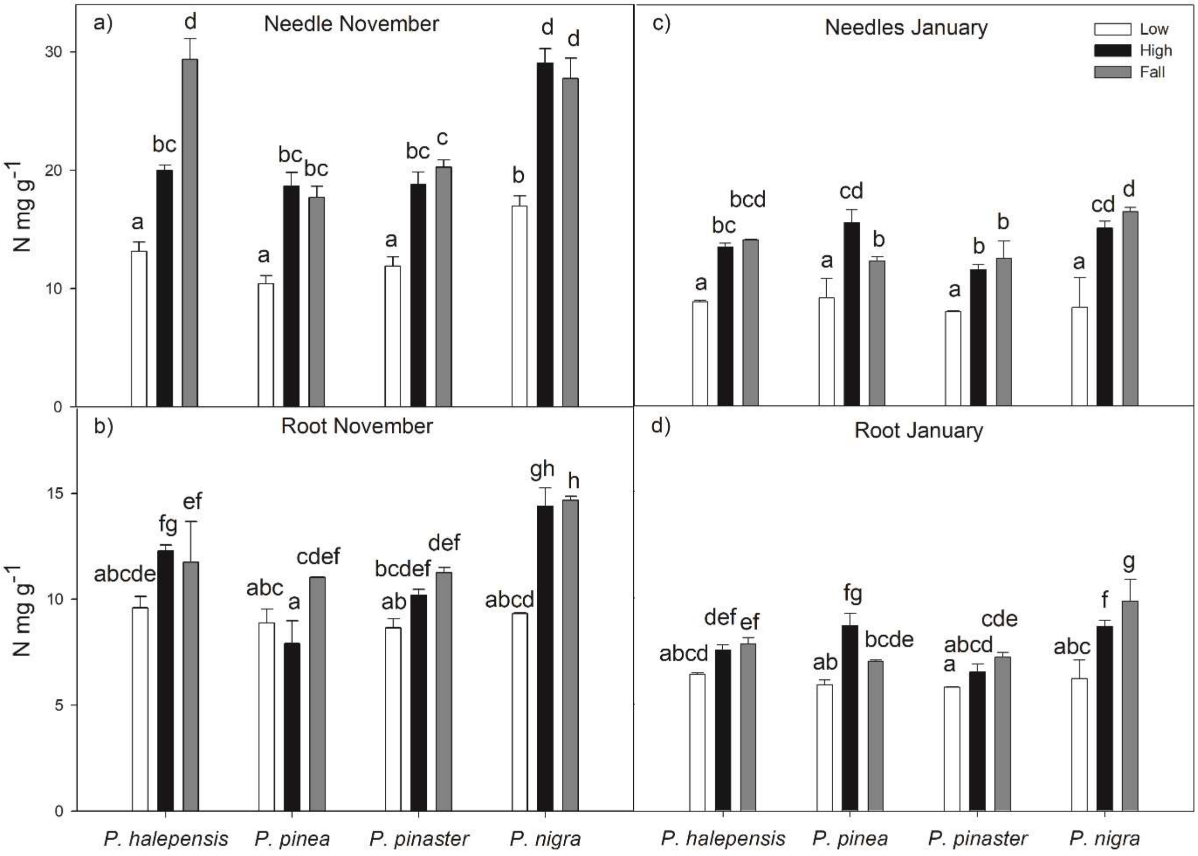

Figure 4: N concentration (Mg/g) in one-year old seedlings needles and roots of four pine species that were cultivated with three N fertilization regimes (pre-hardening low and high N, and fall fertilization) in November (a,b) and January (c,d). Data are means ± 1 SE. Mean values not sharing common letters are significantly different.

Nitrogen and soluble carbohydrates concentration

Needle N concentration was highest among the fall and high fertilized seedlings and lowest in the low fertilized plants in all species and in both dates. However, in both freezing dates, the fertilization effect on needle N concentration was mediated by an interaction between Species and Fertilization, (F=3.4, P<0.008). In November, fall fertilized *P. halepensis* seedlings showed higher needle N concentration than high fertilized seedlings (Figure 4a) while in January, needles of high fertilized *P. pinea* seedlings had higher N concentration than the fall fertilization treatment (Figure 4b). Root N concentration was lower than needle N concentration but differences among species and fertilization treatments followed an overall similar pattern as described for needle N (interaction Species × Fertilization at both dates, F=3.4, P<0.007; Figure 4c and 4d), especially in *P. pinaster* and *P. nigra*.

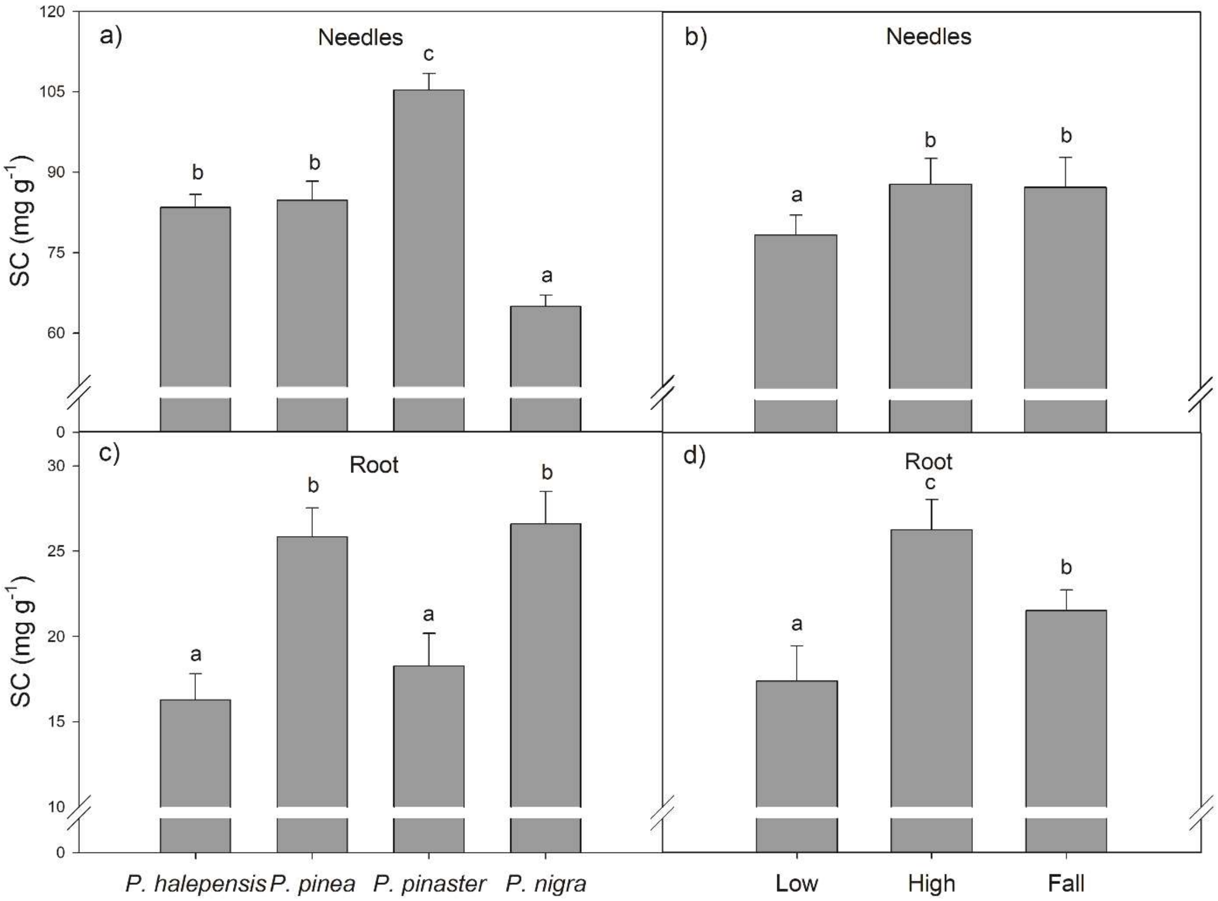

Figure 5: Species (left figures) and N fertilization treatment (right figures) differences in soluble carbohydrates (SC; Glucose+fructose+sucrose) concentration in needles (upper row) and roots (lower row) in one-year old seedlings of four pine species sampled in November. Data are means ± 1 SE. Mean values not sharing common letters are significantly different.

Irrespective of fertilization treatment, N concentration differed among species in November. By this date, species ranking in both needle and root N concentration was *P. nigra* > *P. halepensis* > *P pinea* = *P. pinaster* (Figure 4). N concentration decreased in both needles and roots from November to January in all species and differences in organ N among species were small in January.

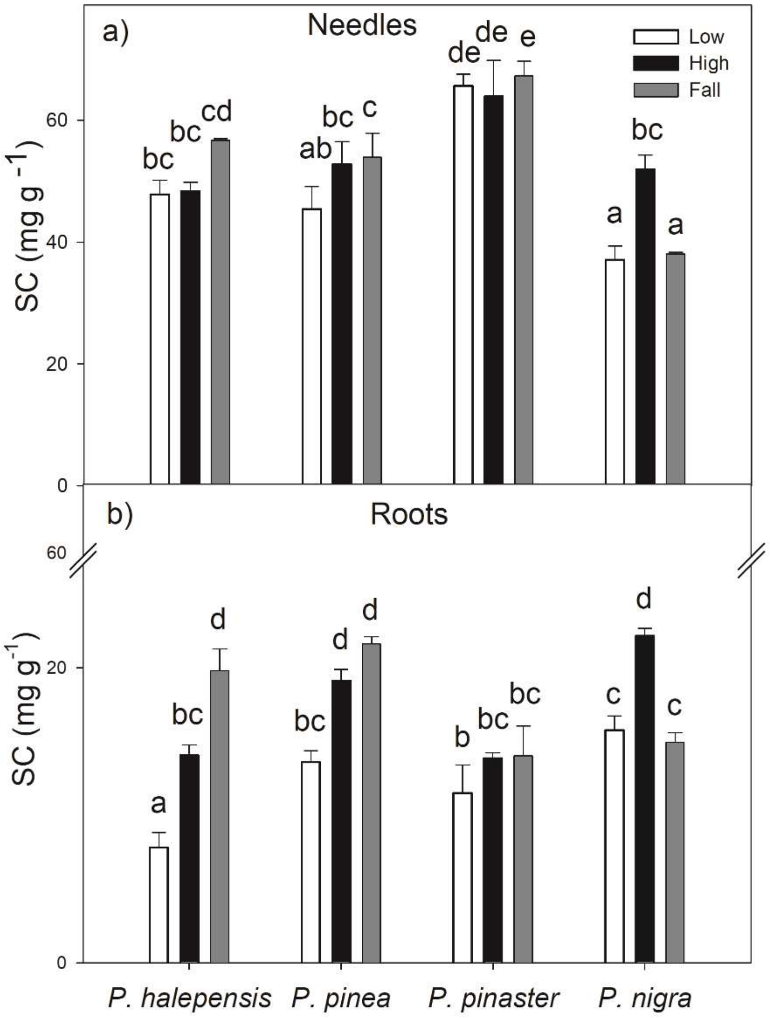

Figure 6: Soluble carbohydrate (SC; Glucose+fructose+sucrose) concentration in needles (a) and roots (b) of one-year old seedlings sampled in January in four pine species that were cultivated with three N fertilization regimes (pre-hardening low and high N, and fall fertilization). Data are means ± 1 SE. Mean values not sharing common letters are significantly different.

Needle and root SC concentration varied significantly among species in November (F= 19.6 $P<0.001$ for roots; F= 57.7 $P<0.001$ for needles). *Pinus pinaster* and *P. nigra* needles showed the highest and lowest needles SC concentration respectively, while *P. halepensis* and *P. pinea* had intermediate values between the former species with no significant differences between them (Figure 5a). In contrast, SC concentration in roots was higher in *P. nigra* and *P. pinea* than in *P. pinaster* and *P. halepensis*, while no significant differences existed between the species within the two previous groups (Figure 5b). On the same date, high and fall N fertilization had similar but higher needle SC concentration than the low N fertilization (F=8.0, $P<0.01$). Root SC concentration in November was highest in the high fertilized plants followed by fall fertilized seedlings, while low fertilized seedlings showed the lowest SC concentration (F=19.9, P<0.001). No interaction between Species and Fertilization occurred for SC.

In January, SC concentration was affected by a significant Species × Fertilization interaction in both shoots and roots (F=3.4, P=0.015 and F=7, P<0.001, respectively). In both fractions, high fertilized *P. nigra* seedlings had higher SC concentration than low and fall fertilized seedlings, which showed no difference between them (Figure 6). In contrast, fall fertilized plants and low fertilized plants had in almost all cases the highest and lowest SC concentration, respectively, in *P pinea* and *P. halepensis*. Finally, *P. pinaster* root and needle SC concentrations were not affected by N fertilization (Figure 6). Root and shoot SC significantly decreased between November and January (F=4.5, P=0.007 and F=7.1, P<0.001, respectively).

Table 1: Statistical results of the effect of species and fertilization treatments on visual damage index values after root and shoot freezing tests at different target temperatures in November and January. Data are F-values and P-values in brackets.

	Root		**Shoot**			
	November	January	November		January	
	-10 ºC	-10 ºC	-8 ºC	-15 ºC	-15 ºC	-23 ºC
Species	9.1 (<0.001)	6.2 (0.001)	32.3 (<0.001)	118 (<0.001)	30.1 (<0.001)	549 (<0.001)
Treatment	5.7 (0.005)	7.0 (0.002)	13.5 (<0.001)	0.6 (0.45)	6.7 (0.002)	4.2 (0.02)
Species × Treatment	2.9 (0.015)	1.8 (0.11)	6.3 (<0.001)	0.2 (0.023)	2.0 (0.065)	4.1 (0.002)

Supplementary material

Table S1: Geographic location and climatic characteristics of the provenances where seeds were collected. MAT= mean annual temperature, TCM= mean of the minimum temperatures of the coldest month. Provenance names follows nomenclature in Alía et al (2009).

Species	**Provenance**	**Latitude (N)**	**Longitude (W)**	**Altitude (masl)**	**MAT (ºC)**	**TCM (ºC)**	**Annual rainfall (mm)**
P. halepensis	Alcarria, ES07	40º24’52''	2º24’33’’	860	12.6	-0.6	580
P. pinea	La Mancha, ES03	39º12’02’’	1º57’59’’	675	14.2	0.7	397
P. pinaster	Cuenca, ES12	39º38’44’’	1º13’52’’	1135	12	-1.5	540
P. nigra subsp. *salzmanii*	Sistema Ibérico Meridional, ES07a	40º15’16’’	1º58’22’’	1515	10.4	-3.2	894

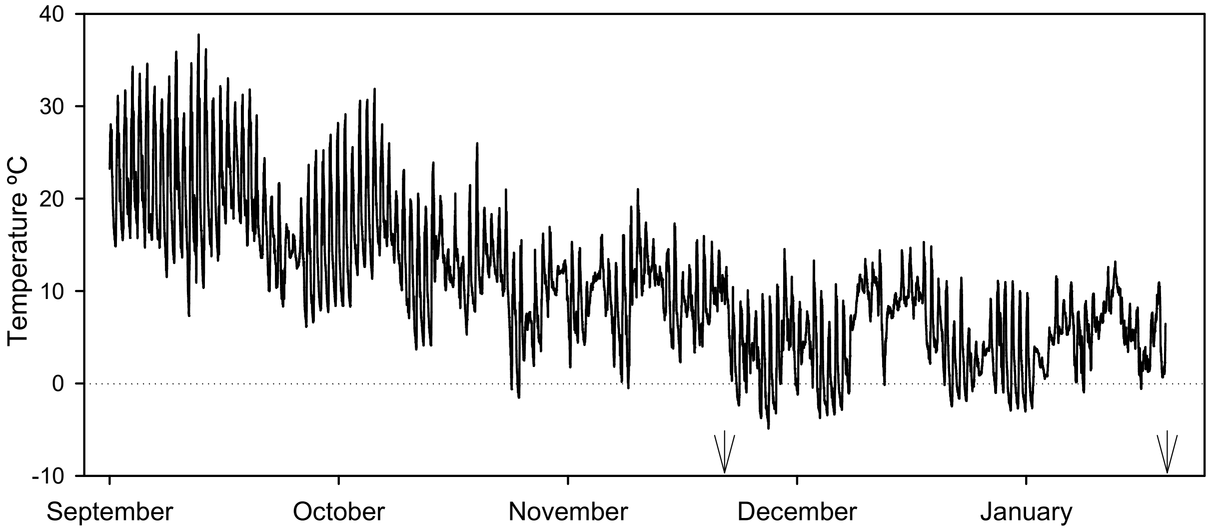

Figure S1: Temperature progression in the nursery from September 2012 to the end of January 2013. The arrows indicate the date of the freezing test

Discussion

Despite the close phylogenetic relatedness of the studied species, N availability differentially affected seedling frost tolerance among them. These differences conform with knowledge of their ecology and frost acclimation physiology. We found three patterns in the frost tolerance responses to N availability during the pre-hardening period: 1) High N increased frost tolerance in *P. nigra,* the psychrophilic species that lives in cold winter locations and has strong endodormant control of growth and cold acclimation (Peñuelas et al. 2013); 2) High N hindered frost tolerance in the thermophilic species, *P. halepensis* and *P. pinea*, the species inhabiting the mild winter sites and having an eco-dormant growth and cold acclimation physiology (Navarro Cerrillo et al. 2013, Puértolas et al. 2013); and 3) N fertilization had little effect on the frost tolerance of the mesophilic species *P. pinaster*. This result supports our first hypothesis and indicates that species ecology and dormancy physiology determine the effect of N availability on plant frost tolerance. A major finding of our study was that despite strong species differences in the frost response to N availability during pre-hardening, N supply in the fall during the hardening period consistently increased the frost tolerance in all species. This result does not support our hypothesis that the timing of nutrient application will have a varying effect among species with contrasting ecology. In contrast to findings linking frost tolerance to cessation of shoot elongation in the fall (Repo et al. 2000, Heredia-Guerrero et al. 2014), SC concentration (Greer et al. 2000, Tinus et al. 2000, Villar-Salvador et al. 2013), and organ N concentration (DeHayes et al. 1989, Rikala and Repo, 1997), we found these attributes to be largely unrelated to frost tolerance differences across species and N treatments. It is possible that the differences among species may be the result of different strategies in using N for frost tolerance and mechanisms of growth control (Charrier et al. 2013). We discuss these species differences in frost tolerance strategies below.

Root and shoot frost tolerance

Shoot and root frost acclimation physiology showed notable differences. First, shoot frost tolerance increased in all species between mid-fall and early-winter as indicated by lower VDI values in January than in November after the -15°C freezing tests. This result agrees with the well-known fall freezing acclimation patterns observed in the aboveground tissues of temperate- and cold-climate trees (Tinus et al. 2000, Bigras et al. 2001, Pardos et al. 2014). In contrast to shoots and consistent with our third hypothesis, frost tolerance of roots remained unchanged between studied dates in all species (Figure 1). A similar lack of root cold acclimation through the fall was found for *P. halepensis* and *Pinus radiata* (Tinus et al. 2000) and *Juglans regia* (Charrier et al. 2013). In contrast to our findings, significant fall root cold hardening was reported for *Pseudotsuga menziesii* (Tinus et al. 2000). Soil insulates the roots from frost under natural conditions, which could explain the low or lack of cold acclimation in some woody species. In climates where snow remains on soil for an extended period, however, the reduction of snow cover and/or earlier snow melting increases the risk of root damage by frost (Groffman et al. 2001, Schaberg et al. 2008).

Second, shoot and root frost tolerance responded similarly to N availability during pre-hardening in the psychrophilic and mesophilic species (*P. nigra* and *P. pinaster*, respectively) and the response changed little between the studied dates. N availability during pre-hardening also affected frost tolerance in the thermophilic pines (*P. halepensis* and *P. pinea*) but unlike the psychrophilic pines, the response of frost tolerance to N availability diverged between organs and dates. While the roots exhibited lowest frost tolerance in mid-fall and in winter with high rate of pre-hardening N fertilization, shoots had the opposite response in November, showing the lowest frost tolerance with the low rate of pre-hardening N fertilization. However, in January shoots had the same response to N as roots. The different effect of N availability on shoot frost tolerance in the thermophilic species through the cold season likely was the result of distinct physiological processes, which importance varied along the cold acclimation process. We suggest that shoot frost tolerance was lowest in low fertilized seedlings in November, likely due to low N-rich cryoprotectant metabolites (Andivia et al. 2012). This hypothesis is supported by the fact that needle N concentration in November in the low fertilized plants was in most cases close to 10-13 mg g^{-1} (Figure 4), which is considered deficient for these species (deficient threshold values for studied species range between 11 to 15 mg g^{-1} (Oliet et al. 2006, Lafuente Laguna et al. 2013, Navarro Cerrillo et al. 2013, Peñuelas et al. 2013, Puértolas et al. 2013). In contrast to results in November, the lower frost tolerance of high N fertilized seedlings in January of thermophilic species might be explained by cold dehardening compared to the other N treatments (Fløistad and Kohmann 2004), thereby reducing their frost tolerance. The earlier cold dehardening in high N fertilized seedlings could be due to the increase in temperatures registered in mid-January (Figure S1, in Supplementary Material). In the cryophilic species, *P. nigra* and to a lesser extent in *P.*

pinaster, frost tolerance in both shoots and roots was lowest for the low N pre-hardening treatment during both fall and winter, which also supports the N deficiency hypotheses. Contrary to shoots, the effect of N availability on root frost tolerance remained similar among species over the cold season in our study, probably because roots do not experience cold hardening in these species (Tinus et al. 2000, Kreyling et al. 2012).

Fernández et al (2017) showed no differences among our studied species in vulnerability to stem xylem freezing-induced embolism. Thus, we presume that differences in stem xylem freezing-induced embolism at interspecific level due to changes in N availability are unlikely and that the differences in frost sensitivity in response to N availability are due to differences in foliage and root frost sensitivity.

Cessation of shoot elongation

Cessation of shoot elongation is essential for frost acclimation to occur (Greer et al. 2000). The idea that high-N fertilized plants are more vulnerable to frost lies on observations that high N availability delays the cessation of shoot elongation in fall (Heredia-Guerrero et al. 2014, Rikala and Repo, 1997). Consistent with this idea, an increase in N availability delayed the cessation of shoot elongation in our study. However, we did not find a clear connection between cessation of shoot elongation and frost tolerance. Several results support this assertion. First, low N availability during pre-hardening advanced the cessation of shoot elongation 20 days in three out of four species, but low N seedlings had the lowest shoot frost tolerance in November (Figure 2). Second, fall fertilization strongly delayed growth cessation in most species but these seedlings showed the highest frost tolerance in mid fall. Third, *P. nigra* and *P. pinaster* showed similar timing of cessation of growth elongation but *P. nigra* was more cold-tolerant than *P. pinaster*. A similar lack of relationship between frost tolerance and growth cessation was found in *Pseudotsuga menziesii* (Hawkins et al. 1995) and *Pinus sylvestris* (Rikala and Repo 1997). Thus, our results illustrate that cessation of shoot elongation may trigger frost acclimation but does not affect the intensity of frost acclimation. Consequently, species with late cessation of shoot elongation such as *P. pinea and P. halepensis* incur a higher risk of freezing damage due to early fall frost than species that cease elongation earlier in the fall, but without additional cascading effects. We believe that the benefit of high N availability during fall may override differences in timing of growth cessation once it has occurred (mid-fall frost tolerance measurements were conducted after shoot growth cessation of all treatments and species), and reinforces the idea that N supplied during hardening is preferentially invested in frost tolerance N-rich metabolites.

Soluble carbohydrates

The general consensus in scientific literature is that SC are involved in plant frost tolerance and an increase in frost tolerance is positively related to SC at a within-species level (Greer et al. 2000, Tinus et al. 2000, Morin et al. 2007, Kreyling et al. 2012). However, we did not find a clear link between frost tolerance across N fertilization treatments and SC. In our study, needle SC concentration was lower in the psychrophilic (most frost tolerant) species *P. nigra,* than in the thermophilic (least frost tolerant) species. Within species, frost tolerance followed a very different pattern compared to SC concentrations under the influence of N treatments. Furthermore, SC concentration in November was higher than in January; however, shoot frost tolerance increased over the same period. The lack of studies that compare the effect of soluble carbohydrates on frost tolerance across species complicates the clarification of this relationship. To our knowledge, this relationship has only been addressed in two interspecific studies (Morin et al. 2007, Charrier et al. 2013); however, the results are not conclusive, especially when broadleaves and conifers are compared under the same environmental conditions. Nevertheless, some studies show that a clear cause/effect relationship does not exist between soluble carbohydrates concentration and frost tolerance (Zhang et al. 2003, Andivia et al. 2011). In agreement with these studies, our results show a similar lack of relationship between soluble carbohydrates concentration and frost tolerance in the tested pine species, reinforcing the idea that other metabolites than soluble sugars such as antifreeze proteins and membrane stabilization metabolites are more important for cold hardening of pines species (see discussion below).

As for the shoot, root SC concentration was unrelated to frost tolerance. Roots of pine species invest most SC in growth during winter (Hansen and Beck 1994) and so it is likely that SC are involved relatively little in frost tolerance; rather, variation in concentration might reflect physiological activities such as storage, remobilization, and fine root growth over winter.

Nitrogen concentration in needles and roots

The role of tissue N in the frost tolerance of plants is controversial (Villar-Salvador et al. 2015). While some studies have shown positive links between frost tolerance and tissue N (Rikala and Repo 1997, Bigras et al. 2001, Andivia et al. 2011) others have shown the reverse trend (Hawkins et al. 1995, Fløistad and Kohmann 2004, Villar-Salvador et al. 2013). In our study, we have found evidence supporting a positive relationship between both variables. For instance, low-N fertilized plants, which showed the lowest tissue N concentration (Figure 4), had overall lower shoot frost tolerance in November than the high-N fertilized seedlings. Many plants invest available N during cold hardening in antifreeze proteins and dehydrins (Kontunen-Soppela et al. 2000, Atıcı and Nalbantoğlu 2003, Griffith and Yaish 2004) and N was found to be critical for repairing root xylem frost-induced embolism in several species (Ewers et al. 2001). Other results of our study, however, do not support a link between frost tolerance and tissue N. For instance, tissue N concentration in November was greatest in the highest and lowest frost tolerant species, *P. nigra* and *P. halepensis,* respectively. Similarly, within species, seedlings showing similar N concentrations varied significantly in their frost tolerance. Specifically, fall fertilized seedlings had higher frost tolerance than pre-hardening high-N fertilized plants, particularly in thermophilic species. Taken together, our results indicate that timing of N supply is critical for frost tolerance and that the variation in frost tolerance between treatments and species are not driven by differences in tissue N concentrations *per se*, but likely by how N is allocated to major plant functions. It is possible that N taken up during the period of cold hardening and cessation of shoot elongation is mainly allocated to N-rich compounds involved in frost tolerance (Andivia et al. 2012), while N supplied during the pre-hardening season is primarily allocated to growth at the expense of frost tolerance and this trade-off may differ among species. This suggestion could explain why some studies have reported that high fertilization applied at pre-hardening reduced frost tolerance of thermophilic species (Puérolas et al. 2005, Villar-Salvador et al. 2013). Future comparative studies of the timing and magnitude of N availability should be designed to: 1) address whether species differ in allocation to N-metabolites involved in frost tolerance; and 2) identify specifically which N-metabolites are involved in these processes.

Conclusions

Four *Pinus* species of close phylogenetic relationship, yet with distinct ecophysiology of cold hardening, showed variable frost tolerance response to N availability at pre-hardening when cultivated under the same environmental conditions. In contrast, N availability during hardening clearly increased frost tolerance of all four species, highlighting the importance of timing of N availability for development of frost resistance of seedlings. Our results can help to design specific fertilization regimes for plant production, with emphasis on the fall fertilization treatment due to its increase of frost tolerance and N reserves. Differences in frost tolerance among species and levels of N availability were not explained by gradients of soluble carbohydrate concentrations nor by timing of growth cessation. Additionally, frost resistance dynamics during hardening varied between roots and shoots. Shoots and roots were sensitive to N availability, but while shoots increased in frost tolerance over winter and (in the thermophilic species) can alter the relationships between N and frost tolerance, roots remained unchanged along the hardening period. These contrasting effects of N on the frost tolerance of roots and shoots among pine species may be a result of physiological differences among species and the frost acclimation strategies of specific organs.

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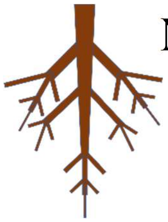
Chapter 3



Nitrogen reserves differentially affect root development in ecologically distinct pine species

Resumen*:*

Introducción y objetivos: La plasticidad del sistema radical permite maximizar la absorción de recursos del suelo bajo condiciones ambientales cambiantes. Sin embargo, el papel del estado fisiológico de las plantas, como las reservas de nitrógeno (N), en la arquitectura y cinética de crecimiento radical son poco conocidas. El objetivo de este estudio es analizar si la ecología de las especies afecta a la influencia del nivel de reservas de N sobre la arquitectura y cinética de crecimiento radical.

Métodos: Se cultivaron plántulas de tres especies de pino de ecología distinta bajo diferentes regímenes de fertilización nitrogenada contrastada y trasplantadas a rizotrones para evaluar su arquitectura y cinética de crecimiento radical.

Resultados: Las especies mostraron distinta arquitectura y cinética de crecimiento radical en respuesta al N. *Pinus pinaster*, la especie que habita climas con moderado nivel de estrés, mostró la mayor plasticidad y eficiencia del uso del N para el crecimiento radical. Las especies de regiones con más estrés ambiental (*P. pinea,* de regiones con una fuerte sequía estival y *P. nigra*, de regiones con inviernos muy fríos) mostraron características opuestas a *Pinus pinaster.* En todas las especies, las plántulas con menor fertilización nitrogenada produjeron raíces con una mayor longitud específica, sugiriendo que el contenido de N de las plantas afecta la arquitectura de las raíces para optimizar la absorción de recursos del suelo.

Conclusiones: La ecología de las especies determina el efecto de las reservas del N sobre la arquitectura y cinética de crecimiento radical. Las diferencias entre las especies podrían reflejar diferencias evolutivas en el compromiso de priorizar el reparto de N hacia el crecimiento radical o hacia mecanismos de resistencia al estrés.

Palaras clave: Arquitectura radical, cinética de crecimiento radical, eficiencia del uso del nitrógeno, longitud radical específica, plasticidad radical, rizotrón.

Nitrogen reserves differentially affect root development in ecologically distinct pine species

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Toca A, Oliet JA, Villar-Salvador P, Martinez Catalan RA, Jacobs DF (2019) Nitrogen reserves differentially affect root development in ecologically distinct pine species. Forest Ecology and Management. Under Review.

Abstract:

Aims: Root plasticity allows plants to maximize soil resource uptake under changing environments. However, the role of plant physiological status, such as nitrogen (N) nutrition, on root architecture and growth dynamics is poorly understood. We assessed if species ecology determines how variation in seedling N storage affects root growth dynamics and architecture.

Methods: Seedlings of three *Pinus* spp. were cultivated under contrasting N fertilization regimes and transplanted into rhizotrons to evaluate root growth dynamics and architecture.

Results: Species showed distinct root growth patterns and architecture in response to N. *Pinus pinaster*, from moderate climatic stress environments, had the greatest root growth plasticity and nitrogen use efficiency. The opposite effect occurred for pines adapted to more stressful environments: *P. pinea,* from sites with strong summer drought, and *P. nigra*, from cold winter areas. New roots of low N content seedlings had higher specific root length than high N seedlings in all species, suggesting that internal N status affects root architecture to optimize resource acquisition.

Conclusions: Species ecology determines how seedling N reserves affect root growth dynamics and architecture. Differences among species may be determined by a trade-off between allocation of stored N to root growth and promotion of stress resistance.

Keywords: growth dynamics, nitrogen use efficiency, rhizotron, root architecture, root plasticity, specific root length.

Abbreviations: N - Nitrogen; SRL - specific root length; NUE - Nitrogen use efficiency; RDPI - relative distance plasticity index

Introduction

Survival of forest tree seedlings in dry ecosystems depends on the capacity to rapidly expand root systems to increase soil foraging capacity (Canadell et al. 1996, Grossnickle 2005). Root morphology and architecture, i.e. root spatial configuration, reflect the functional division of roots (Hutchings and De Kroon 1994). Fine roots, which have short life spans, are mostly responsible for nutrient and water uptake (Sands et al. 1982, Rüdinger et al. 1994). Conversely, coarse roots have long life span, transport water absorbed by fine roots, are nutrient storage sites and provide anchorage (Kalliokoski et al. 2010, Villar-Salvador et al. 2015). The rooting depth and volume of soil explored by coarse roots determine the capacity of plants to access stable soil water reserves, which is critical for plant survival during dry seasons (Canadell et al. 1996, Padilla and Pugnaire 2007). Hence, the ability of seedlings to simultaneously grow many fine and coarse roots is important for tree performance (Simpson 1990, Grossnickle 2005).

Root characteristics vary widely across plant species and these differences are due, in part, to adaptations to the environmental conditions of plant habitats (Canadell et al. 1996, Lopez et al. 1998, Pregitzer et al. 2002, Comas and Eissenstat 2004). For similar plant growth forms, root system size and the shoot:root proportion tend to decrease along aridity gradients (Jackson et al. 1996, Schulze *et al.* 1996). Root system lateral and downward growth also varies across these aridity gradients and among growth forms (Schenk and Jackson 2002). Low soil temperature can reduce water and nutrient uptake and damage root systems (Toca et al. 2018), which inhibits plant development (Carles et al. 2011). In a global review, Freschet et al. (2017) reported that specific root length (SRL), a proxy of fine root production, was greater in plants from cold and temperate climatic areas than in plants from arid and tropical areas. Consequently, species segregation along aridity and temperature gradients could be driven by differences in root growth and architecture (Canadell et al. 1996, Schulze et al. 1996; Valladares and Sánchez-Gómez 2006, Freschet et al. 2017).

The environmental conditions to which plants are exposed along their life also affects root properties. For instance, soil drought hinders root growth (Heiskanen and Rikala 1998, Torreano and Morris 1998, Sayer et al. 2005) but simultaneously increases resource allocation to roots (Reader et al. 1993). Similarly, when exposed to low temperatures, plants reduce the allocation of resources to root growth and prioritize resource allocation to increase cold stress tolerance (Bigras et al. 2001). Soil fertility is another environmental factor that influences root growth and morphology (Lambers et al. 2008, Wang et al. 2017). Most terrestrial ecosystems are N-limited (LeBauer and Treseder 2008). Higher soil N can increase, reduce or have no effect on the belowground biomass and number of growing roots (Mackiedawson et al. 1995, Trubat *et al.* 2006, Wang et al. 2017) and it usually reduces SRL (Ostonen et al. 2007). The internal N status of the plant can also determine the growth of roots (Forde and Lorenzo 2001, Millard and Grelet 2010, Uscola et al. 2015). Plants remobilize stored N to support new organ growth (Millard and Grelet 2010, Villar-Salvador et al. 2015). Consequently, high N remobilization usually enhances root growth (Uscola et al. 2015, Villar-Salvador et al. 2015). Because N remobilization is a source-driven process (Millard and Grelet 2010), plant N content is a more important driver for root growth than N concentration (Cuesta et al. 2010a). Several studies suggest that both plant and soil N could simultaneously regulate root growth and architecture (Forde and Lorenzo 2001, Forde 2014). Unlike the vast literature on the effect of soil N status on root growth and architecture (Forde and Lorenzo 2001, López-Bucio et al. 2003, Wang et al. 2013), the effects of internal N on root growth dynamics and architecture are poorly known for forest trees. Plant NO_3^- concentration modulated root branching in *Arabidopsis thaliana L. (Heyn)* by determining the timing of lateral root development (Zhang et al. 1999). Similarly, Cuesta et al. (2010a) found that high N content *Pinus halepensis* Mill. seedlings developed a larger root system than low N content seedlings due to a higher number of growing roots. Because N is involved in both plant stress resistance (DeHayes et al. 1989, Saneoka et al. 2004, Villar-Salvador et al. 2013, Taulavuori et al. 2014) and growth (Lambers et al. 2008), plants cannot maximize resource allocation into two main functions simultaneously (Herms and Mattson 1992). Therefore, the influence of internal N on root growth and architecture might be constrained by the adaptive strategies of a given plant species. As far as we know, however, no study has compared how differences in N content affect root growth dynamics and architecture in forest tree species with contrasting ecology.

The objective of this study was to assess how variation in seedling N status affects early root growth dynamics and architecture in three ecologically distinct pine species: *Pinus pinea* L., *Pinus pinaster* Ait. and *Pinus nigra* J.F. Arnold. These species are widespread across southern Europe but occupy distinct habitats and exhibit contrasting growth patterns (Barbero et al. 1998, Climent et al. 2011). Their distribution is segregated along an altitudinal gradient where aridity and temperature vary strongly. *Pinus pinea* thrives at low-altitude semiarid sites under mild winter conditions and long, dry summers (Navarro et al. 2013). *Pinus nigra* mainly occurs in high mountain sites in southern Europe, where low temperature rather than summer drought is the main limiting abiotic factor for plant life (Barbero et al. 1998, Peñuelas et al. 2013). Finally, in Mediterranean-climate areas, *P. pinaster* is usually found at mid-elevation sites in habitats with moderate summer drought and cool winter conditions. The climatic niche of the latter pine species slightly overlaps with that of the other two species (Lafuente et al. 2013). All these trees are closely related, which reduces potential phylogenetic bias in assessing the relationship among root development, plant N content and species ecology (Fitter and Stickland 1991, Pregitzer et al. 2002).

We hypothesized that the effect of N reserves on root growth dynamics and architecture will depend on species ecology. Species from moderate stress environments (*P. pinaster*) will show large differences in root growth dynamics and architecture in response to variation in N reserves. Conversely, species from high stress environments (i.e., drought for *P. pinea* and cold for *P. nigra*) will be less affected, as root growth is more constrained according to their ecological acclimation and the development of stress resistance mechanisms. Moreover, we hypothesized that root growth would be more rapid for those species adapted to drier habitats. To test this hypothesis, we cultivated seedlings of these species under three fertilization regimes, promoting varying N reserves (see picture 2-4, photographical section). Then we transplanted the seedlings into rhizotrons to compare root growth dynamics and architecture.

Materials and methods

Plant material and experimental design

Seeds of the three pines were collected from the Sistema Ibérico Meridional (eastern Iberian Peninsula) provenances (ES07a, ES12 and ES03 for *P. nigra, P. pinaster* and *P. pinea* respectively, Alía et al. 2009). Climate of these provenances and coordinates of seed sources are described in Table S1. Seeds were sown on February 1, 2012 at the Centro Nacional de Recursos Genéticos Forestales "El Serranillo" (Central Spain, 40º40'N, 3º 10'W, 650 m a.s.l.) into thermo-formed plastic trays (Plasnor® 190/300-45, Legazpi, Spain, 45 cells of 300 ml, cultivation density 283 plants$\cdot$m^{-2}). Growing medium was sphagnum peat moss pH=4.7, enriched with 16-10-20 NPK fertilizer, 0.9 kg$\cdot$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 May 17, 2012 seedlings were transferred to the School of Forestry, Technological University of Madrid (40°27'N; 3°43'W, 664 m a.s.l.) where the plants remained outdoors for the remainder of the experiment.

Plants received three fertilization treatments designed to promote a gradient of N reserves (Luis et al. 2009, Oliet et al. 2009): (1) High fertilization and 2) Low fertilization, where each plant was supplied with 150 and 20 mg N, respectively, from May 22 to September 19, 2012, and 3) Fall fertilization, where each plant was supplied with 60 mg N prior to September 19, 2012 plus 40 mg N$\cdot$plant^{-1} during the fall, from September 26 to November 8, 2012. Fertilizer was applied weekly at a constant rate using a water-soluble fertilizer. For fall fertilization, we used only ammonium nitrate, while for the other treatments the fertilizer was 20N–20P_2O_5–20K_2O (Scotts Co., Marysville, OH, USA) with N sources being ammonium nitrate (10%) and urea (10 %). Each fertilization treatment had three trays, resulting in a total of nine trays per species. Trays were randomly allocated on the nursery bench, and their positions were rotated every 15 days. After the fertilization treatments were completed, seedlings were watered regularly until the beginning of the rhizotron experiment.

Root growth dynamics and root system architecture was assessed using rhizotrons (see picture 17-23, photographical section), allowing non-invasive and non-destructive measurements of root architecture and growth dynamics over time (Huck and Taylor 1982). Rhizotrons consisted of methacrylate tubes 1 m in length, 12 cm in exterior diameter, and a wall thickness of 0.6 cm. The bottom end was closed with a perforated PVC lid to allow water drainage and prevent substrate loss. Five randomly selected seedlings per species and treatment were transplanted on April 27, 2013 into the rhizotrons placing the root plug against the transparent wall (one seedling in each rhizotron). The rhizotron was filled with a mix of 2:1 sieved and washed sand and perlite. The lowest 10 cm of the rhizotron was filled with gravel. Rhizotrons were inserted into a second opaque plastic tube, 1.1 m in length and 16 cm in diameter buried in the plot soil to maintain a similar temperature to the surrounding soil, while allowing for easy extraction. The opaque tube was buried with a 60º inclination to force the roots to grow along the transparent wall (Cuesta et al. 2010a) (see picture 23, photographical section). A portion of the opaque tube and the rhizotron protruded above the soil 5-10 cm. To prevent the heating of the top of the rhizotron, the protruding portion of the opaque tube was wrapped in an insulating white sheet. The experiment began with the substrate completely hydrated, mimicking wet soil in spring. During the experiment, seedlings were watered on three occasions with 0.5 L tap water (May 8 and 15, and June 6). A 20N–20P_2O_5–20K_2O (Scotts Co., Marysville, OH, USA) water-soluble fertilizer was supplied at low concentration (7 mg/L) together with the 0.5 L irrigation on June 6, 2013.

Plant nitrogen status evaluation

At the beginning of the rhizotron experiment, six seedlings per species and treatment (two per tray) were randomly harvested and frozen to -20 ºC until processing to determine seedling N concentration. Once defrosted, shoots were cut 5 mm below the cotyledon insertion point and separated into needles and stems, and roots were carefully washed from the growing medium with tap water. Samples were rinsed in distilled water for 3 min to remove any trace of nutrients on plant surfaces. Then, leaves, stems and roots were dried at 60 ºC for 48 h and weighed to assess their mass. The leaves, stem and root 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). Determination of leaf, stem and root N concentration of these samples was done by the standard Kjeldahl method using an auto-analyzer (CFA SAN++, Skalar, Breda, The Netherlands). The N content of organs was calculated as the product of organ mass and its N concentration. Plant N content was calculated as the sum of the root, stem and foliage N content.

Root and shoot growth measurements

Shoot height was measured weekly, beginning after planting until the end of the experiment (11 measurements). Shoot height was measured from a set point 5 mm below the cotyledon insertion scar to the apex of the bud (if present) in *P. nigra* and *P. pinaster,* and to the tip of the shoot in *Pinus pinea*, which did not develop a bud.

Root elongation measurements started 2 weeks after transplanting, when most of the seedlings had some visible roots growing against the rhizotron wall and were made weekly for 10 weeks. The elongation and depth of the root system was drawn on acetate sheets that were placed on the rhizotron wall in identical position on each measurement date (see picture 15-19, photographical section). New roots were identified, and new root growth was recorded with different colors each week. The minimum root length registered on acetates was 0.5 cm and roots that did not grow for more than 2 weeks were considered dead. Root growth dynamics was assessed through image analysis of the roots. Sheets were scanned (Lide 120, Canon, Tokyo, Japan) and analyzed with the software *SmartRoot* (Lobet et al. 2011) to obtain the number and length and of root units per week. This information was used to calculate root system elongation rate, rooting depth, number of growing roots, and total root growth. The root system elongation rate during a time lapse was calculated as the sum of all new root elongations per time unit. The number of growing roots was measured as the roots that showed elongation in the last 2 weeks. Rooting depth at a specific date was measured as the vertical distance from the bottom of the plug to the tip of the deepest root. Finally, the elongation rate per root unit was calculated as the total root elongation rate divided by the number of growing roots.

On July 16, 2013, seedlings were extracted carefully from the rhizotrons by gently removing the growing medium with water (see picture 22, photographical section). Plants were separated into needles, stem, old roots, and new roots, and washed with tap water. We considered new roots to be those that protruded out of the plug into the surrounding growing medium. After separating root fractions, plants were stored in the freezer at -20 ºC. Once defrosted, new roots were scanned with EpsonScan v.304S 10000XL 3.4 (Epson, Tokyo, Japan) and the images analyzed with the software WinRhizo Pro 2007.d® (Regent Instruments, Canada) to obtain the total length, diameter, and volume 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 ºC for 48 h and weighed to assess their mass. The specific root length (SRL) was calculated as the ratio between length and mass of new roots. Finally, nitrogen use efficiency for root growth (NUE) was calculated as the ratio between total root length or total root mass and the N content of the plants at time of transplanting to the rhizotrons.

A relative distance plasticity index (RDPI) for the root system was calculated following (Valladares et al. 2006) using the R package Plasticity (Ameztegui 2017). The index was calculated for seven root characteristics: Root system elongation rate, number of growing roots and depth in the tenth week of growth in the rhizotron, NUE, SRL, total root length, and percentage of fine roots.

Data analysis

Two-way ANOVA was used to analyze data from N content and concentration, total root development, SRL, NUE, and RDPI after checking that the data met the statistical assumptions for the analysis. Data with repeated measurements were analyzed via generalized linear mixed models. Species and N treatments were included in the model as fixed effects and the seedling individual that was subjected to repeated measurements was included as a random effect. While rooting depth data followed a normal distribution, number of growing roots, root elongation rate and the elongation rate per root unit showed a gamma distribution. Data were analyzed with R version 3.3.0 (R Core Team, 2016). *Lme4* package (Bates et al. 2015) was used to perform the linear mixed effect models. Distributions were tested by Shapiro-Wilk and Kolmogórov-Smirnov tests. P-values of the models were obtained using the R package *LmerTest* (Kuznetsova et al. 2017). We considered results significant when P-values were ≤0.05. Differences within species and treatments were determined via post hoc tests in R using the *lsmeans* package (Lenth 2016).

Results

Nitrogen content of seedlings prior to transplant

Overall, N content in *P. pinaster* and *P. nigra* was highest among the high fertilized seedlings and lowest in the low fertilized plants, while fall fertilized seedlings had intermediate values between high and low fertilized plants (Figure 1). However, the pattern was different in *P. pinea* (Species × Fertilization interaction, Table 1): the lowest N content occurred in the low fertilized plants, while the plants with high and fall fertilization showed no statistical differences. On average, *P. nigra* and *P. pinaster* seedlings had a lower N content (56 ± 9 and 55 ± 7 mg, respectively) than *P. pinea* (93 ± 13 mg). Seedling N concentration was higher in *P. nigra* than in the rest of the species and higher in the fall fertilized seedlings compared to the other two treatments, although this pattern varied slightly across species and treatments as shown by the significant Species × Fertilization interaction (Figure 1 and Table 1). Specifically, high fertilized seedlings had a higher N concentration than low-fertilized seedlings in *P. nigra*, while the other species did not show significant differences between these two fertilization treatments.

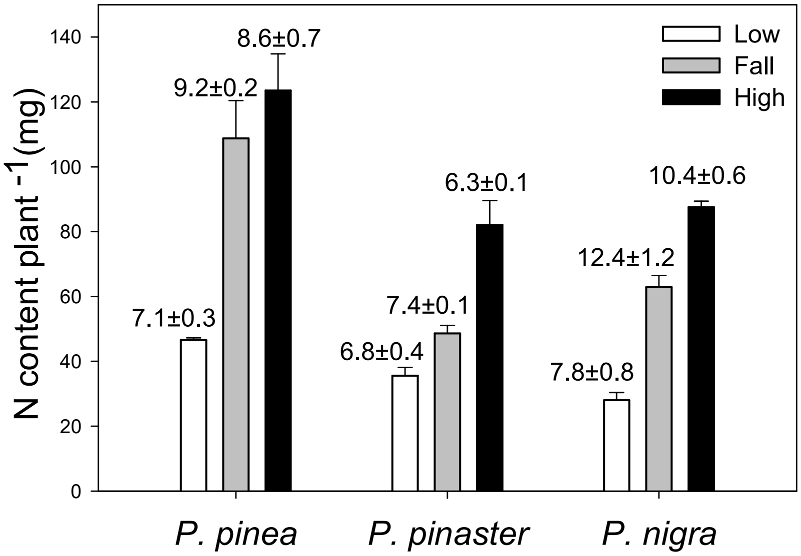

Figure 1. Nitrogen content in one-year-old seedlings of three pine species that were cultivated with three increasing N fertilization regimes (Low, fall and high fertilization). Numbers above the bars are corresponding plant N concentrations (mg·g^{-1}). Data are means ± 1 SE.

Rooting depth

Species differed significantly in rooting depth over time, but these differences depended on N treatment (Species × Fertilization interaction, Table 1). Low fertilized plants had less maximum rooting depth than the fall- and high-fertilized plants in all species except for *P. pinea*, where fertilization treatments showed no differences (Figure 2). On average, *P. pinea* had the deepest roots during the entire study period followed by *P. pinaster* and *P. nigra*, which showed no statistical differences. For instance, in the sixth week *P. pinea* roots were 30% deeper than *P. pinaster* roots and 51% deeper than *P. nigra* roots (Figure 2).

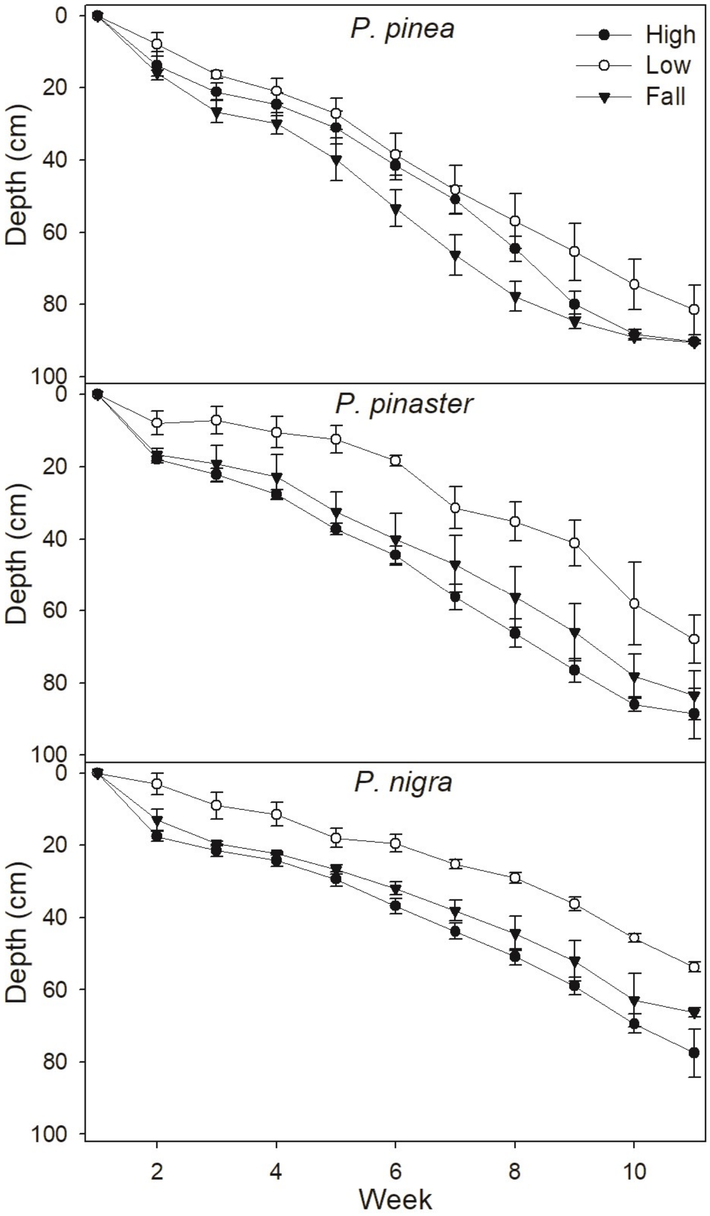

Figure 2. Rooting depth over time in seedlings of three pine species that were transplanted into rhizotrons after being cultivated under three increasing N fertilization regimes (Low, fall and high fertilization). Data means ± 1 SE.

Root growth dynamics

Elongation rate of the root system steadily increased over time, reaching maximum values during the tenth week in most cases and then slightly decreasing. This reduction in elongation rate coincided with the time that many plants reached the bottom of the rhizotron. High and fall fertilization significantly increased the root elongation rate in seedlings of all species compared to low fertilization. However, the magnitude of this effect was greatest in *P. pinaster,* lowest in *P. pinea* and intermediate in *P. nigra* seedlings (Table 1, Figure 3). Root system elongation rate of low fertilized seedlings in *P. pinaster* and *P. nigra* was almost negligible for the first 5 weeks.

Number of growing roots followed a similar pattern to the elongation rate of the root system. The increase in the number of growing roots over time was gradual in *P. nigra* and abrupt in *P. pinaster* and *P. pinea* (starting in week 7 and increasing until at least week 10, Figure 3). The effect of N fertilization treatments on the number of growing roots followed similar patterns among species, although the magnitude of the effect differed (Species × Fertilization interaction, Table 1). High and fall fertilized plants had a greater number of growing roots than low fertilized plants in all species. The number of growing roots in low fertilized *P. pinaster* and *P. nigra* seedlings was almost negligible for the first 5 weeks. The differences between the low fertilized plants and the high and fall fertilized plants were greater in *P. pinaste*r than in the other species. After week 10, the number of growing roots declined in most treatments coinciding with the time that many plants reached the bottom of the rhizotron, similar to the effect observed for root elongation rate.

Overall, elongation rate per root unit peaked between the 6th and 8th week and then decreased until the end of the experiment. The elongation rate per root unit did not differ among fertilization treatments or species (Table 1; Figure S1).

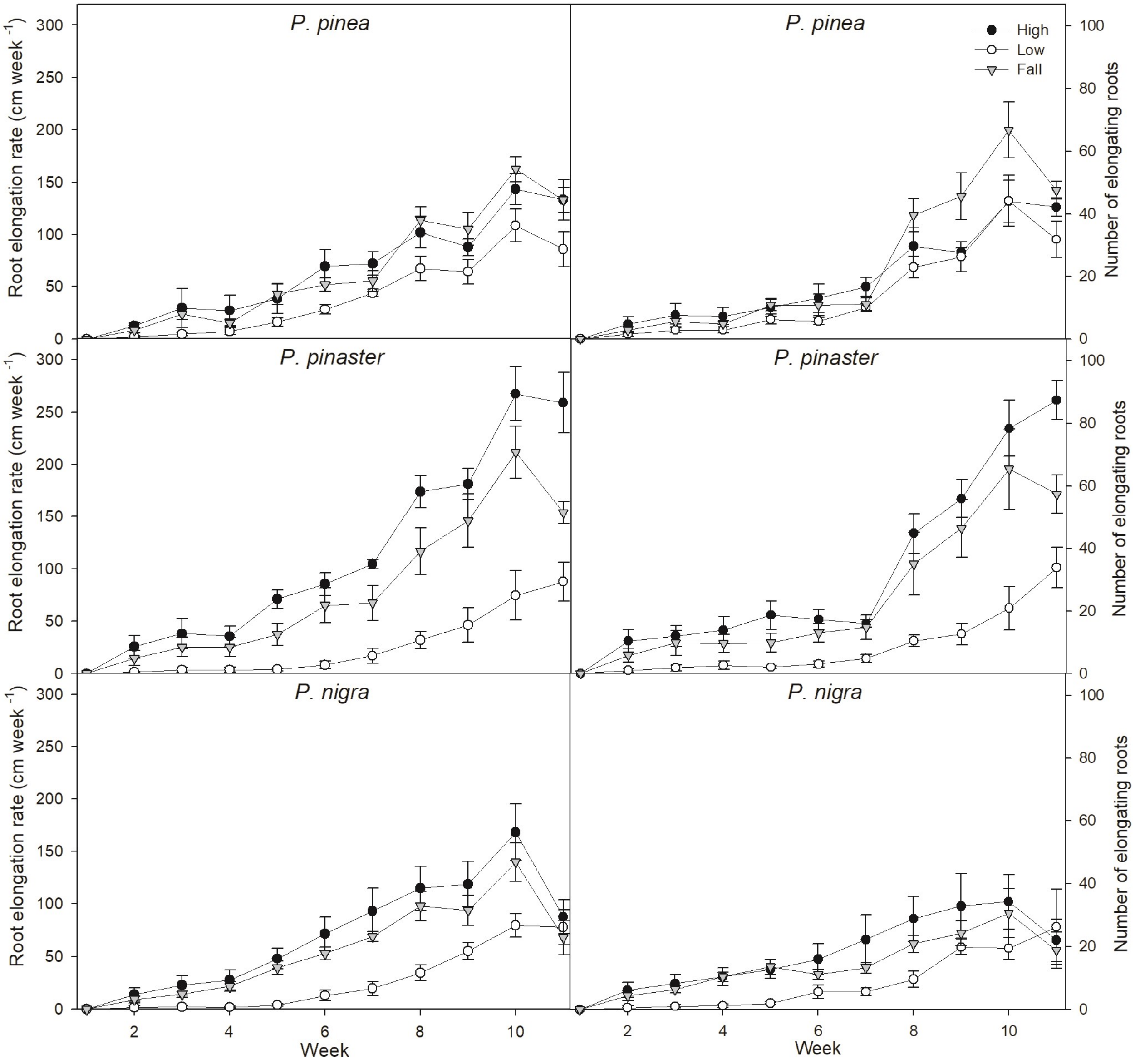

Figure 3. Elongation rate of the root system (left) and number of elongating roots (right) over time in seedlings of three *Pinus* species that were transplanted into rhizotrons after being cultivated under three increasing N fertilization regimes (low, fall and high fertilization). Data are means ± 1 SE.

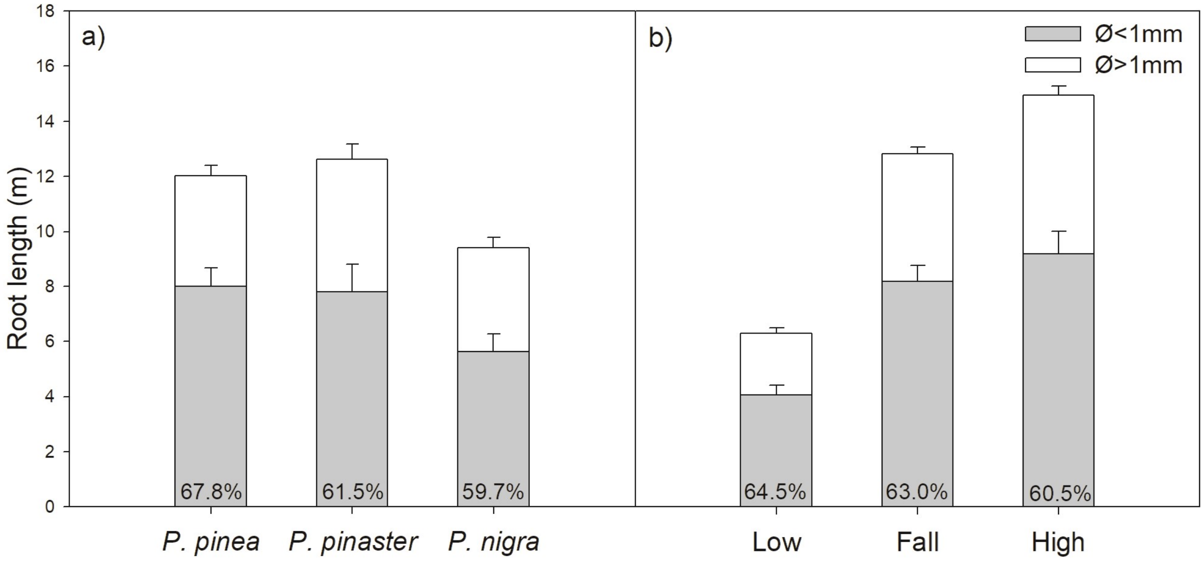

Figure 4. Total root length per root diametrical classes of species (a) and fertilization treatments (b) in one-year old seedlings of three *Pinus* species after 11 weeks growing in rhizotrons following cultivation under three increasing N fertilization regimes (low, fall and high fertilization). Numbers in the grey bars are the percentage of fine root length. Data are means ± 1 SE.

Total root and shoot development

At the end of the experiment, the total length of roots varied among species (Table 1 and Figure 4a). Total and fine root length was significantly lower in *P. nigra* than in the other two pine species, which were similar to each other. Final length of coarse roots was higher in *P. pinaster* than in the other two species, which did not differ from each other. Total root length was also highly dependent on N fertilization for all species (Table 1, Figure 4b). Across species, total root length of low N fertilization plants was less than the half of the root length measured in high and fall fertilized seedlings, which did not differ from each other. Similarly, the length of fine and coarse roots of low-fertilized seedlings was significantly lower than in the other treatments. High-fertilized seedlings, however, developed longer coarse roots than fall fertilized seedlings, but the difference between them was smaller than that with low fertilized seedlings. The fraction of fine roots was higher in *P. pinea* than in *P. nigra* and *P. pinaster*, but no differences occurred among fertilization treatments (Table 1, Figure 4b). Seedlings had very little shoot growth during the experiment. On average, over the 10 weeks, *P. pinea, P. pinaster, and P. nigra* grew 0.9, 1.2, and 2.8 cm in shoot height, respectively.

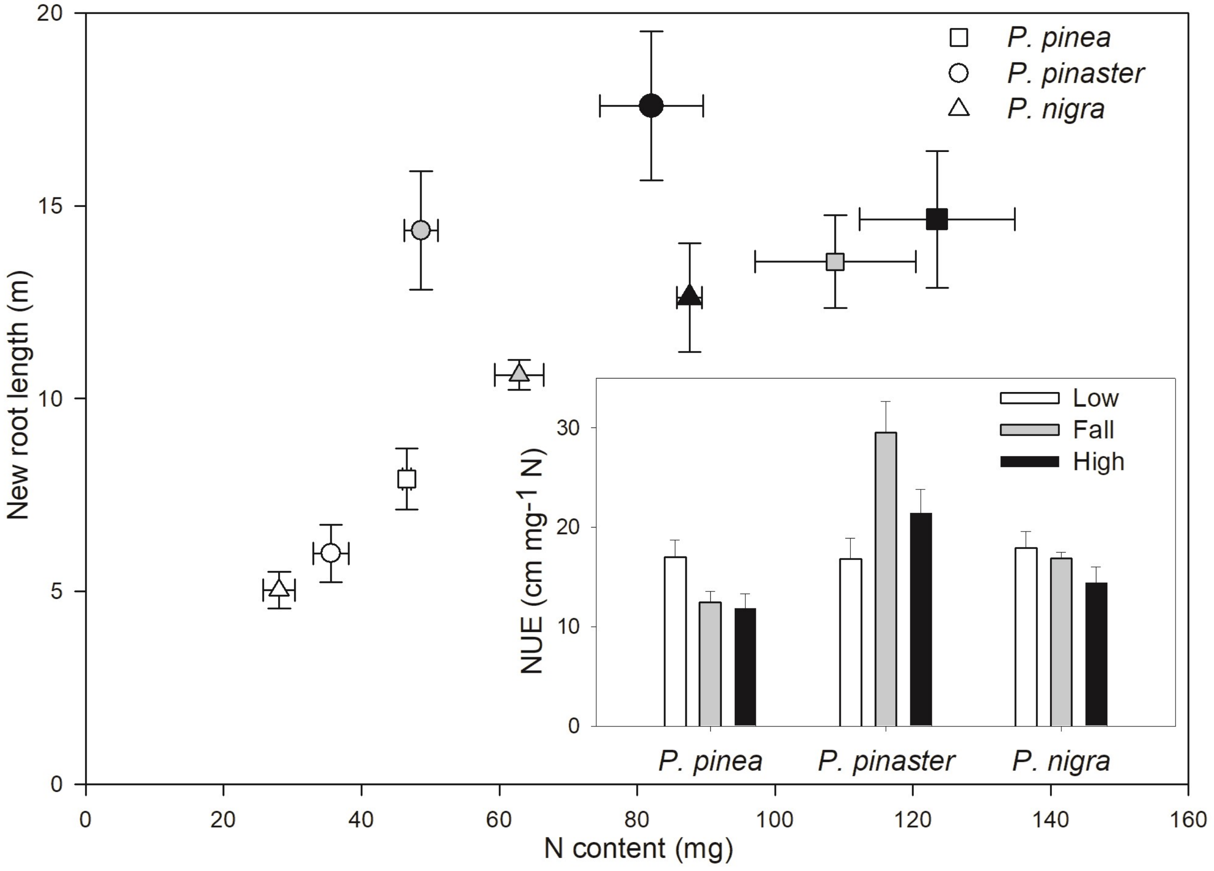

Figure 5: Relationship between new root length after 11 weeks growing in rhizotrons and the initial N content of the plant at the beginning of the experiment. One-year old seedlings of three pine species were cultivated under three increasing N fertilization regimes (low, high fertilization). The inset figure represents the root length nitrogen use efficiency by fertilization treatments and species. Data are means ± 1 SE.

Specific root length and root growth nitrogen use efficiency

On average, low-fertilized seedlings had 60% higher SRL (13.7 ± 1.0 m g^{-1}) than high- and fall-fertilized plants (8.9 ± 0.4 and 8.1 ± 0.5 m g^{-1}, for the high and fall fertilization treatments, respectively), which were not significantly different (Table 1). SRL was similar among species and was not affected by the interaction between species and N fertilization treatments.

Table 1: Statistical results of the effect of species and fertilization treatments on several plant variables. Data are F-values (first seven variables) or Chi-square (last four variables) and P-values in brackets. Bold font indicates significant differences.

	Species	Treatment	Spp. × Treatment
N content	**30.2 (<0.001)**	**63.9 (<0.001)**	**3.72 (0.024)**
N concentration	**22.2 (<0.001)**	**12.8(<0.001)**	**3.29 (0.036)**
Total root length	**5.6 (0.008)**	**38.5 (<0.001)**	1.1 (0.37)
Fine roots length	**5.4 (0.009)**	**23.0 (<0.001)**	0.67 (0.61)
Coarse roots length	**6.0 (0.006)**	**62.5 (<0.001)**	1.83 (0.14)
Percentage of fine roots	**3.77 (0.033)**	1.08 (0.35)	0.06 (0.99)
Specific root length	1.8 (0.17)	**18.5 (<0.001)**	0.47 (0.76)
NUE root length	**17.4 (<0.001)**	3.0 (0.062)	**5.89 (0.001)**
NUE root mass	**24.9 (<0.001)**	**10.1 (<0.001)**	**6.16 (0.001)**
Rooting depth	**114 (<0.001)**	**24.6 (<0.001)**	**16.2 (0.003)**
Number of elongating roots	0.66 (0.71)	**102 (<0.001)**	**32.8 (<0.001)**
Root system elongation rate	1.6 (0.44)	**220 (<0.001)**	**29.9 (<0.001)**
Elongation rate per root unit	2.4 (0.10)	2.6 (0.08)	0.35 (0.83)

The length and mass of new roots at the end of the study was positively related to seedling N content at planting in all species (Figure 5 and Figure S2). However, root length and mass values in *P. pinaster* sharply increased with increasing N content compared to *P. pinea* and *P. nigra*. Consequently, species differed in root length and mass NUE, but these differences depended on fertilization treatments (Species × Fertilization interaction, Table 1). Fall and high fertilized *P. pinaster* seedlings showed the highest NUE values, while little variation with fertilization was observed in the other species (Figure 5 and Figure S2).

Relative distance plasticity index

Species varied significantly in their RDPI values for all the studied plant characteristics (Figure S3 and Table S2). Overall, *P. pinaster, P. nigra,* and *P. pinea* had the highest, intermediate, and lowest RDPI values, respectively.

Supplementary material

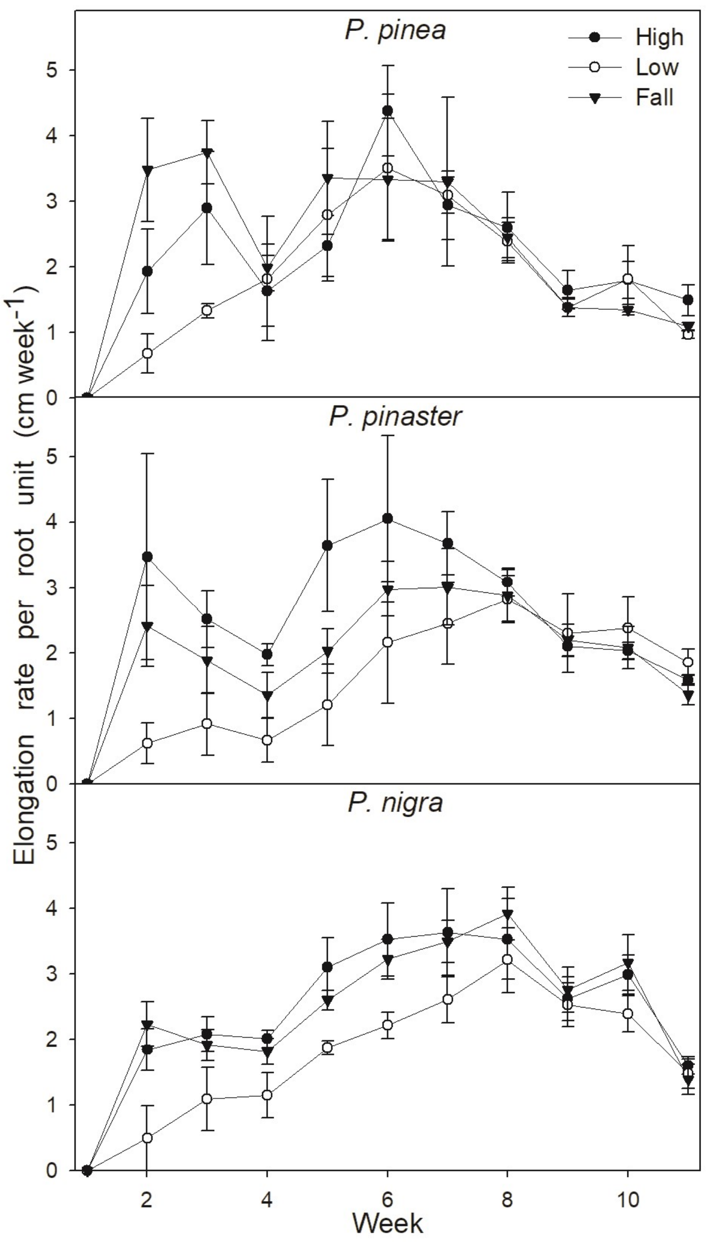

Figure S1. Weekly elongation rate per root unit in one-year old seedlings of three Pinus species that were transplanted into rhizotrons after being cultivated under three increasing N fertilization regimes (low, fall and high fertilization). Data are means ± 1 SE.

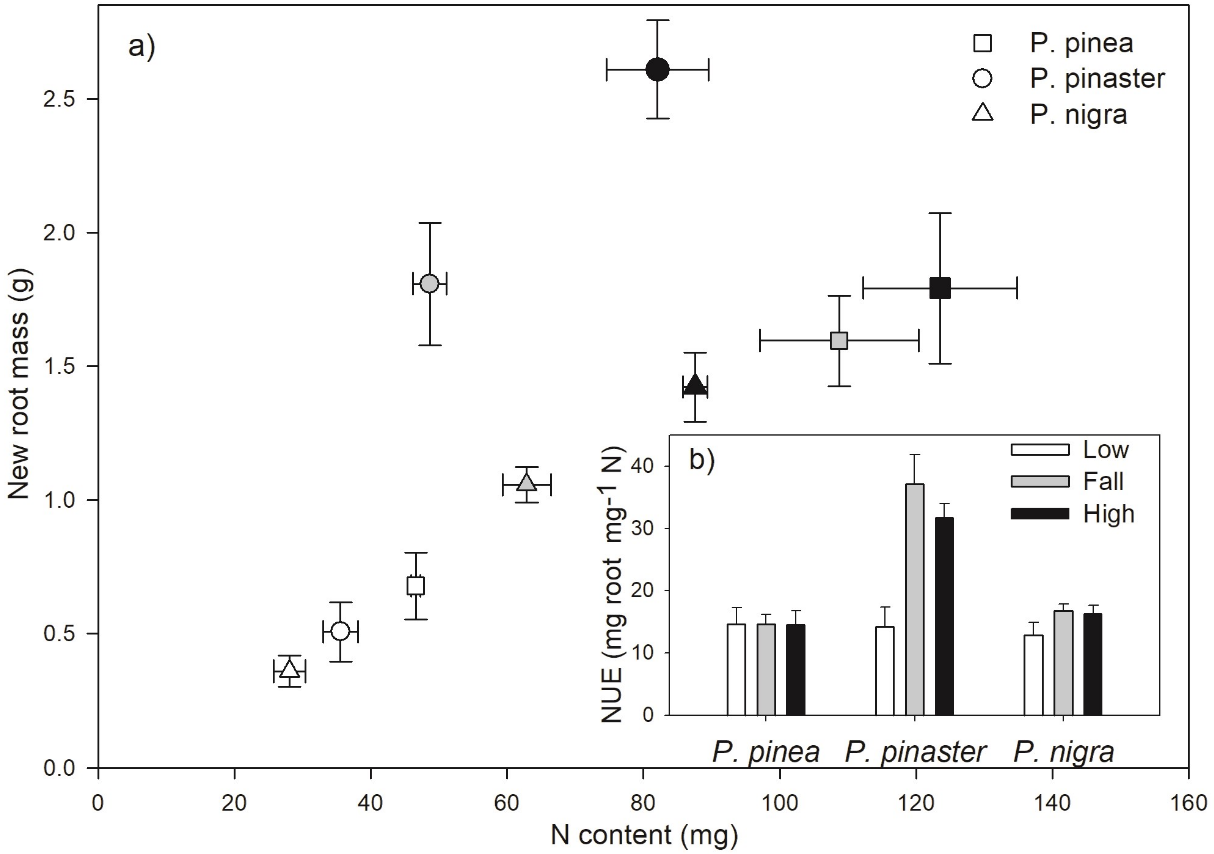

Figure S2: Relation between new root dry mass and the initial N content of the plant at the beginning of the experiment, in one-year old seedlings of three pine species that were cultivated under three increasing N fertilization regimes (Low, fall and high fertilization). The insert figure represents the nitrogen use efficiency by fertilization treatments and species. Data are means ± 1 SE

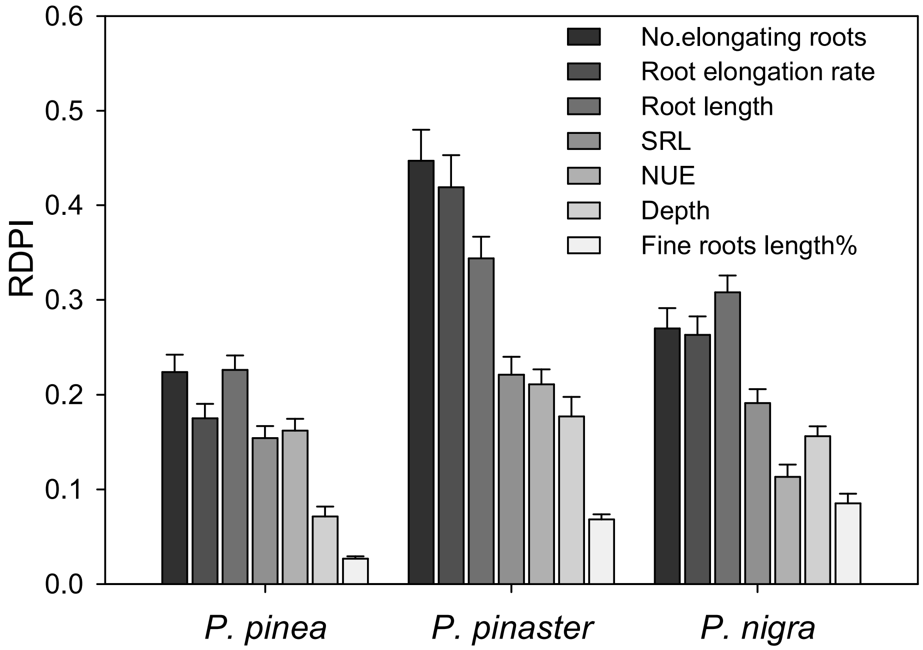

Figure S3: Relative distance plasticity index (RDPI, Valladares et al. 2006) of several variables in one-year old seedlings of three *Pinus* species after 11 weeks growing in rhizotrons following cultivation under three increasing N fertilization regimes (low, fall and high fertilization). Indexes abbreviations were: SRL, specific root length; NUE, nitrogen use efficiency. All variables had significant statistical differences in RDPI among species.

Table S1: Geographic location and climatic characteristics of the provenances where seeds were collected. MAT= mean annual temperature, TCM= mean of the minimum temperatures of the coldest month. Provenance names follows nomenclature in Alía et al (2009).

Species	Provenance	Latitude (N)	Longitude (W)	Altitude (m asl)	MAT (ºC)	TCM (ºC)	Annual rainfall (mm)
P. pinea	La Mancha, ES03	39º12'02''	1º57'59''	675	14.2	0.7	397
P. pinaster	Cuenca, ES12	39º38'44''	1º13'52''	1135	12	-1.5	540
P. nigra subsp. *salzmanii*	Sistema Ibérico Meridional, ES07a	40º15'16''	1º58'22''	1515	10.4	-3.2	894

Table S2: Analysis of variance (ANOVA) of the difference among species in the relative distance plasticity indexes (RDPI) of several root variables in one-year old seedlings of three pine species that were cultivated with contrasting N fertilization regimes. For all variables degrees of freedom = 2.

RDPI variable	Sum of squares	Mean Sq	F	P
Total root length	0.552	0.276	10.3	<0.001
Fine roots length percentage	0.135	0.067	19.7	<0.001
Specific root length	0.168	0.083	4.4	0.012
NUE root length	0.357	0.179	12.6	<0.001
Rooting depth	0.465	0.232	14.6	<0.001
Number of elongating roots	2.086	1.043	22.3	<0.001
Root system elongation rate	2.292	1.146	25.8	<0.001

Discussion

Overall, increasing plant N content (i) increased root elongation rate and final root system size, (ii) decreased SRL and (iii) had varying effects on root growth N use efficiency. Despite their close phylogenetic relatedness, however, pine species showed marked differences in the plasticity of root properties and in root growth NUE to variation in seedling N reserves. In agreement with our hypothesis, we found that differences in root responses are linked to the ecology of these pine species. Specifically, pines species seem to have different strategies for using surplus stored N for root growth depending on the cold and drought stress intensity to which they are exposed in their range. Nitrogen is a critical nutrient for plant growth (Millard and Grelet 2010) and stress resistance (Saneoka et al. 2004, Villar-Salvador et al. 2012, Taulavuori et al. 2014). Therefore, species differences in root properties and in root growth NUE in response to variation in N storage might be driven by a trade-off between allocation of N to growth and to stress resistance (Herms and Mattson 1992).

On one hand, *P. pinaster*, which occurs in sites with less drought and cold limitations than *P. pinea* and *P. nigra,* respectively (Lafuente et al. 2013), had the highest plasticity in root properties with variation in seedling N content. Compared to the other pines species, it is possible that *P. pinaster* prioritizes the allocation of stored N to root growth rather than to stress resistance mechanisms. This could explain why NUE for root growth strongly increased with seedling N content in *P. pinaster*, while it decreased in *P. pinea* and *P. nigra*. Moreover, Uscola et al. (2015) found that fast growth among woody species involves high dependence on stored N. Consistent with this observation, *P. pinaster* showed a higher root elongation rate than *P. pinea* and *P. nigra* (Figure 3), which could also be an indication that *P. pinaster* proportionally could rely more on N reserves for root growth than the other pine species. On the other hand, *P. pinea* and *P. nigra,* the species that live in high stress habitats, had the lowest plasticity in root properties and root growth NUE in response to varying seedling N content. This indicates a low flexibility in the use of surplus of stored N for root growth that, along with low NUE, probably reflects a preference of N allocation to other high N-demanding physiological processes, such as stress resistance. Thus, *P. pinea*, which thrives in semiarid areas with mild winters developed the deepest root system yet showed the lowest plasticity in rooting depth and root elongation rate with variation in seedling N reserves among fertilization treatments (Figure 1). Similar to our findings in *P. pinea*, rooting depth in *P. halepensis,* which thrives in areas where water stress is a major bottleneck for juvenile recruitment (Barbero et al. 1998, Puértolas et al. 2013), was also independent of seedling N content (Cuesta el al. 2010a). Growth of deep and extensive root systems is a key trait for avoiding water stress (Levitt 1980, Grossnickle 2005) but has a high cost in N reserves (Pregitzer et al. 1997) that can, however, help to compensate the plant if it leads to higher drought survival (Padilla and Pugnaire 2007, Villar-Salvador et al. 2012). Finally, *P. nigra*, which inhabits the coldest but wettest areas, had notably lower root plasticity than *P. pinaster* but slightly higher plasticity values than *P. pinea* (Figure S3). In the conifer *Juniperus thurifera* L., which occupies a similar climatic niche to *P. nigra*, high N content seedlings had deeper roots than low N content seedlings (Villar-Salvador et al. 2012). It is possible that the low root plasticity in *P. nigra* compared to *P. pinaster* reflects a prioritization in the allocation of N reserves to frost tolerance physiological mechanisms (DeHayes et al. 1989), such as the formation of antifreeze proteins (Atıcı and Nalbantoğlu 2003, Griffith and Yaish 2004). Although our study reports a link between species ecology and how seedling N reserve variation affects root growth, the limited number of species used constrains the generalization of our conclusions. Therefore, future studies should test our hypothesis with a higher number of ecologically contrasting species.

Differences in the elongation rate and final size of root systems among fertilization treatments and species were more closely related to the number of elongating roots (and consequently to branching intensity) than to the elongation rate of individual roots. This idea is supported by the following facts: increasing seedling N content increased the number of growing roots in all species while the elongation rate per root unit was similar among fertilization treatments (Figure 4), and the temporal variation in the total root elongation rate mirrored the temporal pattern of the number of growing roots (Figure 3). A similar root growth pattern was found in *P. halepensis* seedlings (Cuesta et al. 2010a), suggesting that pines show a common root elongating strategy to enhance soil foraging capacity of root systems (Fitter et al. 1991, Paula and Pausas 2011). This pattern of root growth based on the production of new root units likely maximizes foraging effectiveness of root systems as they can explore more soil volume.

The greater root growth in seedlings with higher N content could be due to increased remobilization of stored N (Maillard et al. 2001, Grelet et al. 2003, Uscola et al. 2015). Nitrogen remobilization is a source driven process (Millard and Grelet 2010) and consequently a higher amount of stored N usually enhances root growth (Uscola et al. 2015, Villar-Salvador et al. 2015). The contribution of remobilized nonstructural carbohydrates to the construction of new fine roots seems to be low in conifers (van den Driessche 1987, Villar-Salvador et al. 2015). High and fall fertilized seedlings had greater leaf mass in all species (data not shown) and tissue N concentration in *P. pinea* and *P. nigra* (Figure 1) than low fertilized plants. Higher foliar N concentration is associated with greater photosynthesis rates (Evans 1989, Reich et al. 1998, Oliet et al. 2013, Zhang et al. 2018). Therefore, it is possible that some of the root growth differences among species may also be explained by differences in photosynthesis.

A major finding of this study is that not only soil N, but plant N status is linked to plant SRL. It is well known that low soil N increases SRL (Ostonen et al. 2007, Wang et al. 2018). In our case, low N-content seedlings had higher SRL than high N-content plants. Other studies have shown that tissue nitrate concentration also controls root branching, root hair growth and nodulation (Zhang et al. 1999, Forde and Lorenzo 2001). However, in these latter studies, differences in tissue nitrate concentration and root growth were measured in plants growing under distinct fertilization rates, which precludes disentangling the influence of internal and external N on SRL. All seedlings in our study were grown in nutrient poor soil (sand and perlite) and fertilization was terminated at least five months before the rhizotron experiment. Therefore, our study lends support to the hypothesis that internal N has *per se* a strong control on SRL.

In contrast to SRL results, variation in seedling N content did not affect the proportion of fine roots in any species. This suggests that variation in seedling N content affected the production and elongation of fine and coarse roots in a similar way, but it likely affected root tissue density (Comas and Eissenstat 2004, Kramer-Walter et al. 2016). At the same time, pine species differed in the proportion of fine roots but not in SRL, which also suggests root tissue density differences among species. *Pinus pinea*, the pine inhabiting the most arid locations, had the highest proportion of fine roots, which might be an adaptation to enhance the uptake of soil resources (Wells and Eissenstat 2002).

Results of this study have practical implications for forest restoration. As roots play a crucial role in seedling survival (Grossnickle 2005, Padilla and Pugnaire 2007) and seedling N content strongly determines root development, our results highlight the importance of building N reserves in seedlings used in forest plantations (Salifu and Timmer 2003, Salifu et al. 2009, Oliet et al. 2013, Villar-Salvador et al. 2015). This explains why many studies show that moderate to high N nursery fertilized seedlings enhance field performance compared to low N seedlings in seasonal dry climates such as in the Mediterranean (Cuesta et al. 2010b, Villar-Salvador et al. 2012). However, results of this study demonstrate that species vary in their root growth responses after N seedling loading, implying that fertilization regimes should be tailored to species' capacity to use N reserves.

Conclusions

Differences in seedling N content drive root growth dynamics and architecture. Overall, an increase in seedling N content enhanced root growth. However, species with close phylogenetic relatedness in our study showed strong differences in root growth plasticity in response to variation in plant nitrogen (N) reserves. We believe that the degree to which increasing seedling N content affects root growth depends on species ecology. *Pinus pinaster*, a species inhabiting moderate to low stress environments, showed higher root growth plasticity and NUE in response to changes in plant N content than *P. pinea* and *P. nigra*, species inhabiting more stressful environments. We have also demonstrated that high N content seedlings developed larger root systems by maintaining a greater number of growing roots rather than by increasing the elongation rate of individual roots, which can be interpreted as a strategy of tested species to maximize foraging efficiency. In addition, although all seedlings grew in an equally nutrient poor soil, seedlings with low N reserves had higher SRL than high N content plants, indicating that internal seedling N status also plays a significant role in specific root length of root systems.

Acknowledgements

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Chapter 4

The interpretation of nitrogen effects on root hydraulics of pine species varies across standardization methods

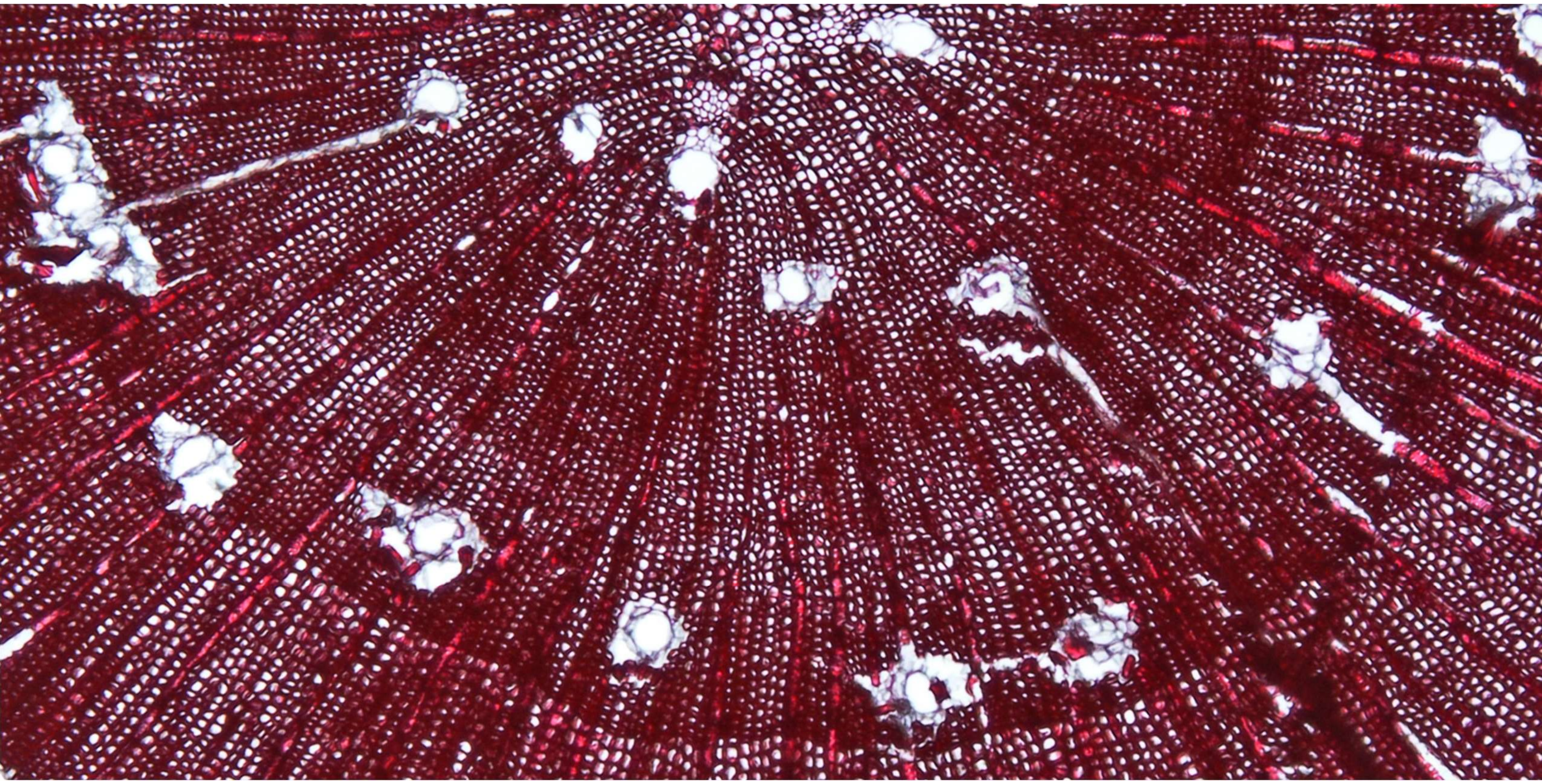

Foto: *Pinus halepensis* by Laura Fernández Pérez

Resumen

Antecedentes y objetivos: El funcionamiento del sistema hidráulico es clave para la supervivencia y el crecimiento de las plantas ya que determina la tasa fotosintética y de transpiración. Las condiciones ambientales pueden influir en la hidráulica de la planta, sin embargo, no existe un consenso claro sobre el efecto de la disponibilidad de nitrógeno (N). El objetivo de este estudio es analizar el efecto del N en las propiedades hidráulicas de la raíz de cuatro especies de pino ecológicamente diferentes.

Métodos: Estudiamos cuatro especies filogenéticamente cercanas, aunque ecológicamente distintas: *Pinus nigra* J.F. Arnold, *Pinus pinaster* Ait., *Pinus pinea* L. y *Pinus halepensis* Mill. Las plántulas se cultivaron bajo regímenes de fertilización de N contrastados (baja: 20 mg N en la temporada de crecimiento; otoñal: 40 mg N en la temporada de crecimiento + 60 mg N en el otoño y alta: 150 mg N en la temporada de crecimiento). La conductancia hidráulica de la raíz se midió con un medidor de flujo de alta presión y los valores se estandarizaron por el área foliar total (conductancia específica foliar, K_l), área transversal del xilema (conductancia específica xilemática, K_s), área total del sistema radical (conductancia específica radical, K_r) y área de las raíces finas (conductancia específica de raíz fina, K_{fr}).

Resultados: El aumento de la disponibilidad de N redujo K_l y K_s, pero aumentó K_r y especialmente K_{fr}. Las especies de pino mostraron diferencias en su eficiencia hidráulica en respuesta al N, pero estas diferencias no siguieron ningún patrón especifico y no se relacionaron con la ecología de las especies.

Conclusiones: El efecto del N sobre la eficiencia hidráulica depende del método de estandarización. El efecto positivo del N en la conductancia radical estandarizada por el área total de la raíz y el de las raíces finas (K_r o K_{fr}) es consistente con los resultados publicados a nivel interespecífico, donde la hidráulica de la planta está positivamente relacionada con el crecimiento y la fotosíntesis. En cambio, la estandarización por el área foliar total y área transversal del xilema (K_l y K_s) reflejó una respuesta antagónica a K_r o K_{fr}. Ello indica que los métodos de estandarización pueden condicionar la interpretación del efecto del N sobre la hidráulica de la planta, lo que puede limitar la comparación de especies y tratamientos.

Palabras clave: Conductancia hidráulica específica, conductancia hidráulica de la raíz, ecofisiología, fertilización nitrogenada, hidráulica de plantas, medidor de flujo de alta presión, *Pinus* spp., relaciones hídricas.

The interpretation of nitrogen effects on root hydraulics of pine species varies across standardization methods

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Abstract

Aims: Plant hydraulics is key for plant survival and growth because it is linked to photosynthesis and transpiration rates. Although the environment influences plant hydraulics, there is no clear consensus on the effect of nitrogen (N) supply. The objective of this study was to analyze the variation in root hydraulics in four ecologically different pine species in response to N supply.

Methods: We studied four closely related, yet ecologically distinct species: *Pinus nigra* J.F. Arnold, *Pinus pinaster* Ait., *Pinus pinea* L. and *Pinus halepensis* Mill. Seedlings were grown under contrasting N fertilization regimes (Low: 20 mg N in the growing season; Fall: 40 mg N in the growing season + 60 mg N in the fall and High: 150 mg N in the growing season). Root hydraulic conductance was measured with a high-pressure flowmeter and values were standardized 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 area of fine roots (fine root specific conductance, K_{fr}).

Results: Increasing N availability reduced K_l and K_s, but increased K_r and especially K_{fr}. The pine species showed differences in their hydraulic efficiency in response to N, but these differences did not follow any specific pattern with N supply and were not related to the ecology of these species.

Conclusions: The effect of N on the hydraulic efficiency depends on the standardization method. The positive effect of N on root hydraulic conductance standardized by either total root or fine roots area (K_r and K_{fr}) is consistent with published results at interspecific level, where plant hydraulics is positively linked to growth and photosynthesis. In contrast, standardization by leaf area and xylem cross-section area (K_l and K_s) reflected an antagonistic response to K_r and K_{fr} indicating that the standardization methods can determine the interpretation of the effect of N on plant hydraulics, which can limit species and treatment comparisons.

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

Introduction

The absorption and transport of water are key physiological processes for plant productivity and survival, especially under water stress (Lambers et al. 2008). Plant hydraulics has evolved concurrently with foliage physiology by placing a limit to the photosynthesis and transpiration rates of plants (Brodribb 2009). Accordingly, photosynthesis is positively correlated with plant hydraulic capacity 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). Consequently, hydraulic traits form part of a unified plant economics spectrum, where high hydraulic capacity is positively linked to a set of attributes associated with high carbon assimilation and fast growth (Reich 2014).

The plant hydraulic system includes roots (facilitates water uptake), xylem (transports the water) and leaves (main evaporative surfaces) (Cruiziat et al. 2002). All these organs vary widely, resulting in large differences in hydraulics among plant species (Mencuccini 2003, Wright et al. 2006, Hernández et al. 2009). Plant hydraulic conductance (K_h), the flow of water through the plant per unit of pressure, scales up with the foliage and root absorption area and the stem water transport capacity of the plants (Cruiziat et al. 2002). Therefore, several standardization methods have been used to compare K_h in plants. Most studies have used the leaf specific conductance (K_l), which standardizes K_h by leaf area. The water transport capacity of an organ can also be standardized by xylem cross-section area (xylem specific conductance, K_s) or by root surface area (root specific conductance, K_r) (Nardini and Tyree 1999, Fichot et al. 2011).

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). Nitrogen 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 increases 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 in the hydraulic system can be interpreted as anatomical and physiological adjustments to meet the water transport capacity to the increasing water demand of N loaded plants, which also tend to augment photosynthesis and transpiration rate (Evans 1989, Oliet et al. 2013, Camarero and Carrer 2017, Zhang et al. 2018). Thus, published literature points out to a coordination among the hydraulic system, photosynthesis rate and overall growth in response to N. Additionally, root growth undergoes significant adjustments with increasing N availability, but changes are not unidirectional. While most studies show that an increase in soil 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 (Kozlowski 1987), and account for a high portion of root surface area, improving the overall water and nutrient absorption of the root system (Green and Clothier 1999, Wells and Eissenstat 2002, Rewald et al. 2011). Changes in fine root proportion, therefore, can have important consequences for plant hydraulics (Brissette and Chambers 1992).

The different K_h standardization methods for calculating and comparing the efficiency of the hydraulic system among plants should integrate all the effects of soil N variation and any other potential source of changes on plant attributes listed above. However, few studies that have analyzed the hydraulic system under varying N availability, and the methods used to assess this relationship have often differed (Lovelock et al. 2004, Samuelson et al. 2007, Wang et al. 2017). A recent meta-analysis by Zhang et al. (2018) reported no effect of N on standardized root K_h; however, many important studies were not included in the analysis. Most studies indicate that K_l and K_s (both K_h measured in roots or shoots) decrease with soil 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). Other studies show that 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 standardization methods and experimental conditions employed, 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 analyze the variation in plant hydraulic properties of four ecologically distinct pine species (*Pinus halepensis* Mill., *Pinus pinea* L., *Pinus pinaster* Ait., *Pinus nigra* Arnold) in response to N supply. Studied species are widespread across southern Europe, are distributed along an aridity and temperature gradient, and show contrasting ecological and growth patterns (Climent et al. 2011, Fernandez-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 to the previous species (Lafuente et al. 2013). These pine species differ in their response of frost tolerance (Toca et al. 2018) to N supply and dynamics of root growth and architecture (Toca et al. 2019) to stored N. Due to their ecological differences, these pine species may have different patterns of biomass allocation 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. We compared the effect of N availability during seedling development on root hydraulic conductance through several standardization methods. 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 propose to standardize root K_h using fine root 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}, will increase in response to N availability in order to match the water transport capacity to the increase in assimilation and transpiration.

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 (Table S1, in Supplementary Material, Alía et al. 2009). Seeds were sown in February 2012 at the Centro Nacional de Recursos Genéticos Forestales “El Serranillo” (Central Spain, 40º 40’N, 3º 10’W, 650 m 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 May 17, 2012 seedlings 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. Plants were assigned to three fertilization treatments: 1) High and 2) Low fertilization, where each plant was supplied with 150 and 20 mg N, respectively, from May 22 to September 19, 2012 (pre-hardening period); 3) Fall fertilization, where each plant was supplied with 60 mg N during the pre-hardening period + 40 mg N supplied in the fall, from September 26 to November 8, 2012. Fertilizer was applied by hand at a weekly constant rate using a water-soluble fertilizer. Fall fertilization extra supply was applied as ammonium nitrate, while for the rest of treatments the fertilizer was $20N–20P_2O_5–20K_2O$ (Scotts Co., Marysville, OH, USA) with N sources being ammonium nitrate (10%) and urea (10%). 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 to field capacity based on gravimetric methods (Timmer and Armstrong 1987). 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. 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 June 20 to September 15, where plants grew under a shading with a 20 % light transmission to reduce evapotranspiration.

Hydraulic conductance and morphology measurements

On April 15, 2013, six seedlings per species and treatment 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. Then, the remaining stem of the root system was connected to a high-pressure flowmeter (HPFM, Dynamax Inc., Houston, TX) after debarking 2 cm. The (see picture 13 and 14, photographical section) method for measuring root hydraulic conductance using the HPFM system was described in detail by Tyree et al. (1995).

Briefly, the HPFM is an apparatus designed to perfuse 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 seconds. The pressure was increased up to 0.5 MPa. The hydraulic conductance (K_h) was calculated from the slope of the linear region of the regression of water flow against of pressure. Later, shoot and roots were frozen to -20 ºC until processing. Once defrosted, plants were washed with tap water and rinsed in distilled water for 3 min. Then, seedlings were separated into needles, stem, and roots. 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 Image J (version 1.46r, Madison, WI, US). 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, Canada) to obtain the total length, diameter, and surface area of roots. Later, roots were differentiated into two categories: fine (≤ 1mm in diameter) and coarse (> 1 mm in diameter) (King et al. 2002). Samples were then dried at 60 ºC for 48 h and weighed to assess their mass. Relative growth rate (RGR) was calculated as:

$$RGR = \frac{Ln(Mass_2) - Ln\ (Mass_1)}{43\ weeks} \qquad \text{……………………………………………(Equation 1)}$$

where $Mass_2$ and $Mass_1$ are final mass and the mass of seedling of the same batch 43 weeks before, respectively. The root maximum K_h values were used to calculate leaf (K_l), xylem (K_s), root (K_r) and fine roots (K_{fr}) specific hydraulic conductance by dividing K_h by total leaf area, xylem cross-section area, total root system and fine root surface area, respectively. Leaves, stems and roots were dried at 60 ºC for 48 h and weighed to assess their mass.

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 10000 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, AT).

Results

Seedling morphology

Nitrogen supply significantly affected seedling growth and biomass allocation; however, for most traits the magnitude of this effect depended on the species (Species × Fertilization interaction, Table 1). High N fertilization consistently increased then content, mass, total leaf area, xylem cross-section area, root surface area and RGR of seedlings compared to low N fertilization (Table 2). *Pinus halepensis* showed the largest differences between high and low N fertilization for all traits, while *P. pinea* showed the lowest difference between high and low fertilization. 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 higher in *P. pinea*, *P. pinaster* and *P. nigra*, respectively. Fertilization also significantly increased the root surface area in all species. However, the differences 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 roots in all species (Table 2). The effect of fall fertilization on growth and morphology varied among species. In all species, traits of fall fertilized seedlings ranged between the values of high and low fertilized plants except for *P. pinea* that showed the highest root surface area with fall fertilization. For some species such as *P. pinaster*, trait values of fall-fertilized plants were closer to low-fertilized plants while for other species such as *P. pinea* values were closer to high-fertilized seedlings.

Pinus pinea and *P. pinaster* had the highest values for all morphological variables except for the root-shoot ratio, while *P. nigra* and *P. halepensis* showed the lowest morphological values and the highest root-shoot ratio. However, *P. halepensis* had higher leaf and root surface area than *P. nigra,* but lower than *P. pinea* and *P. pinaster* (Table 2).

Hydraulic conductance

K_h increased with N fertilization in all species (Table 1, Figure 1). However, in all species low fertilized seedlings showed the lowest K_h values 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 and fall-fertilized plants had slightly but significantly higher K_h than low fertilized plants. K_l and K_s significantly differed among species (Table 1) in the following order: *P. nigra* ≥ *P. halepensis* ≥ *P. pinaster* > *P. pinea* (Figure 2a and 2c). 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 Figures 2a and 2c).

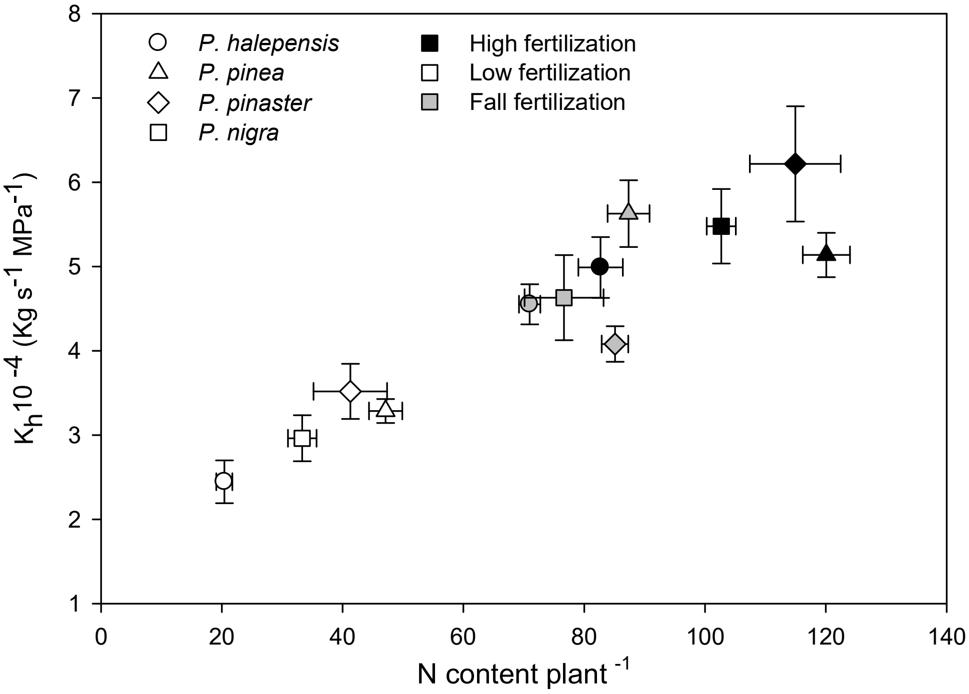

Figure 1: Relationship between seedling N content and hydraulic conductance in four pine species. Seedlings were cultivated under three increasing N fertilization regimes (prehardening low and high fertilization and fall fertilization). Data are means ± 1 SE.

K_r also significantly varied among species, though the differences were small. *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 and 2d). In contrast to K_l and K_s, fertilization increased K_r. Fertilization had a similar effect on K_{fr} and 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_r and 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_r and K_{fr} in *P. pinea* and *P. nigra* than low fertilized plants.

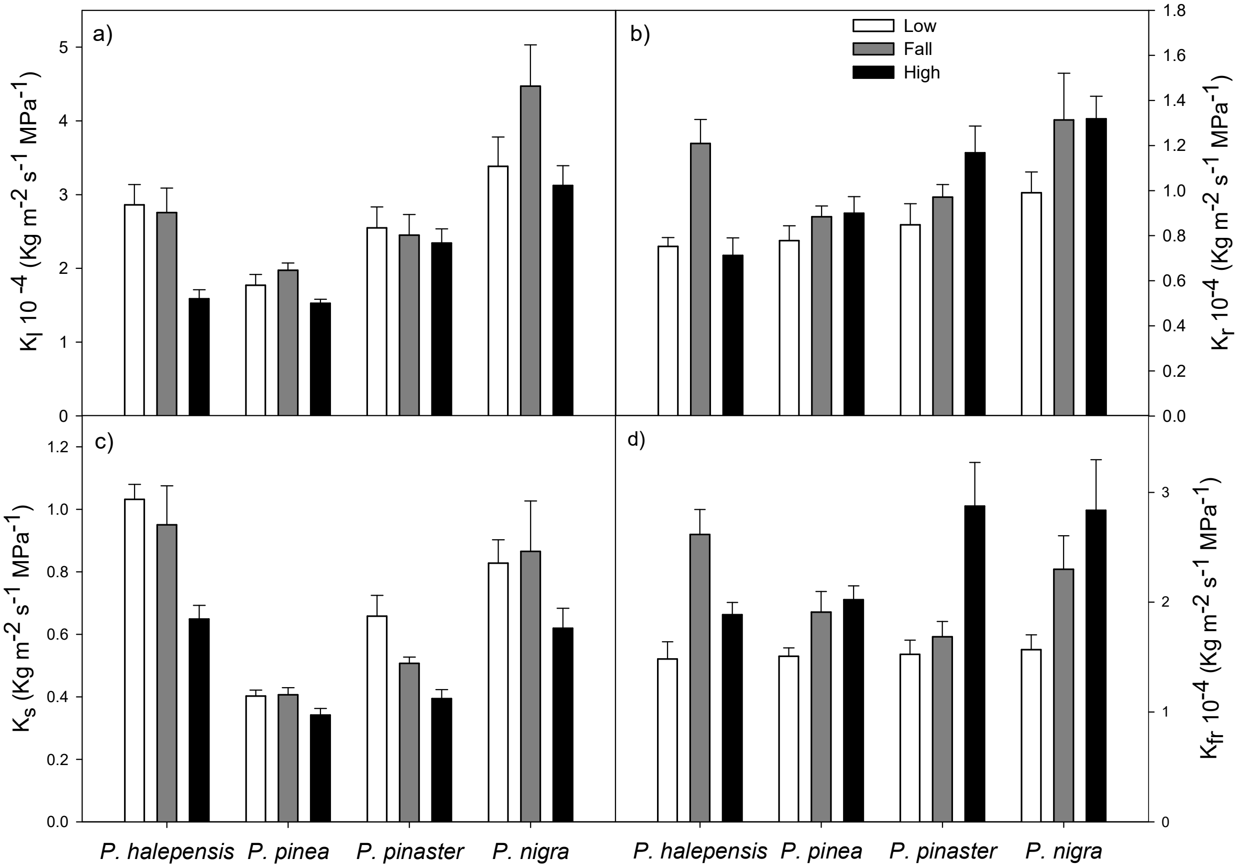

Figure 2: Hydraulic conductance per leaf surface area (K_l) (a), root surface area (K_r) (b), xylem cross-section area (K_s) (c) and fine root surface area (K_{fr}) (d) in one-year-old seedlings of four *Pinus* spp. that were cultivated with three nitrogen fertilization treatments (Low, high and fall fertilization). Data are means ± 1 SE.

Table 1: Statistical results of the effect of species and fertilization treatments on plant variables. Data are F-values and P-values in brackets.

	Species	Treatment	Spp. × Treat
Seedling mass	**39.4 (<0.001)**	**105.5 (<0.001)**	**2.8 (0.020)**
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)**
RGR	**28.2 (<0.001)**	**122.8 (<0.001)**	**3.0 (0.012)**
Root-Shoot ratio	**61.7 (<0.001)**	**40.5 (<0.001)**	1.6 (0.163)
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)**

Table 2: Plant morphological and nutrient traits of one-year-old seedlings of three *Pinus* spp. that were cultivated with contrasting N fertilization regimes. Data are means ± 1 SE.

Specie	Treatment	Seedling mass (g)	Leaf area (cm^2)	Xylem surface (mm^2)	Root surface (cm^2)	Root shoot^{-1}	Fine root surface (%)	RGR 10^2 ($week^{-1}$)
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
	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
	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
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
	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
	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
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
	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
	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
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
	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
	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

Supplementary material

Table S1: Geographic location and climatic characteristics of the provenances where seeds were collected. MAT= mean annual temperature, TCM= mean of the minimum temperatures of the coldest month. Provenance names follows nomenclature in Alía et al (2009).

Species	Provenance	Latitude (N)	Longitude (W)	Altitude (m asl)	MAT (ºC)	TCM (ºC)	Annual rainfall (mm)
P. pinea	La Mancha, ES03	39º12’02’’	1º57’59’’	675	14.2	0.7	397
P. pinaster	Cuenca, ES12	39º38’44’’	1º13’52’’	1135	12	-1.5	540
P. nigra subsp. *salzmanii*	Sistema Ibérico Meridional, ES07a	40º15’16’’	1º58’22’’	1515	10.4	-3.2	894

Discussion

Root hydraulic efficiency in pine seedlings in response to N fertilization showed different results depending on the standardization method. 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}. Apparently, there is an antagonistic effect of N on hydraulic efficiency at the root and shoot level. However, if the purpose of the specific hydraulic conductance is to represent the functional performance of the hydraulic system, the antagonistic output of the standardization methods is controversial. We discuss the effects of N on the organs involved in hydraulic conductance and the details of the different standardization methods below.

According to Ohm's law analogy for plant hydraulics, leaf transpiration rate is directly related to the efficiency of plant hydraulics (standardized K_h), under steady state conditions (Cruiziat at al. 2002). In accordance with the Ohm's law analogy for plant hydraulics, efficiency of K_h across species is positively related to plant gas exchange (Brodribb 2009) and growth capacity (Reich 2014). Therefore, at the intraspecific level and in response to an increase in N availability, it should be expected that the standardized K_h should match the increase in transpiration rate (Zhang et al. 2018). In our study, N increased plant RGR and modified the biomass allocation pattern to the main components of plant hydraulics: roots, stems and leaves (Table 2). Similarly, other studies have shown that high N fertilized seedlings usually have a higher photosynthesis rate (Evans 1989, Villar-Salvador et al. 2013, Oliet et al. 2013, Camarero and Carrer 2017, Zhang et al. 2018), and field survival and growth (Kontunen-Soppela et al. 2000, Oliet et al. 2009, Villar-Salvador et al. 2012) than low N fertilized plants. However, the observed reduction of root hydraulic efficiency with increasing N availability in pine species, estimated as K_l or K_s, seems to conflict with the high growth, metabolism and field performance capacity observed in high N fertilized seedlings. Therefore, results for K_l and K_s at the intraspecific level did not concur with the positive relation between the hydraulic efficiency and other functional attributes linked to plant carbon and water economy (i.e., photosynthesis, transpiration and growth rate) at an interspecific level (Brodribb and Feild 2000, Santiago et al. 2004, Brodribb et al. 2004, Maherali et al. 2008, Choat et al. 2011, Reich 2014).

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

Similar to our findings, other studies have shown a negative effect of N on root K_l (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 a different method 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 a clear trend (Lovelock et al. 2004, Samuelson et al. 2007, Hacke et al. 2010). Considering all the results collectively, we suggest that K_l and K_s may have some limitations for the comparison of hydraulic efficiency. These limitations occur because Kl and Ks do not consider other leaf physiological processes and xylem anatomy features intimately linked to water transport and N availability but that covary with leaf and xylem cross-section area in response to N availability. For instance, plants increase their photosynthesis rate with N availability (Evans 1989, Villar-Salvador et al. 2013, Oliet et al. 2013, Camarero and Carrer 2017), which is contrary to the reduction in K_l at the intraspecific level observed in this and in other studies (see references before). N fertilization also increases water use efficiency (Hernández et al. 2009, Zhang et al. 2018) at an intraspecific level, which is due to stomatal conductance, and consequently transpiration rate, increasing at lower rate than does photosynthesis rate with increasing N supply (Zhang et al. 2018). Therefore, at leaf level stomatal conductance or transpiration rate might be more important drivers of plant hydraulic conductance than leaf area *per se*. However, according to a meta-analysis by Zhang et al. (2018), stomatal conductance is in general little affected by N availability. Consequently, the maximum plant transpiration, calculated by multiplying maximum stomatal conductance (or transpiration rate) by total leaf area, might increase at a lower or similar rate than K_h (and at a lower rate than leaf area) in response to an increase in N availability (Figure 3). Hence the standardization of K_h by plant transpiration, rather than by leaf area, might provide a more precise picture of plant´s hydraulic architecture and likely showing a positive or no effect of N fertilization on hydraulic efficiency. At the same time, high N availability increases the density of conduits per sapwood area (Borghetti et al. 2016) and the diameter of the conduits (Hacke et al. 2010). The conducting potential of a conduit, according to the Hagen-Poiseuille law, is proportional to the forth power of its radius and inversely proportional to water viscosity and length of the conduit (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 conductivity is variable (see Cruiziat at al. 2002); small changes in xylem anatomy and aquaporins with N addition could have a high impact on its conductivity, augmenting the capacity of shoot xylem to supply high demanding leaves of N rich seedlings.

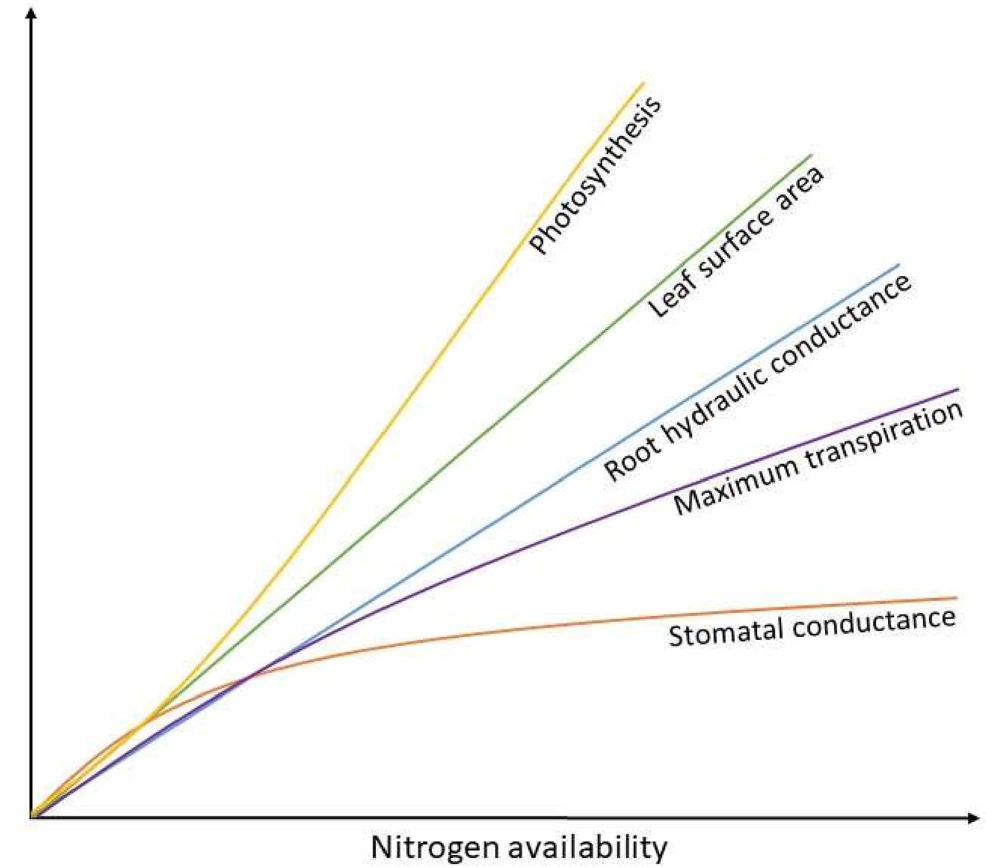

Figure 3: Theorical framework for for the relationship between N and plant stomatal conductance, maximum transpiration, root hydraulic conductance, leaf surface area and photosynthesis along a N availability gradient.

Nitrogen fertilization increased the root surface in all species (Table 2). Contrary to results in leaves, the increase in total and fine root surface was lower than the increase in K_h. This explains the positive effect of N fertilization on K_r and K_{fr}. 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). Accordingly, K_r 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 have a strong effect on 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, standardization of root K_h by total root surface (K_r) may also not account for some other root physiological and structural processes affected by N availability. Specifically, N can reduce the fine to coarse roots ratio (Li et al. 2015) (Table 2) and increase the expression on aquaporins (Hacke et al. 2010), potentially impacting the absorptive capacity and hydraulic conductance of plants. Consequently, differences in the effect on N on K_r observed among most studies seem to stem from variation in how root growth is affected by N availability.

We suggest that the standardization of root K_h by root morphology should consider only the fine roots (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). The positive effect of N on K_{fr} concurs with the higher photosynthesis of high fertilized seedlings (see references above). In addition, results of K_{fr} at the intraspecific level agree with those at the interspecific level, whereby hydraulic conductance is positively related to photosynthesis and growth 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 to explain the higher survival rate of high N fertilized plants over low N fertilized plants under natural conditions (Kontunen-soppela et al. 2000, Oliet et al. 2009, Villar-Salvador et al. 2012).

The four pine species showed differences in their root hydraulic efficiency in response to N (Figure 2). However, no estimation of hydraulic efficiency was related their ecology. This contrasts with results on frost tolerance and root growth dynamics and architecture in these same species, where the effect of N supply was linked to species ecology (Toca et al. 2018, 2019). The standardization methods used in this study to assess the effect of N supply on root hydraulics potentially have the same limitations for comparing species. Species differ in their 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) and root architecture (Pregitzer et al. 2002, Toca et al. 2019). Therefore, more research is needed to develop methods that accurately describe and compare the functional performance of hydraulic systems.

Conclusions

We found that N affected the root hydraulics of pine species. However, the different standardization methods of K_h gave different pictures of the efficiency of the plant hydraulic system. In some cases, the relationships between N and plant hydraulics were antagonistic: high N availability had a negative effect on K_l and K_s, but a positive effect on K_r and especially on K_{fr}. These antagonistic results of the various standardization methods 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.

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. This study provides a rationale to help explain changes in hydraulic properties of seedlings in response to variation in N status.

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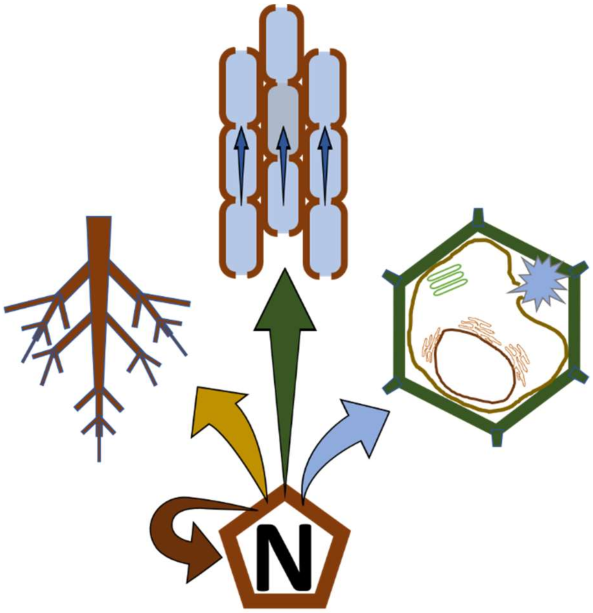
Chapter 5

Thesis discussion



Thesis discussion

This results from this PhD Thesis demonstrate that species ecology plays an important role in determining the effect of N on the capacity for stress resistance in pine species. Many plant species are exposed to environmental stresses such as drought and cold. Plants have developed resistance mechanisms (Bigras et al. 2001, Schulze et al. 2005, Brunner et al. 2015), which permits keeping their fitness under stressful conditions. However, adaptations to stress demand high amount of resources and energy that can potentially limit the outcome of other main plant functions that also require resources and energy. Consequently, plants may show trade-offs between growth and drought or frost resistance (Greer et al. 2000, Darychuk et al. 2012). Our results suggest that this trade-off in pine species occurs because these plant functions are connected by their common demand for N, an essential resource for plant life, which is limited in most terrestrial ecosystems (Figure 1). From an evolutionary point of view, plants may face the dilemma (*sensu* Herms and Mattson, 1992) of how to use N: prioritize the investment of N for fast growth to outcompete neighbors and avoid stress, such as summer drought, or to invest N into metabolic processes to enhance stress tolerance. This dilemma may be particularly important at the seedling stage when plants have low nutrient storage capacity, reduced competitive ability and are highly vulnerable to stress factors.

According to our results, *Pinus halepensis* and *P. pinea* seedlings reduced their frost tolerance with high N supply during the growing season and showed fast root growth (see Cuesta et al 2010 for *P. halepensis*) but low plasticity in root development in response to variation in N reserves (Figure 2). These results support the general hypothesis of the Thesis because the pattern of cold acclimation physiology and root growth dynamics of these pine species match their ecology. *Pinus halepensis* and *P. pinea* thrive in areas where summer drought is long and intense. Survival under arid conditions greatly depends on the rapid expansion of root systems to maximize soil exploration for water and nutrient uptake and rooting depth to reach soil water sources that remain stable during the dry season (Padilla and Pugnaire 2007, Andivia et al. 2018). Accordingly, *P. pinea* prioritized root growth in depth showing similar rooting depths irrespective of the size of N reserves. This result was also observed in *P. halepensis* (Cuesta et al. 2010). Other root characteristics of *P. pinea* and *P. halepensis* (root system elongation rate, number of growing roots and depth in the tenth week of growth in the rhizotron, NUE, SRL, total root length, and percentage of fine roots, see Figure 2) also showed low plasticity in response to variation in N reserves. Root growth has a high investment in carbon and N reserves for plants (Pregitzer et al. 1997), but in dry regions the development of deep and extensive root systems is a key trait for avoiding water stress (Grossnickle 2005) and drought survival (Padilla and Pugnaire 2007, Villar-Salvador et al. 2012). In accordance with our hypothesis, the high nutrient investment due to a high and fixed root development irrespective of seedling N reserves is probably compensated by a higher survival under arid conditions.

Plants showing a less tight control of winter dormancy, can reactivate growth during mild winter spells (Puertolas 2005, Climent et al. 2011, Puértolas et al. 2013). This can potentially extend growth during the wet season, which facilitates seedling establishment before the summer drought onset and increases competition capacity. Low control of winter dormancy implies that high soil N availability can delay the cessation of growth in fall and increase photosynthesis but at the expense of reducing and delaying cold hardening (Hawkins et al. 1995, Heredia-Guerrero et al. 2014). Accordingly, *Pinus halepensis* and *P. pinea*, which have low winter dormancy control (Navarro Cerrillo et al. 2013, Puértolas et al. 2013) decreased frost tolerance in response to high N availability supplied during the growing season. Therefore, these species follow an "opportunistic" strategy of taking advantage of occasional mild conditions in winter that can be reinforced by high N availability (see cessation of shoot elongation and Figure 3, chapter two). This strategy, however, involves a high risk of frost damage (Lockhart 1983) which might become significantly more important due to unusual fall and spring frosts events that are expected to increase with climate change (Augspurger 2013). This probably limits the capacity of these species to colonize cold winter areas (Fernández-Pérez et al. 2018).

In contrast to *P. halepensis* and *P. pinea*, low winter temperature is the main limiting abiotic factor for *P. nigra* in its habitat (Peñuelas et al. 2013). Unlike *P. halepensis* and *P. pinea*, *P. nigra* increased frost tolerance with N availability irrespective of supply timing. Moreover, it showed intermediate values of root development plasticity (Figure 2) in response to variation in N reserves. We interpret the increase in frost tolerance with high N availability as a conservative survival strategy. In this species, N does not reduce frost tolerance because cold hardening is under strong endodormant control (Kreyling et al. 2012, Peñuelas et al. 2013). Thus, the positive relationship between N and frost tolerance likely occurs because plants invest the surplus of N during cold hardening in the synthesis of proteins involved in frost tolerance (Griffith and Yaish 2004), inhibition of the growth of ice crystals (Atıcı and Nalbantoğlu 2003, Lambers et al. 2008) and osmotic adjustment (Zwiazek et al. 2001, Kosová et al. 2007). Water stress is not a main limitation for *P. nigra* forests. Accordingly, rooting depth of *P. nigra* was the lowest among all species and also showed low plasticity (slightly higher than *P. pinea* and *P. halepensis*, Figure 2) in response to variation in stored N. This indicates that *P. nigra* might prioritize the allocation of N reserves to frost tolerance mechanisms (DeHayes et al. 1989) rather than root development.

Nitrogen had little effect on the frost tolerance of *P. pinaster*, but variation in seedling N reserves translated into strong changes in root development (high root development plasticity). Compared to the other pine species, *P. pinaster* seems to prioritize the allocation of stored N to growth rather than stress resistance mechanisms such as frost tolerance or rooting depth. This could explain why NUE for root growth strongly increased with seedling N content in *P. pinaster*, while it decreased in *P. pinea* and *P. nigra* (see Figure 2 in Chapter 3). Unlike *P. pinea, P. halepensis* and *P. nigra*, which are located at opposite ends of the temperature and water availability gradient (see Figure 4 of the general Introduction of the Thesis) and must overcome strong environmental stresses, *P. pinaster* has less abiotic limitations for growth in their habitat (Lafuente Laguna et al. 2013), which permits higher N investment in growth rather than stress resistance.

Nitrogen availability and species ecology is not the only factor affecting plant frost tolerance. The timing of N supply is also critical for frost acclimation. Particularly in thermophilic species, fall fertilized seedlings had higher frost tolerance than pre-hardening high N fertilized plants indicating that frost tolerance is also affected by how N is allocated to major plant functions. It is possible that N taken up during the period of cold hardening and cessation of shoot elongation is mainly allocated to N-rich compounds involved in frost tolerance (Andivia et al. 2012), while N supplied during the pre-hardening season is primarily allocated to growth at the expense of frost tolerance. This trade-off seems to differ among thermophilic and psychrophilic species. In thermophilic species fall fertilization might have a “legacy” effect of the low pre-hardening fertilization (60 mg plant^{-1}) that determines how N is allocated to frost tolerance despite the increase in N availability during fall. We observed a similar legacy effect on root development, where plant N content determined the SRL of roots in seedlings growing in nutrient poor soil (sand

and perlite) despite that fertilization was terminated at least five months before the rhizotron experiment.

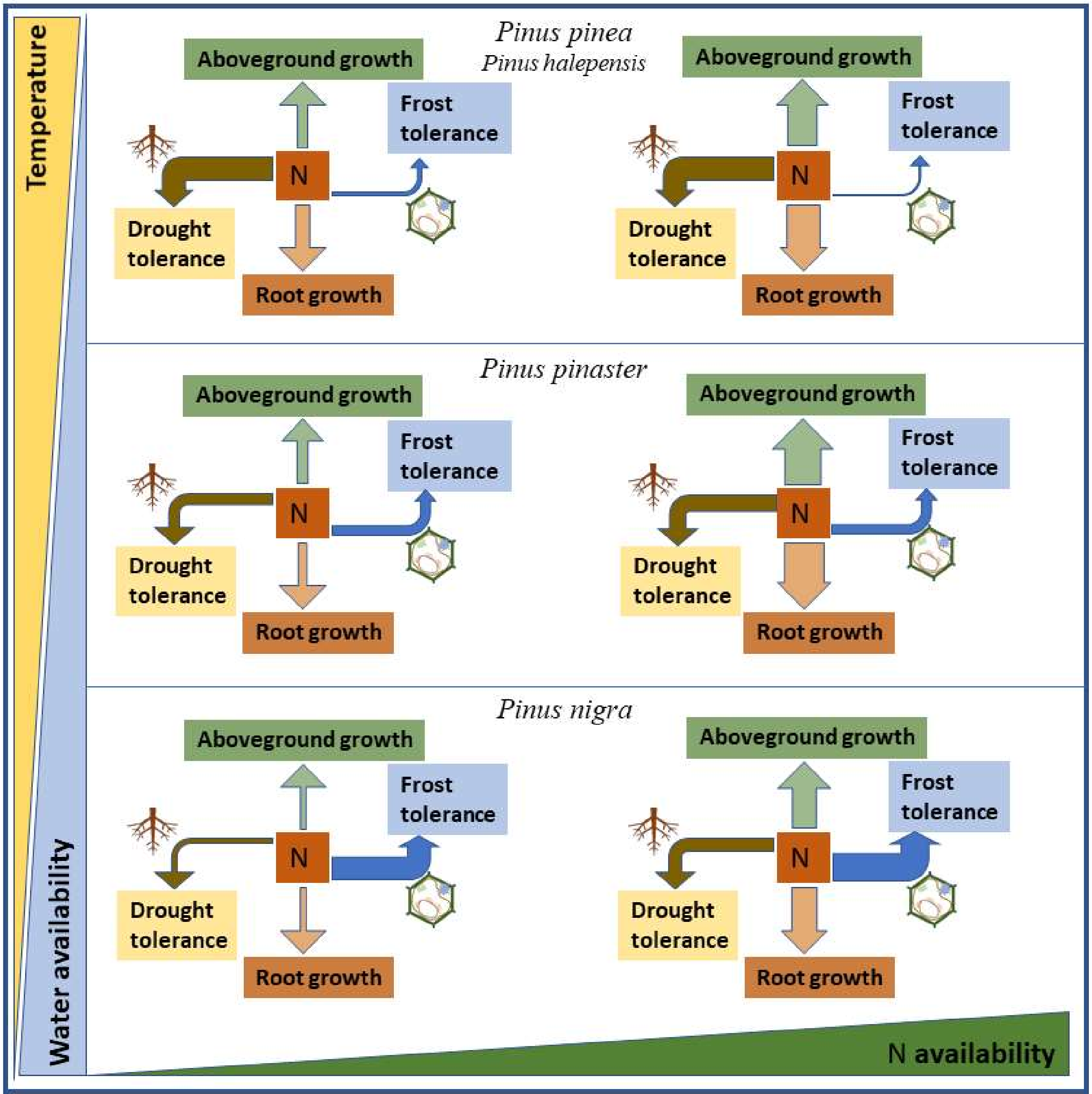


Figure 1: Synthesis of the results on the relationship of nitrogen (N) availability with plant development, stress tolerance, and root growth dynamics and architecture of pine species which distribute across a temperature, water availability and N availability gradient. Arrows indicate the direction of N investment and arrow width represents the magnitude of the process.

Symbols: Frost tolerance (Chapter two) Root growth dynamics (Chapter three)

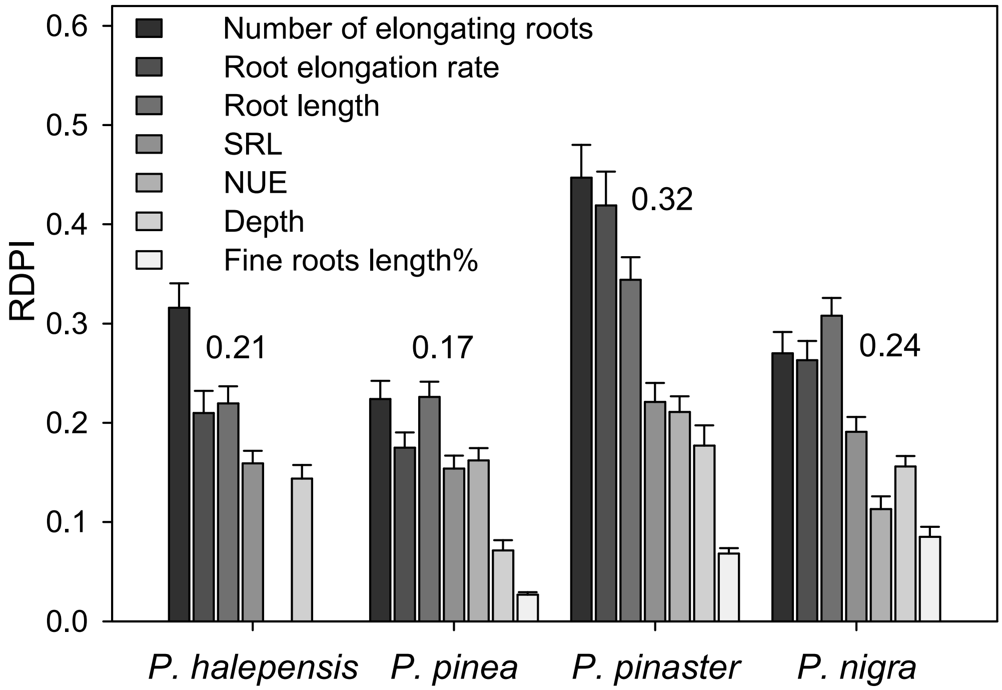


Figure 2: Relative distance plasticity index (RDPI, Valladares et al. 2006) of several variables in one-year old seedlings of four *Pinus* species. The results for *P. pinea*, *P. pinaster* and *P. nigra* belong to the data of the present Thesis (see Chapter 3) while data of *Pinus halepensis* are form Cuesta et al. (2010). The data of P. halepensis do not include NUE and fine root length percentage. Numbers above bars are mean species RDPI and do not include NUE and fine root length percentage. Indexes abbreviations were: SRL, specific root length; NUE, nitrogen use efficiency. All variables had significant statistical differences in RDPI among species.

Contrary to results on root growth dynamics and architecture, as well as frost tolerance, we did not find a clear influence of species ecology on the relationship between N and root hydraulic efficiency. Furthermore, the differences in hydraulic efficiency among species did not concur with their distribution along the temperature or water availability gradient. A possible explanation for the lack of differences among pine species is that their close phylogenetic relatedness implies high similarities in the hydraulic architecture. For instance, the diameter of tracheids is very similar among these species (Fernández-Pérez et al. 2018). Similarly, Creese et al. (2011) found that within *Pinus* species K_S was not related to variations in precipitation in their distribution range. *Pinus* species show a strong phylogenetic signal in some functional traits (He et al. 2012) and plant hydraulics may have evolved conservatively. However more research is needed to develop methods that describe and accurately compare the functional performance of hydraulic systems.

The design of the experiments reinforces the strength of the main findings of the Thesis. All experiments share the same species, we used seedlings cultivated with the same seed lot and experimental conditions and N treatments were the same. This reduced the uncertainties when deriving conclusions from qualitative and quantitative reviews of scientific literature. In addition, the use of four closely related pine species reduces the phylogenetic bias that can occur when comparing phylogenetically heterogenous species (Swanborough and Westoby 1996, Pregitzer et al. 2002, Wright et al. 2007).

The impact of methods applied in this PhD on results

In this Thesis that aimed to assess the response of the functional variables to N availability, we designed the experiments to include more than one methodological approach to study each phenomenon (methodological triangulation). In Chapter two, frost damage was measured through visual damage index (percentage of withered needles; VDI), together with (although not included in the results) the fluorescence method (photochemical efficiency of photosystem II, F_v/F_m, see picture 9) to assess the needle frost damage and the electrolyte leakage (E_L) of root tissue to assess damage in the roots (see picture 7 and 8, photographical section). In Chapter three, we measured root growth dynamics in the rhizotron but also scanned the root system at the end of the experiment and analyzed the images to obtain the total length and diameter of roots. Finally, in Chapter four, we presented and discussed the results of several standardization methods for plants hydraulic conductance. The different methods used in the Thesis resulted in variable results rather than showing a common pattern (Figure 3). The study of Quentin et al. (2015) provides an illustrative example on how the methodological approach can affect the final results of a biological phenomenon. They showed that different methods of quantifying non-structural carbohydrates can yield important differences among laboratories. We discuss some of the results of F_v/F_m and E_L compared to results obtained on the same individuals using VDI.

F_v/F_m was measured in the apical part of the shoot immediately before each freezing test and again 24, 48 and 130h after the end of the test, following Puertolas et al. (2005). Measurements were made with a fluorometer (FMS 2, Hansatech Instruments, Norfolk, UK) after 30 min of dark adaptation using leaf clips. The chlorophyll fluorescence frost damage index (CFDI) was calculated as:

$$\mathrm{CFDI} = \frac{(\mathrm{Fv/Fm})_0 - (\mathrm{Fv\,/Fm})\mathrm{i}_i}{(\mathrm{Fv/Fm})_0} \times 100 \text{ ....(\%)}$$

where $(\mathrm{Fv/Fm})_0$ and $(\mathrm{Fv/Fm})_\mathrm{i}$ are the maximum photochemical efficiencies of photosystem II before the frost test and at time *i* after conducting frost test (*i*=24,48 and 130h), respectively.
Root damage was measured by E_L using the methodology in Earnshaw (1993). E_L is a well-known method widely used to estimate frost damage (Folk et al. 1999, Taulavuori et al. 2014, Fernández-Pérez et al. 2018). Briefly, E_L was assessed immediately before and after the freezing test in each seedling. Between 200 and 300 mg of roots were cut from two opposite sides (one before and the second after the frost test) at mid-height in the plug and washed twice in distilled water for 20 min. Root samples were then placed in a vial with 20 ml distilled water, on an illuminated bench at laboratory temperature and periodically shaken. After 24h the electro conductivity (EC_i) of the solution was measured with a conductivity meter (Crison® CM 35+, Spain) at 25ºC.The electro conductivity of the distilled water was also measured and used as a blank solution. The same samples were then placed in an autoclave at 120 ºC and for 10 min to produce cell membrane destruction and complete electrolyte release (EC_f). Finally, the two values of E_L were used to calculate the relative electrolyte leakage damage index (ELDI)

$$\mathrm{ELDI} = \frac{100(R_t - \mathrm{R}_0)}{(1 - \mathrm{R}_0)} \text{ ....(\%)}$$

Were $R_t=EC_i/EC_f$ of frozen roots and $R_0= EC_i/EC_f$ of roots before the freezing test.

The pattern of frost damage across species and fertilization treatments was very different if measured by VDI, or by F_v/F_m in the case of leaves or E_L in the case of roots (Figure 3). The differences in frost tolerance among species using F_v/F_m or E_L did not concur with their distribution across the temperature gradient. Both methods showed that *P. pinea*, *P. pinaster* and *P. nigra* had very similar root and foliage frost tolerance. In addition, the effects of N on frost tolerance measured by E_L and Fv/Fm showed a pattern that varied among species without a specific trend except for fall fertilization, which clearly increased frost tolerance in most cases. Therefore, the effect of N on frost tolerance and the relative frost tolerance among species varied depending of the method of evaluation. This should be considered when evaluating a controversial relationship such as the effect of N on frost tolerance (Taulavuori et al. 2014).

Faced with the dilemma of the different frost damage results reported by the three methods, we choose VDI over the others because it measures the performance of the seedling as a whole after a frost event. In addition, the results obtained by VDI agree with other frost tolerance studies on the same species (Climent et al. 2009, Fernández-Pérez et al. 2018).

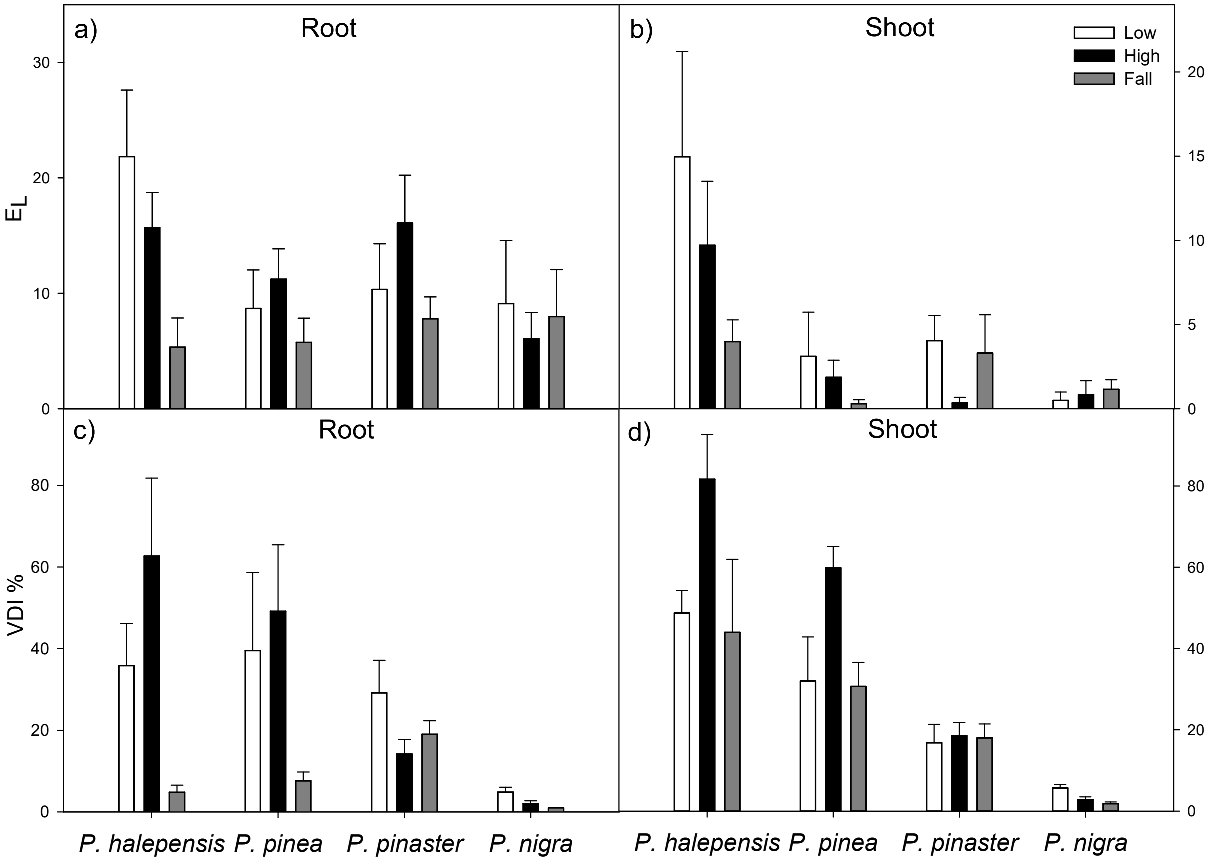


Figure 3: Root (a and c) and shoot (b and) freezing damage in one-year-old seedlings of four pine species. Measurements were taken in January after root freezing test at -10 °C and after shoot freezing test at -15 °C. Damage measurements were made using the Visual Damage Index (VDI, subfigure c and d), the chlorophyll fluorescence damage index (CFDI, subfigure b) and electrolyte leakage (E_L, subfigure a). Seedlings were cultivated with three N fertilization treatments (pre-hardening low and high N, and fall fertilization). Data are means ± 1 SE.

Applied insights

The results of this Thesis have direct implications for forest restoration and seedling cultivation. Nitrogen had varying effects on the frost tolerance, root development and hydraulic conductance of the different pine species, suggesting that dose response studies should be carried out to identify the optimal fertilization regime for each species (e.g., Uscola et al. 2015). However, we found that regardless of ecological differences among species, fall fertilization stimulated the overall highest frost tolerance, highest N concentration and very similar root development to high fertilized seedlings. The positive effects of fall fertilization may be because N taken up during fall is mainly allocated to N-rich compounds involved in frost tolerance, rather than in stimulating new growth during this season. This supports our hypothesis of a trade-off for N allocation to stress resistance versus growth. Nitrogen accumulated during fall fertilization can also be remobilized in spring to support root growth and to promote summer establishment (Villar-Salvador et al. 2012).

Future Research

As a result of this Thesis, we established some new questions for future research:

Although we reported a link between species ecology and how variations in N availability affect plant frost tolerance and root growth dynamics and architecture, the low number of species used limits generalization of our conclusions. Therefore, future studies should test our hypothesis with a higher number of ecologically contrasting species.

Moreover, future comparative studies on the effect of N on frost tolerance should be designed to identify the relationships between N rate, timing and form, on the synthesis of specific N-rich metabolites involved in frost tolerance and whether species differ in the response to these N treatments. In particular, fall fertilization increased seedling performance in most traits we studied compared to high and low fertilization supplied during the growing season.

We suggest exploring the ecological trade-off relationships between the main plant functions and other macronutrients such as phosphorus. This nutrient is also very limiting in some ecosystems and plays a significant role in root growth (Oliet et al. 2011), one of the limiting factors for plants.

We identify an important knowledge gap in appropriately standardizing plant hydraulic conductance. More research and new perspectives are needed for accurately measuring the hydraulic architecture of plants and its relationship with species ecology, biomass allocation, stomatal conductance and water use efficiency.

Finally, this Thesis shows that the influence of N on root development and frost tolerance varies according to species ecology. Therefore, it is possible that the response to N of other ecophysiological processes such as heat tolerance, water relations and herbivory defense may be modulated by the ecology of species. Thus, we recommend exploring these relationships.

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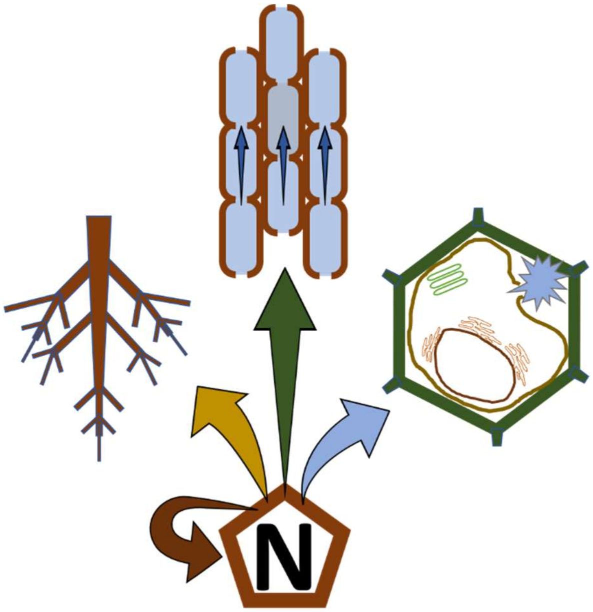
Chapter 6

Thesis conclusions



Conclusions

I. Despite the close phylogenetic relatedness of the studied pine species, and with the exception of hydraulics, the effect of N on seedling function differed among species. Such differences were related to species ecology, suggesting a possible evolutionary trade-off between the allocation of N to growth and to stress resistance.

II. *Pinus* species with distinct ecophysiology of cold hardening showed variable frost tolerance response to N availability during the growing season (pre-hardening) when cultivated under the same environmental conditions. In contrast, N availability during fall (hardening) clearly increased frost tolerance of all four species, highlighting the importance of timing of N availability for development of frost resistance of pine seedlings.

III. Differences in frost tolerance among species and levels of N availability were not explained by differences in organ soluble carbohydrate concentration nor by timing of shoot elongation cessation.

IV. Frost tolerance dynamics during hardening and the effect of N availability on frost tolerance varied between roots and shoots. While shoots increased in frost tolerance over winter, the frost tolerance of roots remained unchanged. In addition, the relationship between N availability and shoot frost tolerance was altered along the hardening period, but only in thermophilic species. These contrasting effects of N on the frost tolerance of roots and shoots among pine species may be a result of ecophysiological differences among species and the frost acclimation physiology of specific organs.

V. Seedling N content drives root growth dynamics and architecture. However, species showed strong differences in root growth plasticity in response to variation in plant N content, as related to species ecology. *Pinus pinaster*, which inhabits moderate to low stress environments, showed higher root growth plasticity and root growth N use efficiency in response to changes in plant N content than *P. pinea* and *P. nigra*, which inhabit more stressful environments than *P. pinaster*.

VI. High N content seedlings developed larger root systems by maintaining a greater number of growing roots rather than by increasing the elongation rate of individual roots. This can be interpreted as a strategy for maximizing foraging efficiency in soil.

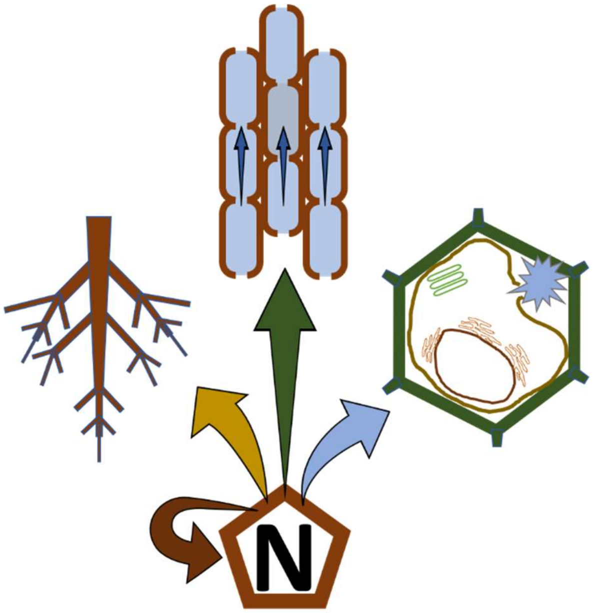
VII. Seedlings with low N reserves had higher specific root length than high N content plants, indicating that internal seedling N status plays a significant role in the architecture of fine roots.

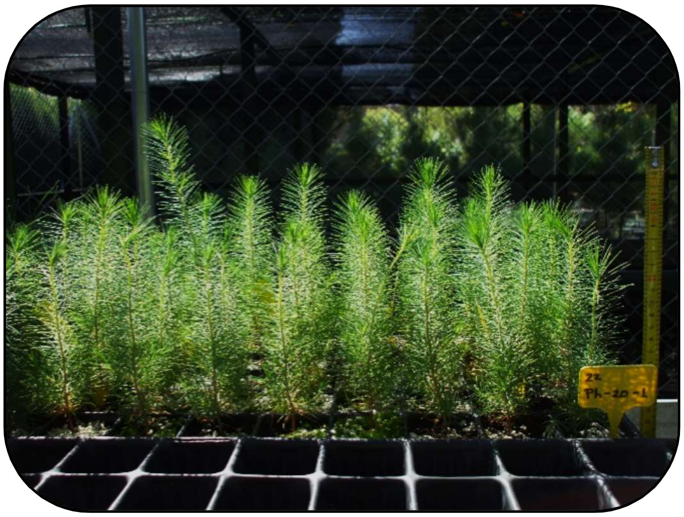
VIII. N availability affected the root hydraulics of pine species. However, the different standardization methods of root hydraulic conductance -K_h- (*i.e* by leaf area, xylem cross section area and root area) gave different pictures of the efficiency of the root hydraulic system. High N availability had a negative effect on specific leaf (K_l) and xylem conductance (K_s), but a positive effect on specific root conductance (K_r) and especially on specific fine root conductance (K_{fr}). These antagonistic results of the various standardization methods were the result of different biomass allocation patterns of plant fractions with N fertilization.

IX. The results of the different methodological approaches used to analyze each phenomenon, indicate that the impact of methods on the results should be considered, especially when studying controversial relationships such as N availability and functional responses of plants.

Chapter 7

Photographical section





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1-2 Nursery seedling production, 3-4 Morphological differences among seedlings *P. halepensis* (3) and *P. nigra* (4) grown under low, fall and high fertilization (respectively from left to right).

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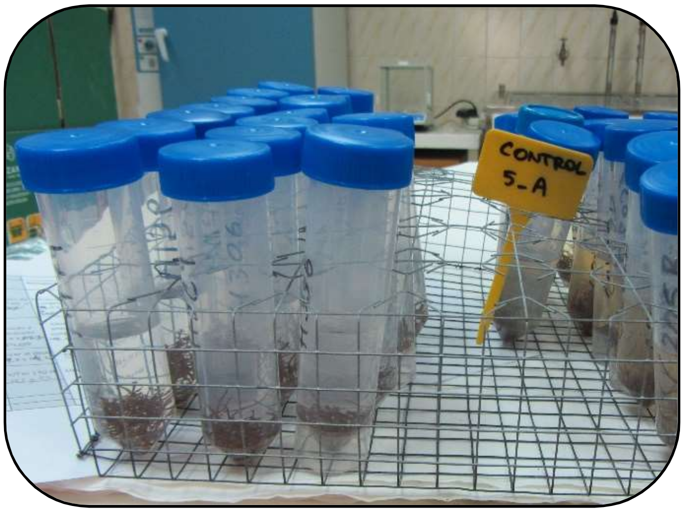

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5-6 Shoot and root freezing test, 7-8 Root preparation for electrolyte leakage, 9 Photochemical efficiency of photosystem II measurements (F_v/F_m)


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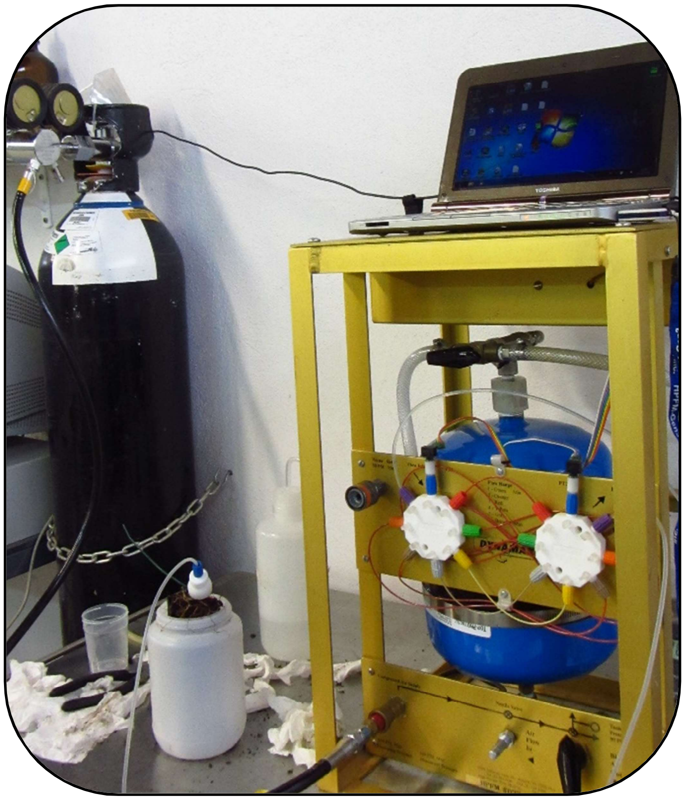
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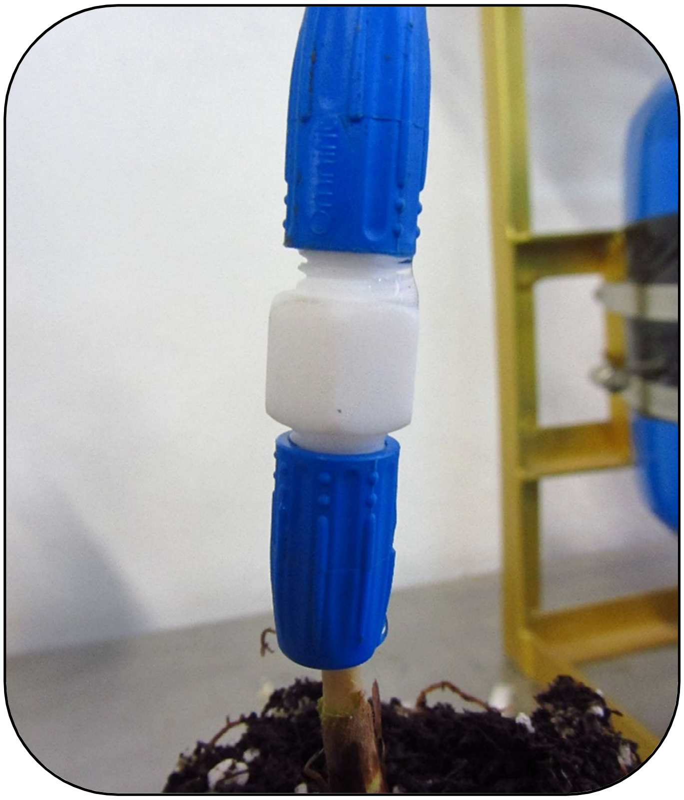
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10-12 Frost damage evaluation through visual damage index (VDI) two months after the freezing test, 13-14 Hydraulic conductance measurements with high pressure flow meter

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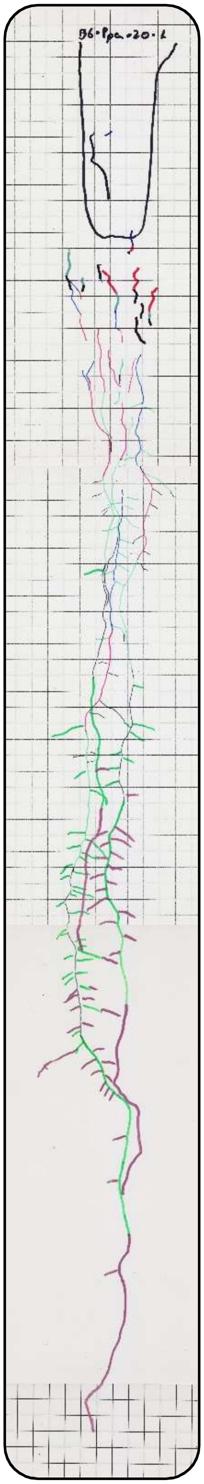

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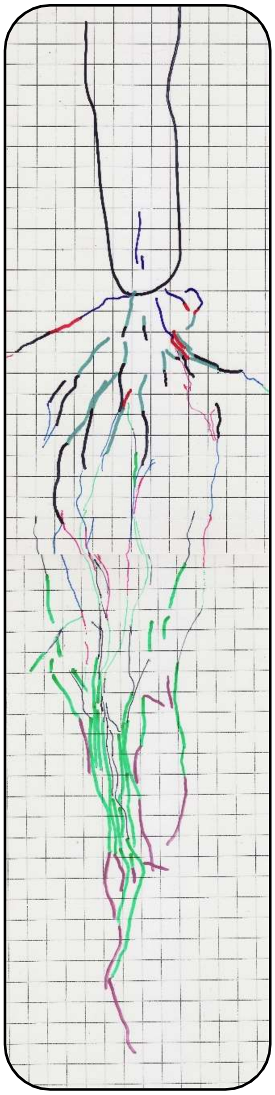

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15-16 and 18-19 Root system development at the end of the rhizotron experiment and the correspondent acetate sheets, 17 Shoot morphological measurements

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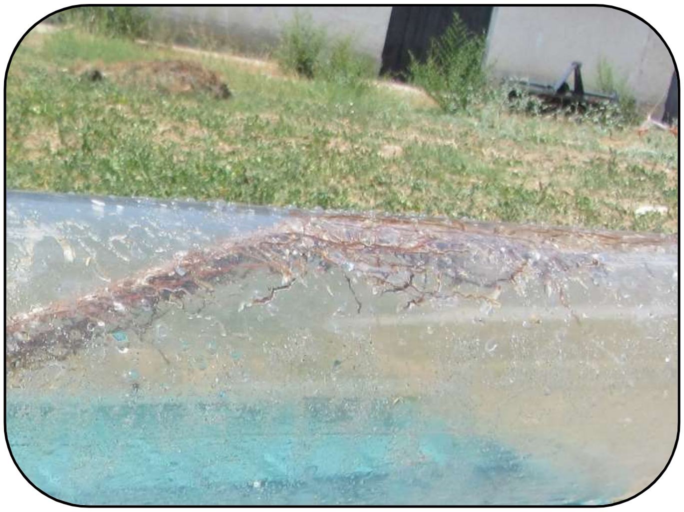

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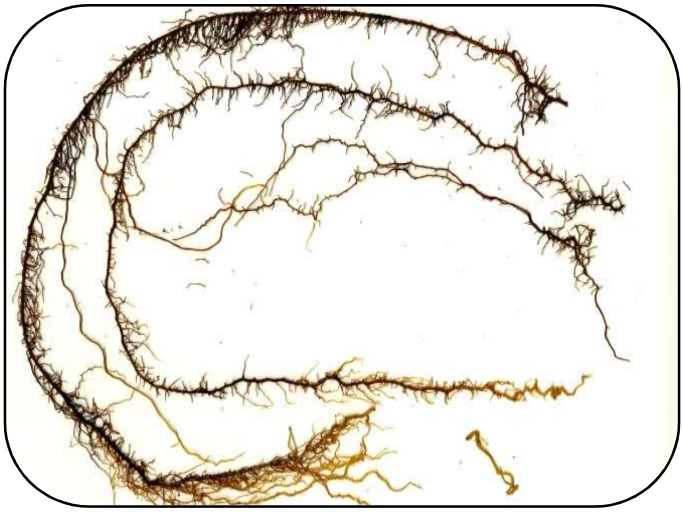

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20 Development of new roots after 10 weeks of growth in the rhizotrons (*P. pinea*), 21 Rhizotron field experiment 22 Root extraction from the rhizotron with water, 23 Roots growing along rhizotron' transparent wall, 24 New roots

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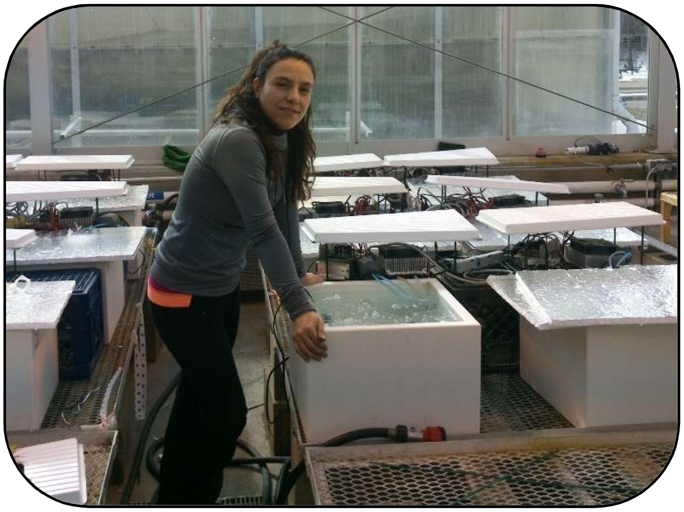
27 Regeneration and Restoration Silviculture Laboratory meeting with Dr. Douglass Jacobs
28-29 Judit Maroto and Luis Fernando Benito-Matías preparing seedlings for freezing test.



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30 Dr. Mercedes Uscola, 31 Rodrigo Martinez Catalan, 32 Best family

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