Ecophysiology of nitrogen in Mediterranean plants: strategies of nitrogen forms absorption, functional responses, and use of reserves for growth

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Mª Mercedes Uscola Fernández

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Ecophysiology of nitrogen in Mediterranean plants: strategies of nitrogen forms absorption, functional responses and utilization of reserves for growth

UNIVERSIDAD DE ALCALÁ, PATRIMONIO DE LA HUMANIDAD

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hace constar:

que el trabajo descrito en la presente memoria, titulado "Ecophysiology of nitrogen in Mediterranean plants: strategies of nitrogen forms absorption, functional responses, and use of reserves for growth", ha sido realizado dentro del Programa de Doctorado "Ecología, Conservación y Restauración de Ecosistemas" (D330), reuniendo todos los requisitos necesarios para su aprobación como Tesis Doctoral, por acuerdo del Consejo de Departamento celebrado el día 03 de Junio de 2013.

Alcalá de Henares, 04 de Junio de 2013.

Dr. Gonzalo Pérez Suárez

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hace constar:

que el trabajo descrito en la presente memoria, titulado "Ecophysiology of nitrogen in Mediterranean plants: strategies of nitrogen forms absorption, functional responses, and use of reserves for growth", ha sido realizado bajo su dirección por Mª Mercedes Uscola Fernández 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.

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Madrid, 04 de Junio de 2013.

Dr. Juan A. Oliet Palá

La Tesis doctoral que se presenta se desarrolla amparada con una beca FPU del Ministerio de Educación y la mayor parte de sus objetivos se desarrollan en el marco del proyecto AGL2006-12609-C02-01/FOR ENCINUT "*Optimización de la nutrición de la encina en vivero: bases fisiológicas, técnicas de fertilización e implicaciones post-transplante*", el proyecto CGL2007-60533/BOS "*Efectos de la revegetación (pasiva y activa) en la dinámica y diversidad de plantas leñosas y aves en paisajes agrarios*" AGL2011-24296/FOR ECOLPIN "*Tolerancia y estrategias ecofisiológicas de los pinos ibéricos durante la fase juvenil en respuesta al estrés hídrico, las bajas temperaturas y la disponibilidad de nutrientes*", y el Programa REMEDINAL-2 S2009/AMB-1783, cofinanciado por la Comunidad de Madrid y el Fondo Social Europeo.

Cover fotography: Mediterranean oak forest in Retiendas (Guadalajara)

By: M. Uscola

One of the first books that I read at the beginning of my PhD was "stable isotope ecology" of B. Fry, and I want to reproduce a fragment of the introduction, which encouraged me during my PhD: *Every scientist is an amateur to start with. ...when a difficult problem was being discussed, Thomas A. Edison said "it was too difficult for any specialist. It would be necessary to wait for some amateur to solve it". In other words amateurs are great for their new thinking and initiatives.*

Let us be grateful to people who make us happy, they are the charming gardeners who make our souls blossom.

Marcel Proust

Agradecimientos

Cuando comienzas la Tesis estás lleno de energía y ganas de aprender. Sin embargo, no siempre es fácil mantener ese nivel de alegría y desempeño. Yo no hubiera sido capaz de lograr llegar hasta aquí sin toda la gente que me ha rodeado, brindándome energía cuando a mi me ha faltado y apoyo cuando lo he necesitado. Llegados a este punto no puedo separar lo que ha sido mi experiencia profesional de la parte mucho más grande e importante que ha sido mi experiencia personal. Por todo ello, son muchas las personas a las que debo y quiero agradecer, y reconocer su contribución a esta Tesis.

En primer lugar quiero agradecer muy especialmente a mis padres científicos Pedro Villar-Salvador y Juan A. Oliet-Palá. Ambos invertisteis (y lo seguís haciendo) muchísima energía, tiempo y dedicación en enseñarme y formarme como investigadora. Me habéis enseñado a perseverar, a focalizar mis esfuerzos, a orientarme entre mareas de datos y detectar los resultados "sexys", pero sobre todo a apasionarme con la ciencia como hacéis vosotros. Pedro, apostaste por mí desde el principio, aún cuando las posibilidades de obtener beca no parecían ser muy elevadas. Recuerdo con mucho cariño y una profunda sonrisa el e-mail que me enviaste de respuesta desde Inglaterra cuando me dieron la beca FPU, "*¡¡¡cojonudo!!!*" el primer taco enfático que te he visto utilizar, y el subsiguiente jarro de agua fría "*¡¡¡ahora puedo sacar el látigo!!!*". Me has permitido ser autónoma y responsable única de gran parte de mi tesis, logro que no hubiera podido alcanzar sin tu desempeño como director. Nunca olvidaré las jornadas encerrados en el despacho discutiendo como homólogos sobre los resultados, perdidos entre artículos y dibujando en la pizarra esquemas, gráficas, etc. Juan has sido parte importante de mi formación.

Siempre has estado disponible para encerrarnos con revisiones interminables, aun pese a las montañas de trabajo que os suelen rodear. Tu distinta forma de abordar las correcciones y tu otra perspectiva me han aportado nuevos puntos de vista y una visión más amplia. No has dejado que desfallezca ante las negativas en revistas o ante fechas límite y me has alentado y apoyado en múltiples ocasiones.

Mi pequeño núcleo familiar científico se incrementa con mis hermanos en ciencia Norberto Heredia y Judit Maroto. Gracias a vosotros he tenido la oportunidad de sentirme parte de un equipo en el que el trabajo y el apoyo han sido recíprocos. Norber, has sido mi co-sufridor de experimentos, y aprendizaje. Juntos nos enfrentamos a la primera comunicación oral en un congreso, a las tomas de datos en campo y a mil otras experiencias. Gracias a ti he podido desarrollar labores ajenas a mi tesis, pero sobre todo me has integrado como parte de tu vida como amiga, aún en la actual distancia. Judit eres como un espejo de cómo me veía cuando empecé la tesis, mirándote me doy todavía más cuenta del largo camino que he recorrido y todo lo que he ganado al hacerlo. Tu entusiasmo de "amateur" renueva mis energías.

Además, he tenido la oportunidad de crecer con investigadores ajenos a la Universidad, del Centro Nacional de Mejora Forestal "El Serranillo" (MAGRAMA) y del Real Jardín Botánico Juan Carlos I de Alcalá de Henares. Desde El Serranillo, y concretamente bajo la dirección de Juan Luis Peñuelas, me han apoyado siempre que lo he necesitado y en múltiples facetas, han colaborado dejándome utilizar sus instalaciones, cediéndome las plantas que con tanta dedicación cultivan y me han permitido acceder a instrumental y equipos de laboratorio inalcanzables de otra forma, como el IRGA o sus magníficos equipos de microscopía, todo ello con la más profunda generosidad. De entre este conjunto de profesionales, muy especialmente quiero agradecer a Luis Fernando Benito-Matías y Amaya Álvarez. Nunca me ha faltado vuestra ayuda, habéis sido una referencia de consulta obligatoria ante muchos de los problemas que se me han presentado. Todo lo que se del maravilloso e infinito mundo de las micorrizas lo he aprendido de vosotros, pero también de otros aspectos del cultivo de especies forestales. No me dejan de sorprender tanto vuestra curiosidad científica como vuestra dedicación a la investigación y vuestra generosidad. Además, no os ha bastado con transmitirme vuestros conocimientos y experiencia profesional, sino que me habéis apoyado a nivel

personal y os habéis incluido en mi vida por derecho propio. Desde el Jardín Botánico, tanto su director de Rosendo Elvira Palacio como todo su equipo, y especialmente Beatriz Díaz González, han sido igualmente generosos; cediéndome espacio en sus invernaderos aún cuando hasta para ellos es escaso, pero también tengo que agradecer toda la ayuda que me han prestado durante la fase de cultivo de todos los experimentos y su preocupación constante por mí y mi trabajo.

Igualmente, mi familia científica no se completa sin mencionar al Departamento de Ecología, o actualmente Departamento de Ciencias de la Vida, Unidad Docente de Ecología. El Departamento, o más bien las personas que lo componen, me han brindado un ambiente único para formarme como investigadora y como persona, y han hecho de mis años de doctoranda un conjunto infinito de buenos recuerdos y ambiente positivo. Son muchas las personas que lo componen y siento no mencionaros a todos y cada uno. Son ejemplos de esta familia: José María Rey Benayas, siempre me has ayudado y apoyado, has estado presente ayudándome en los grandes pasos de mi doctorado, desde la entrada en el Máster hasta la defensa del practicum y mucho más allá. Ana Guerrero, los trámites administrativos, dudas mil y pequeños problemas diarios no habrían sido resueltos sin tu incondicional dedicación; y no solo eso, sino que tu preocupación personal por todos nosotros, tu sonrisa y tus ánimos han sido una pieza más del inicio de mi vida investigadora. Lorenzo Pérez, en tu papel de técnico de laboratorio, me ayudaste ante el que era un mundo desconocido y a veces temido, y posteriormente con tu infinita paciencia en los mil pedidos y peleas con proveedores. Julio Camargo y Miguel A. Rodríguez durante vuestros periodos respectivos de directores de departamento cuidasteis de que fueran accesibles seminarios, cursos y recursos que nos elevan por encima de otros centros. Julio nunca podré olvidar tu generosa oferta de tesis (pero Pedro se te adelantó); aunque finalmente no fui tu doctoranda nunca me falto tú apoyo.

Y por último, aunque no por ello menos importante al grupo de Becarios Eco-geo-mate-físicos o como con la nueva filosofía de nomenclatura departamental recomienda geo-ecólogos y matefísicos de la vida (bicharracos, hierbajos), los números y las piedras. Desde que empezamos siendo tres gatos (Germán, Noe, Cris) hasta la gran multitud que somos actualmente (Sarita, Juan, Natalia, Arantxa, Alfonso, Nacho, Joaquín, Silvia, Isa, Noe, Cris, Guille,

Mila, Laura, Mª Jesús, Uge, Judith, Antonio, Germán, Giovanni, Jesús, Lupe, Hector, Belén, Jaime, Borja, José A., Paloma, Judit y alguno que probablemente se me olvida) han pasado muchas cosas. Cada uno habéis aportado vuestro granito; enriqueciéndome, apoyándome y enseñándome; haciendo los años de tesis mucho más divertidos y amenos. No puedo dejar de pensar que "la temida tesis" habría sido mucho más dura sin los cafés de desahogo, las frases del día, los ratos de esparcimiento y el continuo flujo de información. Siempre guardaré con mucho cariño los recuerdos de las múltiples fiestas de navidad, el cariño y la creatividad para crear regalos maravillosos ante DEA´s o tesis, las interminables jornadas de trabajo en verano solos en la facultad (que lejos de ser tristes, se sobrellevaban con descansos sentados en el césped) y los viajes varios (como el que hice con Cristina en esa fatídica nevada, el que hicimos por Australia Nachete y yo, o el multitudinario a Riaza).

I also want to thank all the professors who taught me and make me feel welcome during the PhD shorts-stays. All of you opened me the doors of your labs and let me join your teams. I am particularly thankful for all the intercultural experiences that I lived, as well as for all the methodologies and new techniques that I learnt under your supervision and for your help in several articles as coauthors. Plus précisément, je veux en remercie a Pascale (Pascale Maillard), et son équipe de l'INRA-Nancy (Dominique Gerant, Patrick Gross, Caroline Plain, Patrick Gross...), aussi à Rana El Zein. Mon stage au Nancy était mon première expérience dans un centre de recherche dehors Espagne, mais je trouvé la collèges et amis. Charlie (Charles R. Warren) thanks for giving me the opportunity for working in your lab at the Sydney University (in spite of that you only knew me through a couple of e-mails). I am very grateful for all your help during the short-stay, and also nowadays, and for all I learned (which is a lot!). Thanks to Doug (Douglass Jacobs) and his team at the Purdue University (Joshua Sloan, Amy Miller, Oriana Rueda Krauss and much more people). Doug you always were more than a colleague, you always encouraged and helped me not only in work areas, also in personal ones.

Aparte de la investigación, mis estudios predoctorales se completaron con actividades docentes. No solo con prácticas, seminarios y clases de las distintas licenciaturas, grados y másteres sino también y quizás más importante, como directora de diversos proyectos. Estas tareas docentes me han

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No solo he tenido apoyo dentro del ámbito científico, sino que en mi vida personal también son muchas las personas que me han apoyado, y requieren un especial agradecimiento, porque siendo ajenos al mundo científico se han esforzado en entenderlo y apoyarme durante todos estos años. Todos ellos han aguantado charlas interminables de laboratorios, experimentos, papers, con paciencia, respeto, buen humor y cariño. En especial quiero agradecer a mis padres (Ángel y Puri), me habéis educado como una cabezona luchadora que siempre aspira a lo mejor y que no se rinde ante las vicisitudes. Os habéis sacrificado para que yo pudiera alcanzar mis sueños y siempre me habéis mostrado lo orgullosos que estáis de mi. Todo ello me ha preparado y dado fuerzas para afrontar cualquier situación, mis mejores virtudes son fruto de vuestro esfuerzo. A Ainhoa (mi "hermana") juntas hemos crecido en edad y madurez, e igualmente has estado conmigo en esta etapa de crecimiento personal y profesional, me es imposible agradecerte algo concreto, cuando es tanto lo que me has dado durante tantísimos años (mira que me siento vieja). A mis amigos Anita, Paloma, Santy, Alber, Sory... desde que entrasteis en mi vida me siento menos sola, me habéis abierto las puertas siempre, aún cuando mis ataques de “hermitañitis” me auto-aislaban de vosotros, habéis sido el apoyo y la recarga energética cuando más lo he necesitado, le habéis dado alegría a mis momentos de ocio, y gracias a vosotros y todas vuestras (y numerosas) cualidades he crecido como persona. A mis amigos y compañeros de juegos (muy especialmente a Germán Navarro pero también a David, Req, Vizo, etc.) que habéis aguantado mis charlas de plantitas y mis crisis informáticas (incluso habéis resuelto muchas de ellas), y todo ello sin quejas (bueno alguna hubo, permitirme la broma, ;-)), con interés y paciencia, y por ayudarme a abstraerme de los agobios de la tesis al menos un rato al día. A mis compañeros del Máster

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Por todo ello, esta Tesis no es más que la guinda del pastel de una experiencia muchísimo más amplia, que solo representa una mínima parte de lo que ha sido mi vida como doctoranda. Mil aspectos (humano, científico, docente, familiar, etc.) suman mi experiencia pre-doctoral y con estos agradecimientos solo muestro algunos ejemplos mínimos. Solo me falta concluir gracias a todos, por todo lo que me habéis aportado y por todos los buenos recuerdos que he acumulado estos años.

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[1]Note: This current PhD Thesis is written in a bilingual format to aspirate to the International Mention.

Resumen

Abstract

"Success consists of going from failure to failure without loss of enthusiasm."

Winston Churchill

Fotography: Mediterranean oak forest in
Retiendas (Guadalajara)

By: M. Uscola

Resumen

El agua y la luz son considerados los principales recursos que limitan la vida de las plantas en los ecosistemas mediterráneos. Las plantas mediterráneas muestran notables diferencias de desarrollo en respuesta a estos recursos, lo cual potencialmente permite la segregación de nichos y, consecuentemente, la coexistencia de las especies. El nitrógeno (N) es un recurso esencial que determina la distribución de las plantas y la productividad primaria en muchos ecosistemas terrestres. Los ecosistemas mediterráneos también están frecuentemente limitados por el N. La mayoría de los estudios consideran el N como un recurso único aunque, en realidad, las plantas son capaces de utilizar distintas formas químicas del N: desde formas inorgánicas, como el amonio (NH_4^+) y el nitrato (NO_3^-), hasta las formas orgánicas, como la urea, aminoácidos, péptidos o pequeñas proteínas. La adquisición, el uso y la respuesta funcional a las distintas formas químicas de N podrían diferir entre las plantas mediterráneas, lo que contribuiría a explicar, junto con las estrategias de adquisición y uso del agua y la luz, la alta diversidad taxonómica y funcional en ecosistemas mediterráneos.

La principal vía de adquisición de N en las plantas es la raíz. Sin embargo, el N depositado en las hojas puede ser también absorbido por éstas y, potencialmente, contribuir a la nutrición de la planta. El N absorbido se distribuye dentro de la planta hacia los puntos de demanda, como los órganos en crecimiento futuro, o es almacenado para ser posteriormente utilizado en el crecimiento futuro, la reproducción, la defensa, o en la recuperación ante perturbaciones. La removilización de reservas para apoyar el nuevo crecimiento variará según la estrategia de vida y el hábito foliar de las plantas. Asimismo, la adquisición y el metabolismo del carbono (C) y del N están estrechamente relacionados. La fotosíntesis depende en gran medida de la abundancia de proteínas en las que el N es un componente fundamental; y al mismo tiempo, la fotosíntesis suministra los esqueletos de C necesarios para la asimilación y el metabolismo del N.

La mayor parte del conocimiento sobre la economía del N en plantas se ha obtenido en estudios con una única especie, y principalmente con especies de los biomas boreales y templados húmedos. Sin embargo, apenas existe información sobre las estrategias de uso del N en especies forestales mediterráneas. El **objetivo general** de esta Tesis es estudiar las estrategias de adquisición de las distintas formas químicas del N, el patrón de distribución y

uso de las reservas de N y C, así como la respuesta morfo-fisiológica a las fuentes de N en plantas forestales mediterráneas. La **hipótesis general** de la Tesis es que el N juega un papel central en la ecología de las especies forestales mediterráneas, ya que éstas difieren en su capacidad de adquisición, distribución, removilización y respuesta al N dependiendo de sus características funcionales. Para abordar el objetivo general se han estudiado tres principales procesos de la economía del N en las plantas: adquisición (tanto por vía radical - *Capítulo 2*- como foliar - *Capítulo 3*), distribución y removilización (*Capítulo 4*) y el efecto de las fuentes de N en el desarrollo vegetal (*Capítulo 5*).

En el **Capítulo 2** se comparan las tasas de *absorción radical* y las preferencias por las distintas formas químicas de N en nueve especies mediterráneas de ecología contrastada y que frecuentemente coexisten en zonas continentales mediterráneas sobre suelos calizos. La tasa de absorción de N de la especie herbácea y los caméfitos fue mayor que la de los arbustos y los árboles. La capacidad de adquisición de N se incrementó con la longitud específica de las raíces y la actividad de nitrato reductasa. No se encontró relación entre la absorción de N y la tasa de crecimiento relativo, la cual estuvo directamente relacionada con la reabsorción de N y la concentración de N de los tejidos. Todas las especies estudiadas fueron capaces de absorber aminoácidos intactos, cuya concentración en el suelo fue tan alta como la de las fuentes inorgánicas de N. La mayor parte de las especies presentaron una clara preferencia por el NH_4^+ y lo hicieron a expensas de la preferencia por el NO_3^-. La preferencia por el NH_4^+ difirió entre especies, siendo mayor en las especies pioneras que en especies intermedias–tardías de la sucesión. No obstante, el patrón de preferencias por las fuentes de N también difirió entre especies que co-existen en una misma etapa sucesional. Entre las especies de etapas intermedias–tardías de la sucesión, los árboles dominantes en el ecosistema presentaron una preferencia por el NH_4^+, mientras que los arbustos subordinados prefirieron el NO_3^- o la glicina. Entre las especies pioneras, las diferencias se debieron tanto a diferencias en la capacidad de absorción de N como a la intensidad de la preferencia por el NH_4^+. Por último, se encontró una relación directa entre crecimiento relativo y la preferencia por el NH_4^+, pero también con la similitud entre la preferencia de absorción de las fuentes de N y la proporción de disponibilidad de éstas en el suelo. Las especies de rápido crecimiento presentaron una elevada preferencia por el NH_4^+, y una baja similitud entre su preferencia y la disponibilidad de las fuentes de N en el suelo. En cambio, la preferencia por las fuentes de N en especies de lento crecimiento fue más similar a las proporciones de las fuentes de N en el suelo y su preferencia por el NH_4^+ fue baja.

En el **Capítulo 3** se compara la *absorción foliar* de NO_3^-, NH_4^+, urea y glicina en *Quercus ilex* y *Pinus halepensis*. *Quercus ilex* tuvo una mayor absorción foliar de N que *P. halepensis*, lo cual parece relacionarse con las diferencias en la densidad de estomas entre especies y a la presencia de tricomas en *Q. ilex*. La permeabilidad de la cutícula al agua no explicó las diferencias entre especies de tasa de absorción de N. Ambas especies absorbieron cantidades notables de todas las fuentes de N, siendo superior en urea seguido de NH_4^+, glicina y NO_3^- en ambas especies. Una parte importante de la glicina fue absorbida intacta. En ambas especies, una mayor conductancia cuticular estuvo asociada con una mayor absorción de todas las fuentes de N, excepto del NO_3^-. La absorción foliar de N incremento la concentración de N de la planta, y el N absorbido se translocó rápidamente a las raíces.

El **Capítulo 4** compara el patrón de *distribución* del C asimilado en invierno, la *contribución del C y N removilizados* a la construcción de nuevos órganos y la importancia relativa de los distintos compartimientos de la planta como fuentes de C y N en plantones de cuatro especies arbóreas perennifolias mediterráneas: *Quercus coccifera, Q. ilex, Olea europaea* y *P. hapelensis*. El C asimilado en condiciones invernales se distribuyó dentro de la planta en función del tamaño del órgano. Sin embargo, las hojas fueron los principales sitios de almacenamiento del C de invierno, ya que contuvieron cantidades de C superiores a las esperadas por su tamaño. El patrón de uso de las reservas C y N para apoyar el crecimiento difirió entre especies. Al principio de la primavera, la construcción de raíces nuevas se realizó principalmente a costa de la removilización de reservas de N, pero el N del suelo fue la principal fuente de N utilizada a mitad de la primavera. Entre especies, la proporción de C y N removilizados para la construcción de los brotes se incrementó con la tasa de crecimiento relativo. Las quercíneas, especies de crecimiento más lento, usaron principalmente C y N recién adquiridos (N del suelo y C de la fotosíntesis del momento), mientras que en *P. halepensis*, la especie de crecimiento más rápido, los brotes se construyeron principalmente con C y N de las reservas. Estas diferencias en la dependencia del C y N removilizado se atribuyeron a una mayor demanda de recursos de los órganos viejos en las especies con alto crecimiento relativo, probablemente debida al crecimiento diametral y a la recuperación de reservas. Las hojas viejas aportaron la mayor parte del C y N removilizados durante la primavera en todas las especies, aunque también existió una contribución muy importante de los tallos y raíces viejas.

En el **Capítulo 5** se evalúa la *respuesta* de dos especies mediterráneas *Q. ilex* y *P. halepensis*, al cultivo con *diferentes fuentes inorgánicas de N* y a la fertilización foliar con aminoácidos. Mientras que a baja concentración las

fuentes de N tuvieron efectos muy pequeños sobre el desarrollo de las plantas, a alta concentración, las fuentes de N condicionaron el desarrollo fuertemente. La fuente de N afectó el crecimiento, el contenido en clorofila, y la arquitectura radical. La respuesta a las distintas fuentes de N dependió de la especie. El pino, una especie pionera que principalmente crece en suelos ricos en NO_3^-, presentó mejor desarrollo cultivado con NO_3^- y una mayor plasticidad a los cambios en el aporte de N. En cambio, la encina, que es un árbol de etapas tardías de la sucesión y que principalmente se desarrolla en suelos ricos en NH_4^+, presentó baja respuesta a las fuentes de N o a la concentración de N. La fertilización foliar con aminoácidos incrementó la fotosíntesis de ambas especies y ligeramente el crecimiento de *P. halepensis*.

La **conclusión general** de esta Tesis es que las especies forestales mediterráneas presentan distintas capacidades de absorción y respuesta a las formas químicas de N, así como diferente uso de las reservas de N para apoyar el crecimiento de los nuevos órganos. Se demuestra que los aminoácidos son una fuente de N potencialmente importante en ecosistemas mediterráneos, ya que su abundancia en suelos es tan alta como la del N inorgánico y las especies mediterráneas son capaces de absorberlos intactos. Finalmente, las diferencias en la utilización de N pueden condicionar la velocidad de crecimiento de las plantas, un atributo clave para su eficacia biológica. Todo ello indica que las plantas mediterráneas tienen nichos fundamentales diferentes en base al uso del N y sugiere que este nutriente juega un papel significativo en la estructura y funcionamiento de las comunidades vegetales mediterráneas.

Palabras clave: Absorción foliar; absorción radical; aminoácido; amonio; crecimiento; estrategias ecológicas; glicina; nitrato; preferencias por las fuentes de N; desarrollo de la planta; removilización; reservas.

Abstract

Water and light are considered as the main resources that limit plant life in Mediterranean type-ecosystems. Mediterranean plants exhibit significant performance differences in response to these resources, which potentially allows for niche segregation and, consequently, species coexistence. Nitrogen (N) is a primary resource determining plant distribution and primary productivity in many terrestrial ecosystems. Mediterranean ecosystems frequently are N-limited. Most studies consider N as a single resource but plants can absorb different chemical forms of N: inorganic, such as ammonium ($NH4^+$) and nitrate (NO_3^-), and organic N, such as urea, amino acids, peptides, or small proteins. Acquisition, use and the functional responses to N chemical forms may differ among Mediterranean plants, and in conjunction with acquisition and use of water and light strategies, might contribute to explain high taxonomic and functional diversity in Mediterranean ecosystems.

N taken up by plants is mainly absorbed by roots. However, N deposited on plant canopies can also be absorbed through leaves and potentially contribute to plant nutrition. Absorbed N is allocated throughout the plant to meet the demand of growing organs or it is stored to support future growth and reproduction, defense or recovery after disturbance. Remobilization of stored resources to support new growth may vary with life form and growth habit of plants. Also, the acquisition and metabolism of carbon (C) and N are closely related. Photosynthesis depends largely on the abundance of proteins in which N is an essential component, that, while photosynthesis supplies C skeletons necessary for N uptake and metabolism.

Most knowledge on the N economy of plants has been obtained from studies with a single species from the boreal and wet temperate biomes. However, there is almost no information on the N use strategies in Mediterranean forest plants. The **general objective** of this Thesis is to study the strategies of acquisition of chemical N forms, the pattern of allocation and utilization of N and C reserves, and the morphological and physiological responses to different N forms in Mediterranean plants. The **general hypothesis** of the Thesis is that N plays a central role in the ecology of Mediterranean forest species, as they differ in N acquisition, allocation, remobilization and response to N depending on their functional characteristics. To address the general objective I studied three main processes of the N economy of plants: acquisition (both root - *Chapter 2* - and foliar absorption -

Chapter 3), N and C allocation and remobilization (*Chapter 4*) and the effect of N forms on plant performance (*Chapter 5*).

In **Chapter 2**, I compared the *root N uptake* rate and the preference for distinct chemical forms in nine ecologically distinct Mediterranean species that frequently coexist in Mediterranean continental areas on calcareous soils. The grass species and the chamaephytes had higher N uptake rate than the shrubs and trees. N uptake across species increased with specific root length and nitrate reductase activity. N uptake was not related to species relative growth rate, which was positively related to N recycling and tissue N concentration. Studied species were able to uptake intact amino acids, which concentration in soil was as much as high as inorganic N concentration. Most species clearly preferred NH_4^+, which was preferred at the expense of NO_3^-. Preference for NH_4^+ differed among species, being higher in pioneer species than in mid–late successional species. N form preference differed among co-occurring species within a successional stage. Among mid–late succesional species, dominant trees in the community had high preference for NH_4^+ while the subordinate shrubs preferred NO_3^- or glycine. Among pioneer species differences in N form preferences, were due to intensity of NH_4^+ preference and different N uptake rates. Finally, we observed a link between relative growth rate and the preference for NH_4^+ and the similarity between the pattern of uptake and the pattern of available N in soil. While preference for N forms was very dissimilar from the pattern of N forms in soil in fast-growing species, which had greater preference for NH_4^+, preferences of slow-growing species for the chemical forms of N were more similar to the pattern of N forms in soil and showed low preference for NH_4^+.

Chapter 3 compares the *absorption* of NO_3^-, NH_4^+, urea and glycine by *foliage* of *Quercus ilex* and *Pinus halepensis* seedlings. *Quercus ilex* had higher foliar N absorption rate than *P. hapelensis*, which was likely explained by differences in stomatal density between species and trichome presence in *Q. ilex*. Permeability of the cuticle to water did not explain species differences in N foliar absorption rate. Both species absorbed significant amounts all N forms but with differences being higher in urea and followed by NH_4^+ glycine and NO_3^-. A significant fraction of sprayed glycine penetrated the leaf as intact glycine. In both species, higher cuticular conductance was associated with faster absorption of all N sources, except of NO_3^-. Foliar absorption of N increased plant N concentration, and absorbed N was quickly translocated to roots.

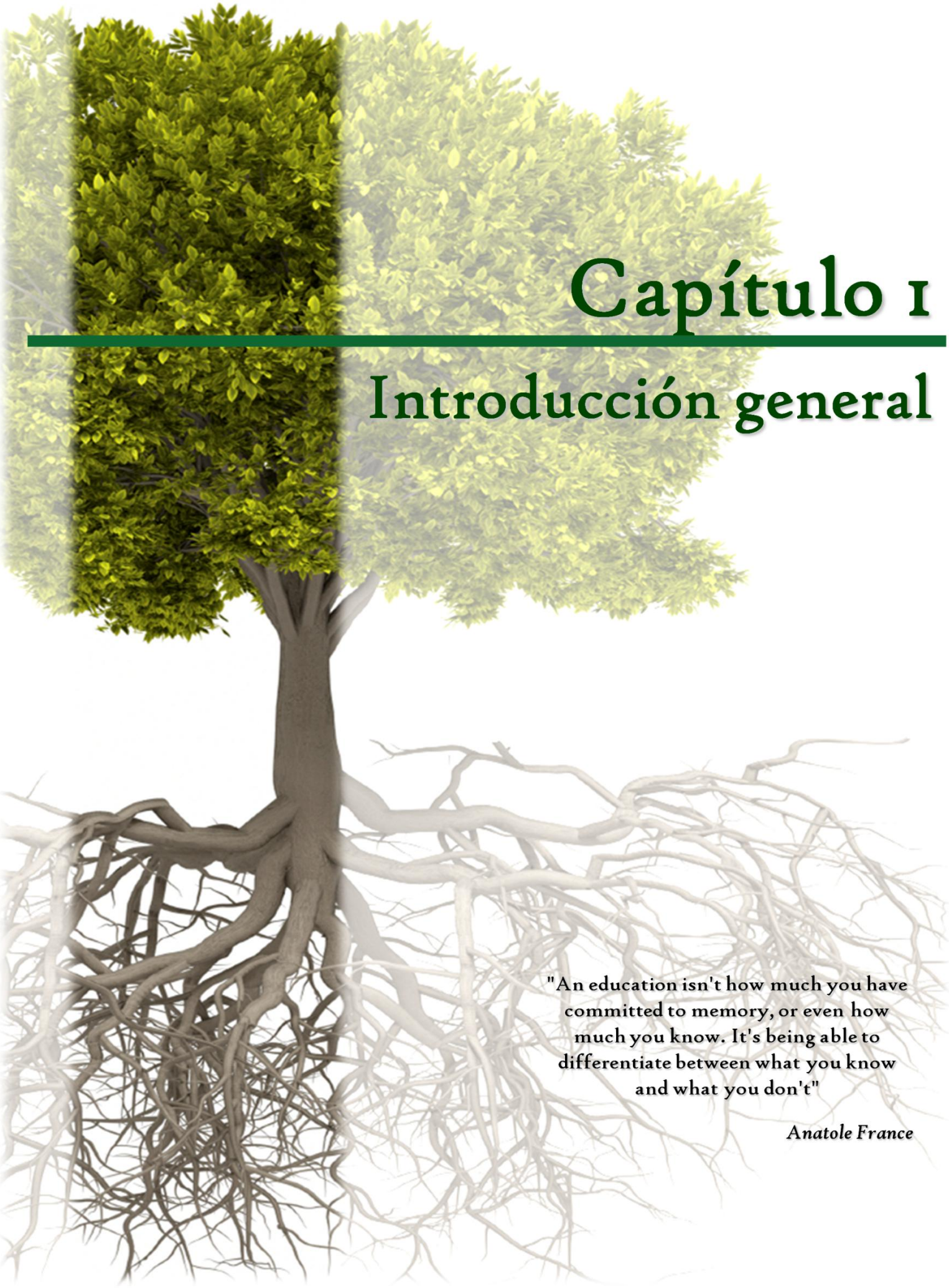
Chapter 4 compares the *allocation* pattern of C assimilated in winter, the contribution of *remobilized C and N* to the construction of new organs and the relative importance of different plant compartments as sources of C and N in seedlings of four evergreen Mediterranean trees: *Quercus ilex*, *Q. coccifera*, *Olea europaea* and *Pinus hapelensis*. Carbon assimilated under winter conditions was partitioned and stored throughout the plant depending upon the size of the plant organs. However, leaves were priority storage sites because they contained more winter C than expected for their size. Patterns of C and N storage and utilization differed among species. Remobilization was the main N source for new fine root growth soon after transplanting in early spring but by mid spring, soil N supplied most of the N in new fine roots. Across species, the proportion of remobilized C and N in new shoots increased with relative growth rate. *Quercus* species, the slowest growing species, primarily used new C and N for shoot growth while in *P. halepensis*, the fastest growing species, shoots were mainly build up by old C and N. Differences in the contribution of C and N remobilization derived from growth differences were linked to an increase in old organs demand in species with high growth rate, probably due to diametrical growth and/or reserves replenishment. Old leaves supplied most C and N during spring growth in all species but also old stems and old roots contributed substantially to growth demands.

In **Chapter 5,** I evaluated the *response* of two Mediterranean trees, *Q. ilex* and *P. halepensis*, to cultivation with *distinct inorganic N forms* and amino acid foliar fertilizer. While N forms at low N concentration had very small effects on plant performance, at high N concentration, N forms strongly affected plant performance. N forms affected growth, clorophyll content and root architecture. However, response to N forms at high N concentration was different between species. The pine, a pioneer tree that grows on NO_3^--rich soils, improved performance with NO_3^- and had strong plasticity to changes in N supply. The oak, a late successional tree that mainly thrives on NH_4^+-rich soils had low responsiveness to N form or concentration. Finally, amino acid foliar fertilization increased photosynthesis in both species and slightly improved growth in pine.

The **general conclusion** of this Thesis is that Mediterranean forest plants show different abilities to absorb and respond to the chemical forms of N and differential use of N reserves for new growth. We demonstrate that amino acids can be an important N source in Mediterranean ecosystems, as availability of amino acids in soil is as high as inorganic N and Mediterranean species are able to absorb them intact. Finally, several differences in N

utilization are linked to plant growth capacity, which is a key trait for plant fitness. This indicates that Mediterranean plants have different fundamental niche based on N use and suggests that N plays a central role in driving the structure of Mediterranean plant communities.

Keywords: Amino acid; ammonium; ecological strategies; glycine; growth; nitrate; N form preference; foliar absorption; plant performance; remobilization; reserves; root uptake.



Capítulo 1

Introducción general

"An education isn't how much you have committed to memory, or even how much you know. It's being able to differentiate between what you know and what you don't"

Anatole France

El ciclo de uso del nitrógeno por las plantas

El nitrógeno (N) es un macronutriente que determina la distribución y el desarrollo de las plantas (LeBauer and Treseder 2008). En muchos ecosistemas terrestres, tanto naturales como manejados, el N es uno de los principales factores limitantes de la productividad primaria (LeBauer and Treseder 2008). Ello se debe a que el N es un constituyente importante de numerosas moléculas clave para el funcionamiento de las plantas, como proteínas, ácidos nucleicos o la clorofila, así como de las paredes celulares. De hecho, a menudo, la capacidad fotosintética se correlaciona positivamente con la concentración de N en las hojas (Warren 2004).

La cantidad de N disponible para las plantas en los ecosistemas terrestres depende de la vía y flujo de entrada, el reciclado de la materia orgánica en el suelo, la competencia con otros organismos y las pérdidas a la atmósfera y lixiviación del suelo. La entrada de N en los ecosistemas terrestres se produce por varias vías. Una entrada es desde la atmósfera a través de procesos de deposición seca y húmeda (Schulze *et al.* 2005) (Figura 1[1]), pero también algunos microorganismos (simbiontes o libres) son capaces de fijar el N_2 atmosférico. El origen de los compuestos de N en la atmósfera es natural, pero la actividad industrial y agrícola (Wilson 1992; Rennenberg and Gessler 1999) ha incrementado en tiempos recientes la concentración de compuestos nitrogenados, constituyendo en algunas regiones, incluida la mediterránea, un importante problema medioambiental (Brumme *et al.* 1992; Sparks 2009).

El follaje puede retener hasta un 70% de la deposición de N atmosférico (Harrison et al. 2000; Sparks 2009). La parte no retenida por el follaje se deposita directamente en el suelo, aunque también parte del N depositado en hojas es lavado y acaba igualmente en el suelo (Adriaenssens *et al.* 2010). Las superficies aéreas de las plantas, especialmente las hojas, pueden absorber directamente una fracción del N depositado sobre ellas. La absorción foliar de N es generalmente inferior a la de las raíces, pero su importancia en la nutrición de la planta no es despreciable (Sanz *et al.* 2002).

[1] Las figuras, cuadros y tablas se han incluido en inglés para facilitar su comprensión a miembros extranjeros del tribunal.

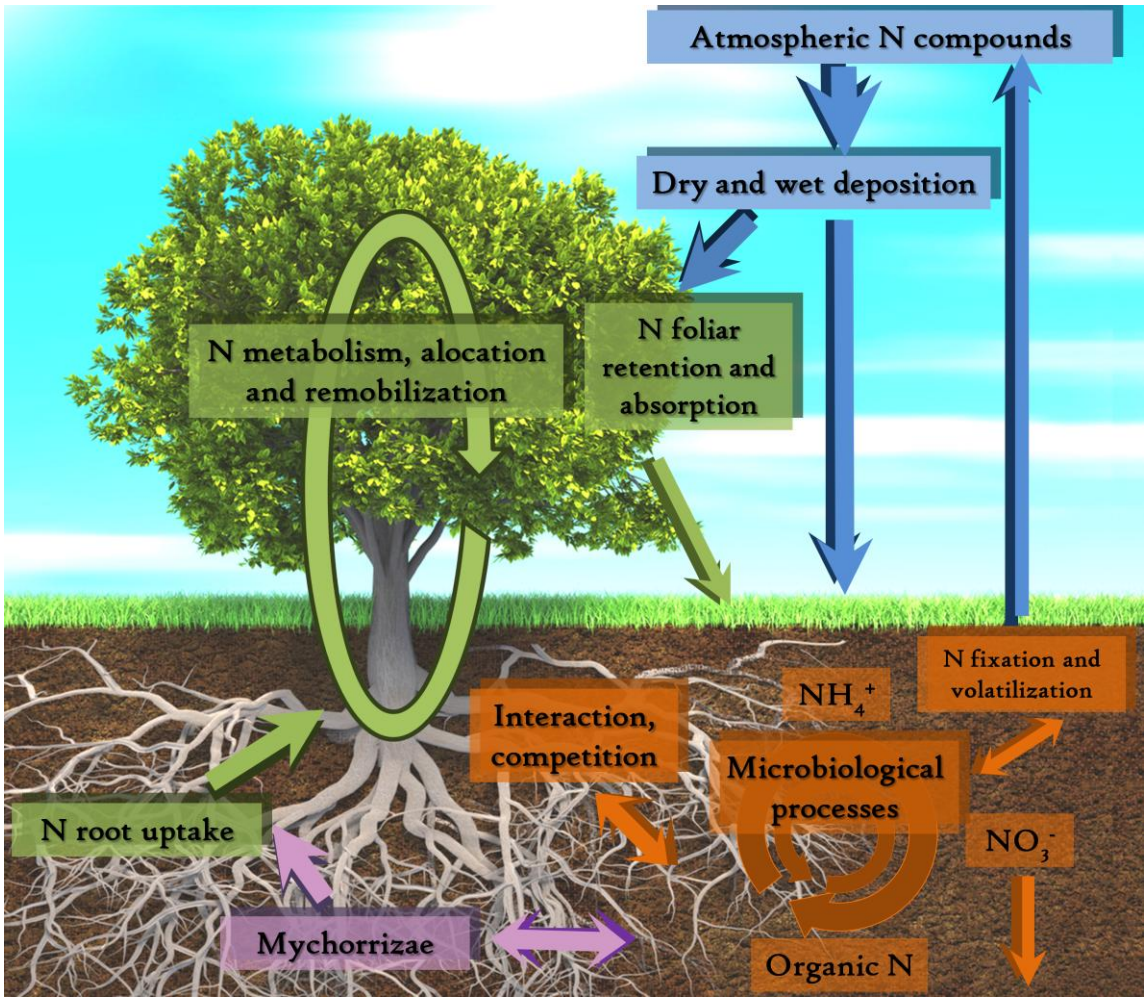


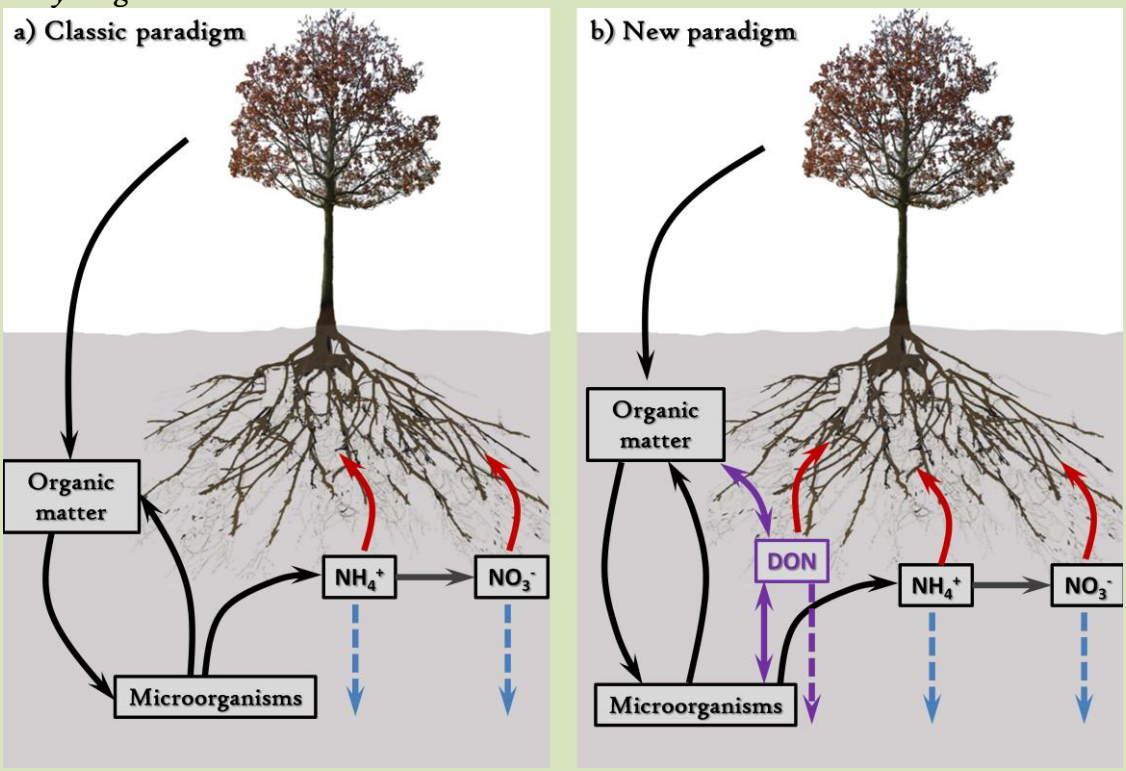
Figure 1. *Main processes that determine N availability for plants in terrestrial ecosystems*. 1) Atmospheric N input from deposition and volatilization from soil (in blue). 2) N cycling and release of N compounds into the groundwater and/or atmosphere due to soil physical-chemical and microbiological processes. 3) Plant-soil microbial community interactions (in orange). 4) Plant and mychorrizae interactions (in purple). 5) Foliar retention and absorption of N compounds, leaves washed by rain and N release to soil. 6) Plant uptake and N metabolization, allocation and remobilization within the plant (in green). Modified from Rennenberg and Gessler (1999).

Además de la fijación biológica de N atmosférico, la descomposición de algunas rocas (Morford *et al.* 2011) y de la materia orgánica aportan N al suelo fácilmente asimilable para las plantas. El N en el suelo se encuentra en distintas formas químicas interrelacionadas a través de procesos químicos y biológicos. El N orgánico (proteínas, péptidos y aminoácidos), proveniente de la descomposición de la materia orgánica, pasa a formas inorgánicas. Primero a NH_4^+ mediante despolimerización (procesos de descomposición de moléculas orgánicas de alto peso molecular a moléculas bajo peso molecular), proteólisis y posterior amonificación de aminoácidos. El NH_4^+ pasa a NO_3^- mediante procesos de nitrificación. La dominancia de una forma o fuente de N vendrá

condicionada por la velocidad de transformación de las formas de N. La composición de la comunidad microbiana que interviene en este proceso varía según el pH del suelo (Raven *et al.* 1992). En suelos con pH neutro o básico dominan los procesos autotróficos (realizados por algas y cianobacterias fotosintéticas y bacterias fijadoras de N) que incrementan la concentración de NO_3^- en el suelo. En suelos ácidos, los organismos autótrofos reducen su abundancia y su actividad se ve limitada (Raven *et al.* 1992), por ello, la nitrificación es más lenta y el NH_4^+ prevalece como forma inorgánica de N (Rennenberg and Gessler 1999; Rothstein and Cregg 2005). El NO_3^- es altamente móvil en el suelo y puede ser fácilmente lixiviado. La magnitud de pérdida de N del sistema dependerá de su cantidad, la capacidad de retención de los suelos y la precipitación (Rennenberg and Gessler 1999), pero también de la absorción radical de la vegetación. Asimismo, durante los procesos biológicos de transformación, parte del N puede quedar inmovilizado en la comunidad microbiana, la cual compite con las plantas por el N (Neff *et al.* 2003; Schimel and Bennett 2004). En resumen, la dominancia de las formas de N en el suelo es el resultado de una interacción compleja de factores ambientales y procesos bioquímicos.

Las principal vía de adquisición de N en las plantas son las raíces pero también pueden captar N por las hojas (Eberhardt and Pritchett 1971; Fageria *et al.* 2009). La micorrización puede favorecer la adquisición de N por las raíces (Read 1991). En los últimos años se ha demostrado que las plantas no sólo son capaces de absorber fuentes inorgánicas de N sino que también pueden absorber aminoácidos y proteínas intactas sin mediación de ningún otro organismo, aunque la micorrización (ver abajo) puede favorecerlo (Persson and Nasholm 2001; McKane et al. 2002; Bardgett et al. 2003; Paungfoo-Lonhienne et al. 2008). Esto implica que las plantas y los microorganismos no sólo compiten por las fuentes inorgánicas de N sino que también competirán por las orgánicas (Cuadro 1) (Schimel and Bennett 2004). Incluso, las plantas pueden incorporar microorganismos intactos como fuente de nutrientes (Paungfoo-Lonhienne *et al.* 2010). La absorción de N orgánico puede proporcionar una ventaja competitiva a las plantas de sitios pobres en N, al acceder a una mayor gama de fuentes de N (Harrison *et al.* 2008). La distinta disponibilidad de fuentes de N entre lugares puede condicionar la distribución de las especies (Aidar *et al.* 2003). Pero las especies que coexisten en un mismo ecosistema, pueden diferir en la eficacia con la que adquieren las fuentes de N, lo que se ha denominado "preferencia" por las fuentes de N. Dichas diferencias de preferencia entre fuentes de N tienen importantes implicaciones ecológicas ya que permiten la

Box 1. *The change in paradigm of N forms use by plants and its implications for soil N cycling.*



The dominant paradigm up to the mid 1990s (a, the classic paradigm) considered that plants only absorbed inorganic N forms (red arrows). The step that limited N availability for plants was the mineralization of organic matter to inorganic N forms.

In the light of the new evidences on the capacity of plants to take up organic N (Näsholm *et al.* 2000, 2009; Harrison *et al.* 2007), a new paradigm emerged (b). In the new model, plants can also take up dissolved organic N (DON) without microbial intervention, short circuiting the classical model. In this new scenario, N cycling in soils should not only consider biological processes, but also physical processes involved in DON cycling that are not mediated by microorganism (purple lines). In this case, depolymerization of organic matter into more simple organic compounds, such as proteins and amino acids, is the main limiting step for N availability and what regulates overall N cycling. DON may also leach from ecosystems despite high plant N demand.

Nevertheless, some processes remain unchanged under both paradigms. During biological N transformation of organic to inorganic N is under microbe control (black lines) and inorganic N forms are susceptible to be lost, which N loss amount depends on plant N absorption (dashed blue lines). N can also be immobilized by the microbial community. Additionally, soil characteristics can determine the importance of nitrification in some soils. Modified from Neff *et al.* (2003) and Schimel and Bennett (2004).

coexistencia de plantas por segregación de su nicho ecológico fundamental (McKane *et al.* 2002).

Una vez absorbido, las plantas deben metabolizar el N, distribuirlo a los órganos que lo demanden o incorporarlo a las reservas internas. El almacenamiento de nutrientes evita su pérdida cuando su disponibilidad en el suelo excede la demanda de la planta. También permite a las plantas acoplar la adquisición de nutrientes con su disponibilidad, incrementando su tiempo de residencia en la planta. Las reservas se utilizan principalmente en momentos de alta demanda, apoyando el crecimiento, la reproducción o la recuperación de la planta tras una perturbación (Chapin III *et al.* 1990; Nambiar and Fife 1991).

Por último, la materia orgánica vegetal retorna al suelo por senescencia o mortandad de los individuos o de sus partes, donde será descompuesta por los descomponedores.

Adquisición de nitrógeno por las plantas

Fuentes edáficas de N y absorción por las raíces

La absorción del N por las raíces incluye una componente pasiva, a favor de gradientes osmóticos, pero también una componente activa que conlleva un gasto energético a la planta. Para optimizar la absorción de N, los sistemas fisiológicos de la planta encargados de su absorción están fuertemente regulados por una compleja interacción entre los niveles de N del sustrato y la concentración interna de las diferentes fuentes de N (Britto *et al.* 2001). La absorción activa de N se produce mediante transportadores de membrana específicos, tanto para fuentes inorgánicas como orgánicas de N (Näsholm *et al.* 1998; McKane *et al.* 2002; Bardgett *et al.* 2003; Schulze *et al.* 2005). La inducción de los transportadores de membrana depende de la disponibilidad y proporción de las distintas fuentes de N. Mientras que a bajas concentraciones de las fuentes de N (<500 μM) funcionan los transportadores de alta afinidad (Williams and Miller 2001; Glass 2009), a altas concentraciones se induce la formación de sistemas de transporte de baja afinidad (Tsay *et al.* 2007; Glass 2009).

El N edáfico puede variar en concentración pero también en la proporción entre formas químicas de N. Las plantas pueden diferir en la capacidad de absorción total y de las fuentes de N. No obstante, una forma de N puede tener una mayor contribución a la demanda de N de la planta porque su

abundancia sea mayor en el suelo, independientemente de la afinidad que tenga una planta por dicha fuente de N (Stark and Hart 1997). La demanda de N de la parte aérea determina, en buena medida, la tasa de absorción radical de N de una especie ya que suele ser el mayor sumidero de N de la planta (Chapin III 1980; Weigelt *et al.* 2005). Mientras que las características morfo-fisiológicas y ecológicas de las plantas que se relacionan con la tasa de absorción de N son bien conocidas, apenas existe información sobre las características funcionales ecológicas que condicionan las preferencias por las fuentes de N.

Las plantas necesitan acoplar la demanda con la absorción, de modo que cualquier factor que incrementa la demanda de la planta por un nutriente tiende a generar un incremento en su capacidad de absorción de éste (Lambers 2008). Por ejemplo, las plantas de crecimiento lento, que suelen ser características de ambientes pobres en nutrientes, generalmente presentan una baja tasa de absorción de N y baja plasticidad a cambios en su disponibilidad (Chapin III 1980; Lambers 2008) y no parecen mostrar diferencias de preferencias por las fuentes de N (Weigelt *et al.* 2005). Por el contrario, las plantas de crecimiento rápido tienen una mayor demanda relativa de N y, consecuentemente, una mayor capacidad de absorción de N (Chapin III 1980; Lambers 2008), éstas además tienen una mayor preferencia por fuentes inorgánicas de N que las orgánicas (Weigelt *et al.* 2005). La utilización de fuentes inorgánicas de N probablemente contribuye a reducir la competencia con la comunidad microbiana, ya que esta está más limitada por el C, abundante en las fuentes orgánicas (Schimel and Bennett 2004; Jones et al. 2005). Sin embargo, otros estudios sugieren que las especies de rápido crecimiento no necesariamente prefieren fuentes inorgánicas de N en todas las condiciones (Dunn *et al.* 2006).

Además de las diferencias en la velocidad de crecimiento, la explicación más extendida de las distintas preferencias por las fuentes de N es que las plantas están adaptadas a la fuente de N más abundante en su hábitat (Kronzucker *et al.* 1997; Warren 2006). La absorción de N orgánico tiende a predominar en plantas de ecosistemas donde la actividad microbiana y la tasa de mineralización de la materia orgánica es baja, como ocurre en ecosistemas árticos, alpinos y boreales (Kielland 1994; Näsholm *et al.* 1998; McKane *et al.* 2002). En estos sistemas, la adquisición de N orgánico proporciona una ventaja competitiva a las plantas. Por el contrario, el uso de N orgánico en ecosistemas templados parece ser comparativamente menor que en ecosistemas de lugares fríos debido a la mayor mineralización de la materia orgánica y disponibilidad de N inorgánico (Owen and Jones 2001; Bardgett *et al.* 2003; Schimel and Bennett 2004).

La proporción de NH_4^+ con respecto a la de NO_3^- se incrementa a lo largo de la sucesión ecológica (Figura 2), debido a un descenso en la nitrificación (Rice and Pancholy 1972; Wolt 1994; Kronzucker *et al.* 1997). Los principales factores ambientales que determinan este patrón son la luz (Smirnoff and Stewart 1985; Gilbert *et al.* 2001), la humedad y la temperatura del suelo (Clements 1928; Finegan 1984; Bonan 1992), y el aumento de la actividad alelopática generada por las especies tardías de la sucesión (Rice and Pancholy 1972). La comunidad microbiana responsable de la nitrificación se ve limitada, además, una menor capacidad competitiva por el N que los microorganismos heterótrofos responsables de la depolimerización y descomposición de la materia orgánica (Lamb 1980) y por una reducción de microorganismos oxidantes del NH_4^+ (Kowalchuk *et al.* 2000). Así, en etapas tempranas de la sucesión, dominadas por hierbas y pequeñas plantas leñosas, donde la mineralización de la materia orgánica es alta, predomina el NO_3^-. En las etapas más maduras, con predominio de árboles y grandes arbustos, la mineralización suele ser menor (Rice and Pancholy 1972; Lamb 1980; Kowalchuk *et al.* 2000), dominando el NH_4^+ y los aminoácidos en el suelo. En este esquema sucesional, las plantas pioneras tienden a tener alta capacidad de absorción de NO_3^-, ligada también a una alta actividad nitrato reductasa, especialmente en las hojas, pero menor capacidad de absorber NH_4^+ y aminoácidos (Stewart *et al.* 1988; Nordin *et al.* 2001). Suelen, además, mostrar rápidamente síntomas de toxicidad ante exposiciones prolongadas al NH_4^+ a altas concentraciones (Kronzucker *et al.* 1997). Por el contrario, las plantas de etapas sucesionales tardías tienden a preferir el NH_4^+ y los aminoácidos como fuentes de N, muestran baja respuesta a cambios en la disponibilidad de N y asimilan el N principalmente en raíces (Reich *et al.* 1995; Kronzucker *et al.* 2003; Weigelt *et al.* 2005).

Plantas de la misma comunidad también pueden variar en su preferencia por las fuentes de N, tal como se ha observado en pastizales de climas templados (Weigelt *et al.* 2003), en la tundra ártica (McKane *et al.* 2002) y en comunidades alpinas (Miller and Bowman 2003). Estos resultados sugieren que la variación en las preferencias entre especies permite la segregación de nichos ecológicos en base al uso de distintas fuentes de N, lo que permitiría reducir la competencia por el escaso N "fácilmente" disponible y consecuentemente la coexistencia de un mayor número de especies (McKane *et al.* 2002; Weigelt *et al.* 2005). Por ejemplo, en pastizales de la tundra en Alaska, las especies dominantes y que acaparan la mayor parte de la productividad primaria utilizan la fuente de N más disponible, mientras que las especies subordinadas y menos productivas utilizan las fuentes de N menos abundantes (McKane *et al.* 2002). La mayoría de los estudios sobre utilización de fuentes de N en plantas se han realizado en

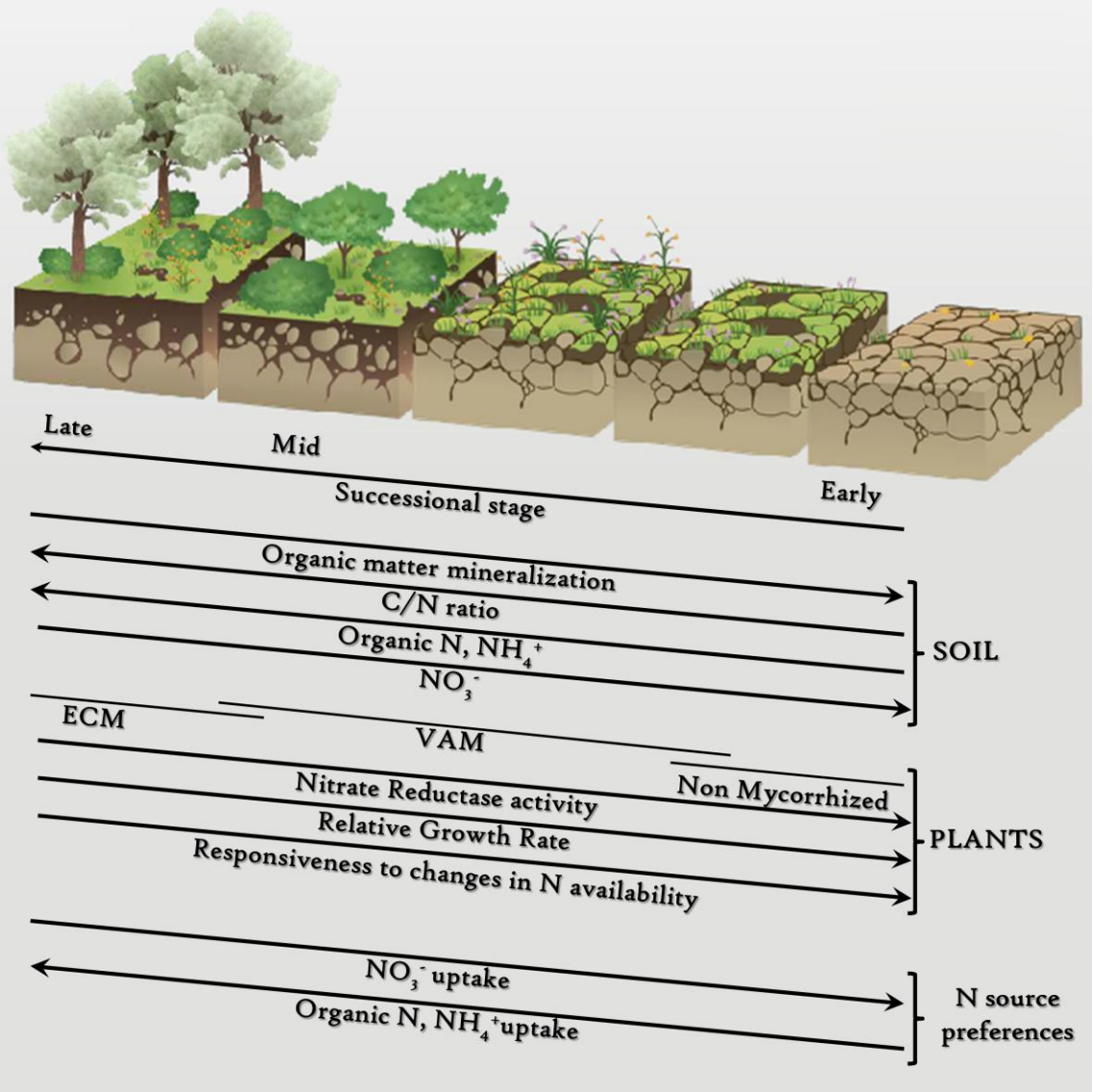


Figure 2. *Conceptual model showing changes in several soil and plant processes along ecological succession in forest ecosystems*. Arrowheads denote de direction of increase. The variables are organized in three groups. The first one is related to soil characteristics: mineralization rates, C / N ratio, and soil organic N, NH_4^+ and NO_3^- concentrations. The second group refers to plant traits: type of mycorrhization (non mycorrhized, vesicular-arbuscular mycorrhizal type - VAM and ecto-mycorrhizae - ECM, as described by Read (1991)), nitrate reductase activity, relative growth rate, and responsiveness to changes in N availability. The last group refers to N source preferences in plants that dominate different successional stages. Modified from Aidar *et al.* (2003).

ecosistemas de climas fríos y principalmente con especies herbáceas (Kielland 1994; Näsholm *et al.* 1998; McKane *et al.* 2002). Aunque la información es escasa, también hay algunas evidencias del uso de N orgánico y/o diferenciación de preferencias entre fuentes de N en plantas de sistemas agrícolas (Näsholm *et al.* 2008). En ecosistemas templados, mientras algunos estudios reflejan ausencia de preferencias entre plantas por las fuentes de N (Warren 2006; Warren and

Adams 2007), otros sí han encontrado diferencias de preferencia ligadas, además, a las etapas sucesionales (Metcalfe *et al.* 2011) y entre plantas que co-existen (Scott and Rothstein 2011). En relación con los ecosistemas mediterráneos, el conocimiento que se tiene de la nutrición y metabolismo del N en plantas es bastante limitado, especialmente, si se compara con otros biomas. En particular, la información disponible sobre el uso de las fuentes de N y sus implicaciones funcionales y ecosistémicas es prácticamente desconocido (Cruz *et al.* 1993; Warren and Adams 2002).

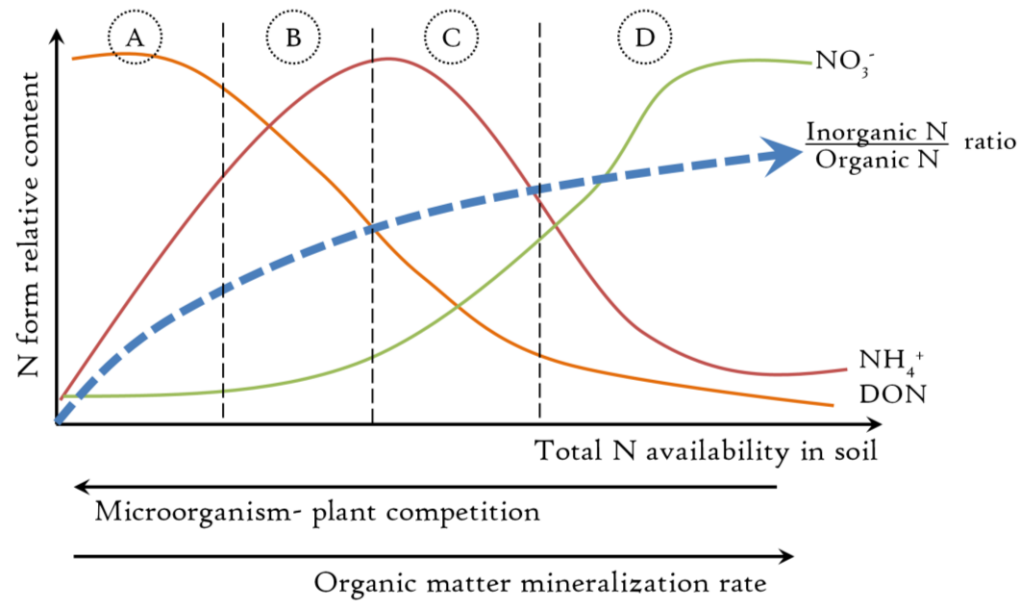
La coexistencia de especies y su ensamblaje en comunidades están determinadas por las interacciones que se establecen entre ellas (Begon *et al.* 2006; Terradas *et al.* 2009). En concreto, la competencia por los recursos es una importante fuerza que moldea la estructura de las comunidades. Las especies que utilizan un mismo recurso pueden coexistir si lo utilizan de manera distinta, es decir que se produce una diferenciación de nichos ecológicos por segregación en el espacio o en el tiempo de las especies (Tilman 1982) o por diferencias funcionales entre especies que condicionen su tolerancia a las variaciones de dicho recurso (Kraft *et al.* 2008). Los ecosistemas forestales mediterráneos son relativamente ricos en especies y tipos funcionales en los que se produce la coexistencia de diferentes formas de crecimiento (herbáceas, caméfitos, arbustos y árboles) y plantas de distinto hábito foliar (perenne *vs.* caduco) (Cowling *et al.* 1996). El agua y la luz son considerados los principales recursos por los que las especies forestales mediterráneas segregan sus nichos ecológicos (Zavala *et al.* 2000; Sánchez-Gómez *et al.* 2006). Pero, además, los ecosistemas mediterráneos están a menudo limitados por la disponibilidad de N (Delgado-Baquerizo *et al.* 2011). Es posible que otros recursos, como el N y sus formas químicas, sean una dimensión del nicho ecológico por el que las plantas mediterráneas también deban segregar su nicho ecológico fundamental para coexistir (Filella and Peñuelas 2003; Kahmen *et al.* 2006; Terradas *et al.* 2009). Así, creemos que en especies que utilizan de una manera parecida el agua o la luz podría darse una complementariedad de nichos ecológicos determinada por un uso diferencial de otros recursos edáficos (Davies *et al.* 1998).

Uno de los principales problemas para medir la tasa de absorción de las fuentes de N es la competencia con los microorganismos del suelo. Las plantas difieren en su capacidad de competencia con los microorganismos por las fuentes de N (Näsholm *et al.* 2000; Weigelt *et al.* 2005). La absorción de N depende de la composición de la comunidad microbiana en la rizosfera. A su vez, las plantas condicionan la composición de la comunidad microbiana en la rizosfera y, por tanto, los procesos dominantes de transformación del N en el suelo (Bardgett *et al.* 1999; Dunn *et al.* 2006; Eskelinen *et al.* 2009). Las plantas

están a menudo asociadas con un tipo específico de micorriza (Vandenkoornhuyse *et al.* 2003), lo que a su vez condiciona fuertemente la capacidad de absorber las fuentes de N (Bardgett *et al.* 2003; Harrison *et al.* 2008). La competencia entre las plantas y la comunidad microbiana por el N depende de la disponibilidad de N (Bardgett *et al.* 2003; Schimel and Bennett 2004) y la proporción C/N en el suelo (Dunn *et al.* 2006), así como de la actividad de los microorganismos (Jones and Hodge 1999).

Schimel and Bennett (2004) relacionan la disponibilidad de N en un suelo en función de la competencia microorganismos-planta, la fuente de N dominante en el suelo y la fuente de N disponible para las plantas (Cuadro 2). De acuerdo con el modelo de Schimel and Bennett (2004), los ecosistemas mediterráneos, que en general se caracterizan por una baja fertilidad edáfica derivada de una lenta descomposición de la hojarasca (Gallardo and Merino 1998), deberían estar dominados por N orgánico, ya que la competencia por el N no permitiría la existencia de elevados niveles de fuentes de N inorgánicas libres en el suelo. Por ello, la segunda fuente de N más abundante sería el NH_4^+ y, finalmente, el NO_3^-. Los resultados Delgado-Baquerizo and Gallardo (2011) apoyan el modelo de Schimel y Bennett demostrando que los procesos de despolimerización dominan sobre la mineralización (amonificación y nitrificación) en suelos pobres en N, mientras que la mineralización domina en suelos ricos en N de ecosistemas mediterráneos.Existen pocas referencias en regiones de clima mediterráneo, sobre la disponibilidad de N orgánico disuelto (DON) y/o datos comparativos con la disponibilidad de fuentes inorgánicas y orgánicas de N. Recientemente, Delgado-Baquerizo *et al.* (2011) en otro artículo, han publicado datos sobre la variación estacional de la concentración de N orgánico y de fuente inorgánicas de N en una amplia variedad de ecosistemas mediterráneos (Tabla 1). La variabilidad tanto en concentración como en dominancia relativa de las fuentes de N entre ecosistemas es muy elevada. Dentro de un ecosistema, la variabilidad estacional de DON es mucho mayor que la de NH_4^+ y NO_3^-. Los valores más altos de DON tienden a corresponderse con los periodos húmedos (principalmente invierno) mientras que en los periodos secos dominan las fuentes inorgánicas de N. Entre fuentes inorgánicas, por norma general, el NH_4^+ es la fuente de N inorgánico dominante, mientras que el NO_3^- predomina en suelos de comunidades típicas de etapas tempranas de la sucesión (retamar con *Stipa tenacissima*). Desafortunadamente, el estudio de (Delgado-Baquerizo *et al.* 2011), no distingue entre fuentes de N orgánico (aminoácidos, péptidos, etc.) y no todo el N orgánico está disponible para las plantas.

Box 2. *Theoretical framework linking N availability with N mineralization, microorganism-plant competition and the dominant N form in soil* (Schimel & Bennett model).



In extremely N-poor ecosystems (case A), where litter inputs and decomposition and N cycling are slow (such as boreal, artic, and alpine ecosystems), plants and microbes compete primarily for N at the organic stage. Microbes are enough limited by N that they retain amino acids and only rarely mineralize N. In this situation, both plants and microbes rely on organic compounds to meet their N demand. As soil N-availability increases, decomposition, N availability, depolymerization, and the release of N containing monomers increases (case B). Microbes are less N limited and begin to mineralize N. If N availability is high enough, it supports the development of small populations of nitrifiers. Increasing amounts of NH_4^+ (and NO_3^-) become available for both plants and microbes. Thus, while plants continue having some access to organic-N compounds, the apparent dominance of NH_4^+ in the overall N cycle increases, but plants and microbes actively compete for the limited amount of NH_4^+. This situation might occur in some temperate forests where litter is N-poor and decomposition is slow (Delgado-Baquerizo and Gallardo 2011). As N availability further increases (case C) a greater fraction of the microbial community meet their N needs using local organic sources. This reduces the competition between plants and microbes for N, and plants have access to mineralized N after microbial demands are met, as assumed by the classical paradigm. Mineralization and NH_4^+ increasingly dominate soil N pools. The combined demands of microbial and plant uptake still limit overall NH_4^+ supply to nitrifiers, thus maintaining NH_4^+ as de dominant N source, though NO_3^- progressively increases. These conditions tend to occur in moderately fertile temperate forests and some grassland. At very high relative N availability, plant and heterotroph competition for NH_4^+ becomes low enough to allow nitrifiers to flourish and the N economy of the system becomes progressively more NO_3^- dominated (case D). Nitrifiers live in close association with mineralizers so NO_3^- becomes the dominant N form and plants rely on NO_3^- to meet their N demand. This is the case of agricultural systems and N-rich tropical forests. Modified from Schimel and Bennett (2004).

Table 1. Dissolved organic N (DON) and inorganic N (NH_4^+-N and NO_3^--N) concentration (mg N kg^{-1} dry soil) in the top 10 cm of the soil profile, in different Mediterranean plant communities and along different seasons. Data are means ± 1 standard error. The soil type and main plant species present are indicated under the name of the plant community. In some communities author provide results under and beneath the dominant tree / shrub. Here I have averaged these data. Similarly, I have averaged the data of several cork-oak forest soils. Riparian, managed systems and dunes have not been included. Values in bold indicate the dominant N source. Source: Modified from (Delgado-Baquerizo *et al.* 2011).

Plant community	Season	DON	NH_4^+-N	NO_3^--N
Bare soil				
	Winter	**3.86±0.78**	1.56±0.36	0.37±0.18
	Spring	**8.99±1.98**	2.23±0.34	0.00±0.00
	Summer	4.94±1.44	1.29±0.14	**5.59±0.72**
	Autumn	1.99±0.20	**3.58±0.87**	3.50±0.82
***Stipa tenacissima* ecosystem**				
Gypsisol (*Stipa tenacissima, Retama sphaerocarpa, biological soil crust*)	Winter	**11.36±5.00**	4.25±1.39	6.50±1.51
	Spring	6.52±3.69	4.24±0.68	**16.69±10.59**
	Summer	1.75±1.69	6.80±1.19	**27.56±10.36**
	Autumn	0.00±0.00	3.80±0.44	**69.86±23.84**
Mediterranean heathland				
Leptosol (*Erica scoparia, Lavandula stoechas*)	Winter	**4.63±0.83**	2.53±0.32	0.00±0.00
	Spring	**4.41±0.38**	2.26±0.15	0.29±0.12
	Summer	**5.75±0.86**	3.69±0.47	0.35±0.20
	Autumn	2.38±0.67	**4.19±0.61**	0.80±0.22
***Juniperus phoenicia* ecosystem**				
Regosol (*Juniperus phoenicia, Lavandula stoecha, Corema album, Rosmarinus officinalis*)	Winter	**10.73±5.23**	1.98±1.54	0.08±0.11
	Spring	**10.06±2.91**	2.09±0.60	0.86±0.91
	Summer	**4.17±0.85**	0.86±0.24	1.36±0.61
	Autumn	0.24±0.34	**1.82±0.10**	0.67±0.64
***Pinus pinea* forest**				
Planisol (*Pinus pinea, Quercus suber, Chamaerops humilis, Ruscus aculeatus, Rosmarinus officinalis*)	Winter	**3.24±0.29**	0.21±0.04	0.00±0.00
	Spring	**2.82±0.13**	0.84±0.11	0.68±0.06
	Summer	**3.26±0.29**	1.62±0.27	1.14±0.08
	Autumn	0.00±0.00	**1.65±0.25**	1.19±0.22
Cork-oak forest				
Cambisol, leptosol (*Quercus suber, Lavandula stoechas, Ruscus hypophyllum, Rosmarinus officinalis, Erica scoparia*)	Winter	2.85±1.04	**5.82±0.58**	1.11±0.47
	Spring	**4.74±0.54**	3.26±0.35	2.13±0.37
	Summer	**4.35±0.40**	2.72±0.43	0.68±0.20
	Autumn	0.46±0.38	**4.42±0.57**	4.32±1.06

Las plantas que forman asociaciones micorrízicas tienen una clara ventaja sobe las plantas no micorrizadas en la adquisición de N, ya que todos los tipos de micorrizas son capaces de absorber N y transferirlo al huésped (Näsholm *et al.* 1998; Talbot and Treseder 2010). La elevada producción de hifas de los hongos aumenta el volumen de suelo explorado y su elevada afinidad por los aminoácidos puede aumentar la capacidad de competencia con los microorganismos del suelo (Chalot and Brun 1998; Leake *et al.* 2004). Sin embargo, la capacidad de acceder a las fuentes de N difiere entre micorrizas. Las asociaciones con micorrizas vesículo arbusculares (VAM) dominan en herbáceas y leñosas de suelos minerales mientras que las ectomicorrizas (ECM) predominan en ecosistemas forestales con una capa de hojarasca potente (Read 1991; Talbot and Treseder 2010). En comparación con las VAM, las ECM suelen presentar un sistema de hifas externas mucho más extendido (Simard and Durall 2004). Además, las VAM tienen menor capacidad de movilizar N orgánico que las ECM debido a su menor actividad proteasa y una mayor preferencia por NO_3^- que por el NH_4^+ o N orgánico (Read 1991; Talbot and Treseder 2010).

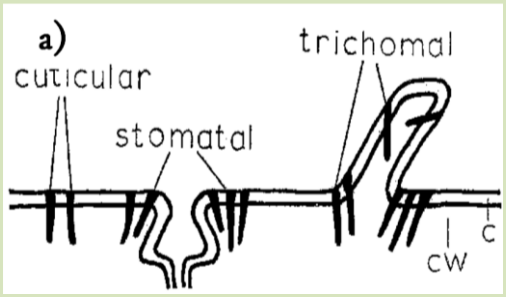
Fuentes atmosféricas de N y absorción foliar

El interés por la absorción foliar de nutrientes, y en particular la del N, ha suscitado una atención creciente por su potencial en los programas de fertilización, así como también por su papel en la entrada de nutrientes y contaminantes en los ecosistemas. A diferencia del N absorbido por las raíces, el absorbido por vía foliar está más disponible para la planta y se incorpora más rápidamente a su metabolismo ya que queda menos inmovilizado por los microorganismos y se pierde menos por volatilización o lixiviación (Rennenberg and Gessler 1999; Dong *et al.* 2002). En cambio, la absorción de nutrientes por vía foliar es mucho menos eficiente que la absorción por las raíces. El proceso de absorción foliar es diferente al de la raíz debido a que la superficie foliar está cubierta por la cutícula, que no se encuentra en la raíz. Existen dos vías de penetración de los compuestos por la superficie de la hoja: la cuticular (Peuke *et al.* 1998) y la estomática (Hull *et al.* 1975; Bondada *et al.* 2006; Eichert and Goldbach 2008); y la importancia relativa de cada vía varía entre especies (Haynes and Goh 1977).

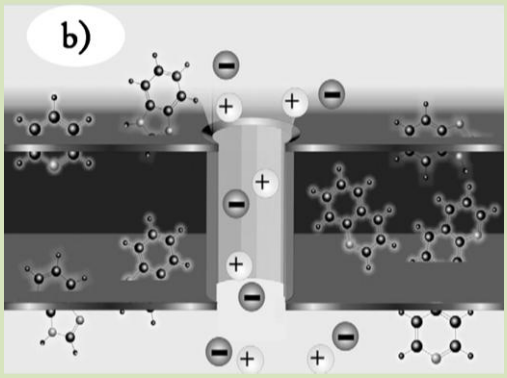
La conquista de las plantas del medio terrestre estuvo aparejada con la aparición de la cutícula, que aísla el medio interno de la planta de la desecación que impone el medio aéreo. No obstante, aunque la cutícula constituye una barrera de protección eficiente, es parcialmente permeable al agua, gases, y a

Box 3. *The cuticular pathway.*

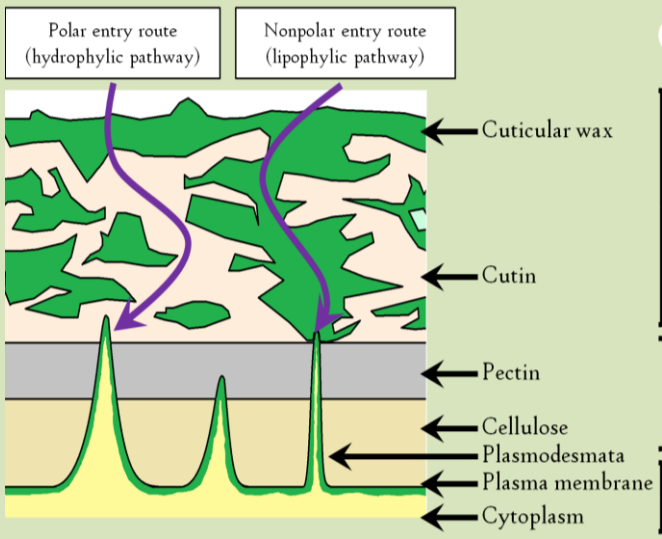
The cuticle is not structurally homogeneous and there are points where its thickness is reduced and /or the presence of **ectodesmata (a)** (physicochemical pathways in the outer epidermal wall extending into the cuticular area above them; black solid lines). Ectodesmata -abundant areas are generally found in ledges of stomata around guard cells, over anticlinal walls of epidermal cells and surrounding trichomes. These points can be considered as cuticular penetration pathways (C: cuticle, CW: cell wall) (Source: Haynes and Goh (1977)).



The lipophilic non-electrolyte compounds dissolve in the membrane matrix while ion penetration is restricted to **aqueous pores**. To maintain electroneutrality, cations and anions must penetrate in equivalent amounts. This two different pathways can be observed in the schematic drawing **(b)** (not to scale) of a solubility membrane (polymer membranes, fluid membranes and cell membranes) crossed by an aqueous pore (Source: Schönherr (2006)).



Detailed cross section **(c)** show the cuticle, it has an epicuticular waxy surface underlayed by a mixed matrix of cutin and wax. Waxes are lipophilic and reduce water loss from the leaf. The **lipophilic** components of the cuticle are painted in green in the subfigure C. Cutin is a **hydrophilic** substance determining the structure of the cuticle. The cell wall occurs under the cuticle and it is made of cellulose, hemicellulose and other hydrophilic substances. The most internal layer is the cell membrane, a liphophilic structure that actively controls the movement of materials both in and out of the cells. Regardless of the penetration pathway, at some point across the cuticle, any compound penetrating the cuticle must move from a lipophilic to a hydrophilic component. This is important since a water soluble compound would associate with hydrophilic portions of the leaf and not penetrate into lipophilic regions, whereas oil soluble compounds would preferentially associate with lipophilic regions (Source: Ashton and Crafts (1981)). Diffusion of oil-soluble compounds across the membrane occurs through molecular-size holes that form temporarily by the movement of cuticular wax and cutin molecules (Popp *et al.* 2005).

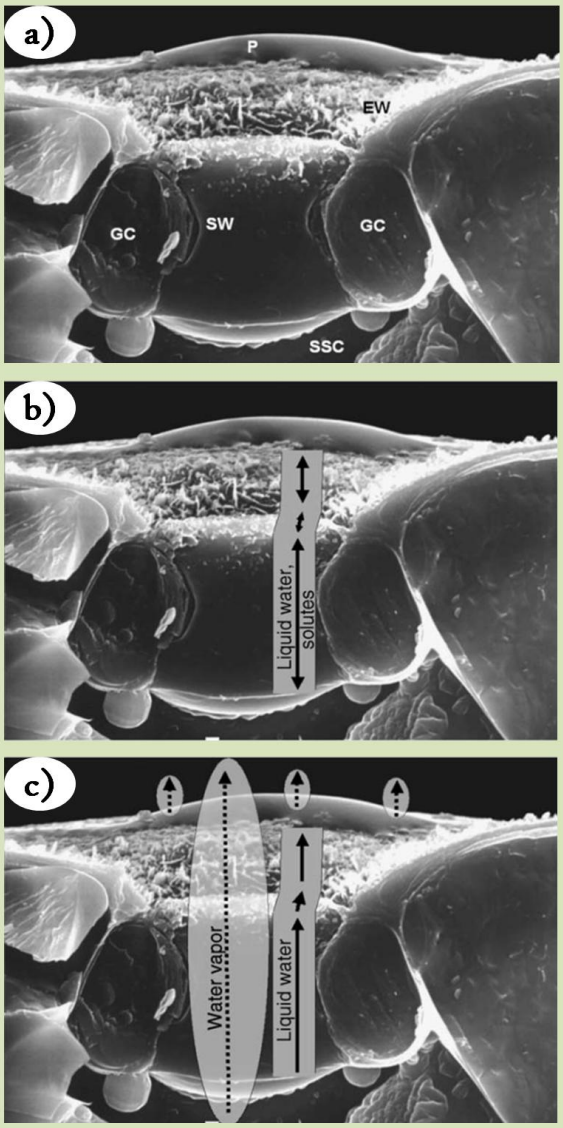


algunos compuestos según distintas rutas de penetración (Cuadro 3) (Wilson 1992; Bondada *et al.* 2006). La eliminación de las ceras cuticulares aumenta muy notablemente la penetración de los compuestos químicos (Santier and Chamel 1998). Sin embargo, la absorción a través de la cutícula depende de las interacciones fisicoquímicas entre la sustancia que penetra y los componentes que forman la cutícula (Tyree *et al.* 1990). Por ello, la composición de la cutícula condiciona más la penetración de los compuestos químicos que su grosor o contenido en ceras (Santier and Chamel 1998; Bondada *et al.* 2006).

La presencia de los estomas aumenta significativamente la absorción foliar, especialmente cuando los estomas están abiertos (Eichert and Burkhardt 2001; Eichert and Goldbach 2008). Parte del efecto de los estomas se ha atribuido a una mayor permeabilidad de la cutícula periestomática que rodea las células oclusivas o estomáticas y que aumentaría cuando los estomas están abiertos (Eichert and Burkhardt 2001; Eichert and Goldbach 2008). No obstante, se ha demostrado la presencia de canales de absorción no cuticulares a través de los estomas asociados a regiones hidrofílicas del poro estomático (Cuadro 4) (Eichert and Burkhardt 2001). La absorción a través de estomas es posible para cualquier sustancia soluble en agua y es menos selectiva al tamaño de las moléculas y a su carga eléctrica que la absorción cuticular (Eichert and Goldbach 2008; Burkhardt 2010).

Al igual que la absorción por raíces, las plantas pueden absorber por las hojas tanto fuentes inorgánicas como orgánicas (urea, aminoácidos o proteínas) de N (Inselsbacher *et al.* 2007; Warren 2009). Sin embargo, la absorción de cada compuesto está asociada a distintas vías de penetración, como se ha indicado. La penetración de los compuestos apolares está determinado por su radio molecular y su capacidad de interacción con los componentes cutícula (Baur *et al.* 1997). La penetración de iones estará determinada por el tipo de carga, solubilidad, capacidad de adsorción y el radio iónico (Tyree *et al.* 1990). Las especies difieren en su capacidad de absorción foliar de los distintos compuestos. Tanto el grosor y composición de la cutícula como la distribución y densidad de los estomas varían ampliamente entre plantas y esto puede explicar en parte las diferencias de absorción foliar entre especies (Baur *et al.* 1997). La mayoría de las adaptaciones a la pérdida de agua foliar disminuyen la capacidad de absorción foliar, como incrementos en el grosor y contenido de ceras de la cutícula (Eichert and Goldbach 2008; Burkhardt 2010). Sin embargo, la pubescencia puede favorecer la absorción foliar (Bussotti *et al.* 2002) ya que pueden retener más agua durante más tiempo en la superficie de la hoja (Kerstiens 1996) y favorecer la deposición de compuestos (Burkhardt 2010). Además, los tricomas

Box 4. *Stomata pathway*.



The stoma structure of *Allium cepa* with an amorphous salt particle on the surface is illustrated with a cryo-scanning electron microscopy picture. **(a)** Details of the epicuticular waxes (EW), guard cells (GC), stoma wall (SW), substomatal cavity (SSC), and particle (P). The formation of a liquid water connection **(b)** along the stoma walls is called "hydraulic activation of stomata" (HAS). HAS enables stomatal transport of solutes and liquid water (solid arrows) in both directions. Once established, a liquid water connection across the stomata will provide a continuous pathway for the flow of water and solutes as long as stomata remain opened. **(c)** Wicking of water caused by HAS and salt on the surface. Liquid water flows along stoma walls to the salt particle on the leaf surface, where it evaporates. This is the "wick" part of a system with split transpiration, in addition to transpired water vapor (dotted arrows). Higher HAS can therefore be expected when hygroscopic particles increase on leaf surface. The thickness of the liquid water connection through stomata is approximately 100 nm or less. Water will be transported more rapidly and efficiently via this hydraulic connection through the stomata than across the cuticule, which has high hydraulic resistance (Source: (Burkhardt 2010).

de algunas especies favorecen la penetración cuticular, tanto por absorción directa como por un menor grosor de la cutícula en su base (Benzing *et al.* 1976; Schreiber 2005).

Aparte de los conocidos efectos de la fertilización foliar sobre el estado nutricional de especies agronómicas, las plantas en ecosistemas "naturales" también pueden adquirir nutrientes a través de las hojas y esto debe ser útil en

circunstancias concretas, ya que reduce la competencia con la absorción radical de otras plantas (Sparks 2009). La absorción foliar puede ser una ventaja en ecosistemas en los que se produce una importante entrada atmosférica de nutrientes, como en muchos ecosistemas áridos, donde la baja disponibilidad de nutrientes y agua en suelo limitan la absorción radical y transporte de nutrientes a las hojas (Burkhardt 2010). Durante las últimas décadas, las actividades humanas han incrementado dramáticamente las concentraciones de N atmosférico. En algunas regiones, incluida la mediterránea, constituye un importante problema medioambiental y aumenta la contribución del compartimento atmosférico al ciclo del N (Brumme *et al.* 1992; Sparks 2009). Por ejemplo, se han detectado valores de deposición de N superiores a 15-22 kg N ha^{-1} $año^{-1}$ en encinares (Avila *et al.* 2002; Roda *et al.* 2002) o 38 kg N ha^{-1} $año^{-1}$ en pinares de pino carrasco (Michopoulos *et al.* 2004). En la Cuenca mediterránea la deposición de N está dominada por N inorgánico, principalmente, óxidos de nitrógeno (NO_x) y amonio (NH_4^+), pero una fracción importante de N depositado es orgánico, alcanzando hasta un 25% del N total depositado. Entre el 20 y el 50% de N orgánico está compuesto por aminoácidos y hasta un 10% es urea (Cornell 2011).

Aparte de los beneficios asociados a la absorción foliar de nutrientes, una excesiva deposición de partículas en la superficie foliar puede tener efectos negativos, como degradación de la estructura foliar y necrosis (Fageria *et al.* 2009; Burkhardt 2010). Por ello, mientras algunas especies maximizan la intercepción de la deposición, otras la minimizan aumentando la hidrofobicidad de la superficie foliar o reduciendo la densidad de estomas y distribuyéndolos en la parte inferior de la hoja (Burkhardt 2010). Estas adaptaciones son muy habituales en especies de ambientes costeros donde los aerosoles salinos son constantes. Muchos de los procesos de decaimiento de masas forestales detectados en Europa se asocian a niveles crecientes de deposición atmosférica (Sparks 2009; Burkhardt 2010). Las especies deben responden ante cambios en los niveles de deposición, aprovechando su máximo potencial y disminuyendo los efectos nocivos. Por ejemplo, se han detectado cambios en los estomas aumentando el contenido de ceras que obstruyen las cámaras epiestomáticas en diversas especies de *Pinus*, y cambios en el contenido de ceras cuticulares ante aumentos en los niveles de deposición (Nicolotti *et al.* 2005). A pesar de la importancia de la absorción de N vía foliar, tanto para procesos de deposición como para diseñar programas de fertilización, el conocimiento sobre los procesos de absorción foliar de N en especies forestales es muy limitado, particularmente en especies mediterráneas.

Metabolización, distribución y removilización del nitrógeno

Metabolización de fuentes de nitrógeno

El metabolismo del N y el del carbono (C) están estrechamente interrelacionados, ya que la asimilación de N requiere la utilización de carbohidratos y la asimilación de C depende de la maquinaria fotosintética rica en N. La asimilación de N, es decir, la conversión de N inorgánico a orgánico, tiene un coste sustancial de C. El coste energético y la velocidad de reacción difieren entre fuentes de N y pueden ser un paso limitante para la absorción de las mismas (Seco *et al.* 2008). El NO_3^- debe ser primero reducido mediante la nitrato reductasa a NH_4^+. El NH_4^+ debe posteriormente ligarse a un esqueleto carbonado antes de poder utilizarse en la biosíntesis mediante la enzima glutamato sintetasa. Además, el NH_4^+ es tóxico para las células vegetales y, por tanto, debe ser asimilado rápidamente a aminoácidos. Como el NH_4^+ no requiere ser reducido para ser asimilado, su metabolismo es más rápido que el del NO_3^- (Calanni *et al.* 1999). Los aminoácidos son precursores en múltiples rutas metabólicas, por lo que su velocidad de transformación es superior a la de las fuentes inorgánicas de N (Maini 2006; Warren 2012). Por todo lo anterior, el coste energético y la velocidad de la asimilación es generalmente superior en el orden NO_3^- >> NH_4^+ > aminoácidos (Zerihun *et al.* 1998). Los lugares de metabolización también varían entre fuentes de N con diferencias entre especies (Bowman and Paul 1992; Campbell 1996; Calanni *et al.* 1999). Los aminoácidos, pueden metabolizarse en toda la planta (Bowman and Paul 1992; Campbell 1996; Calanni *et al.* 1999). Igualmente, las plantas metabolizan el NH_4^+ en toda la planta. Por el contrario, el lugar principal de metabolización del NO_3^- depende de la especie. La mayoría de las especies suelen tener una mayor capacidad de metabolización de NO_3^- en raíces (Andrews 1986). Sin embargo, hay evidencias de que la capacidad de reducción de NO_3^- en raíces tiene un límite máximo y ante una elevada disponibilidad de éste, la enzima nitrato reductasa se induce también en hojas donde la metabolización tiene lugar en presencia de luz y por tanto es más eficiente. Por ejemplo, las gimnospermas generalmente tienen un bajo contenido de nitrato reductasa en las hojas y reducen la mayoría del NO_3^- en las raíces. En cambio, las plantas herbáceas ruderales tienen una alta capacidad de reducción de NO_3^- en hojas. Aunque, por norma general, la metabolización del NH_4^+ en hojas es superior que la del NO_3^-. La síntesis de las enzimas metabólicas se puede inducir por la presencia y concentración de las distintas fuentes de N (Bowman and Paul 1992; Campbell 1996; Calanni *et al.* 1999). Por ello, el incremento en la exposición a una fuente de N aumenta la capacidad de metabolización de la planta.

Distribución y removilización de N

Una vez adquirido y metabolizado, el N y el C se ubicarán en la planta en múltiples posibles destinos: pueden distribuirse a los puntos de alta demanda (tejidos en crecimiento u órganos de reproducción), acumularse como reservas para un uso posterior, y/o quedar secuestrado sin posibilidad de reutilización (Chapin III *et al.* 1990; Millard and Grelet 2010) (Figura 3). Una parte del C y N se pierde por la senescencia de órganos y la secreción de exudados. La removilización del C y N almacenado en las reservas permite apoyar el crecimiento y la reproducción de la planta. La reabsorción (reciclado) ocurre cuando una parte de los nutrientes de los órganos senescentes se recupera previa a su abscisión y se almacena o se usa inmediatamente para apoyar el crecimiento y la reproducción (Millard and Grelet 2010). Las plantas difieren tanto en la capacidad de adquirir nutrientes como en la demanda, la concentración de nutrientes de sus tejidos, el momento y eficacia de la reabsorción (Lambers 2008).

El crecimiento primaveral constituye un importante sumidero de recursos para las plantas perennes. La demanda de C y N de los tejidos en crecimiento puede ser cubierta mediante la adquisición de recursos externos

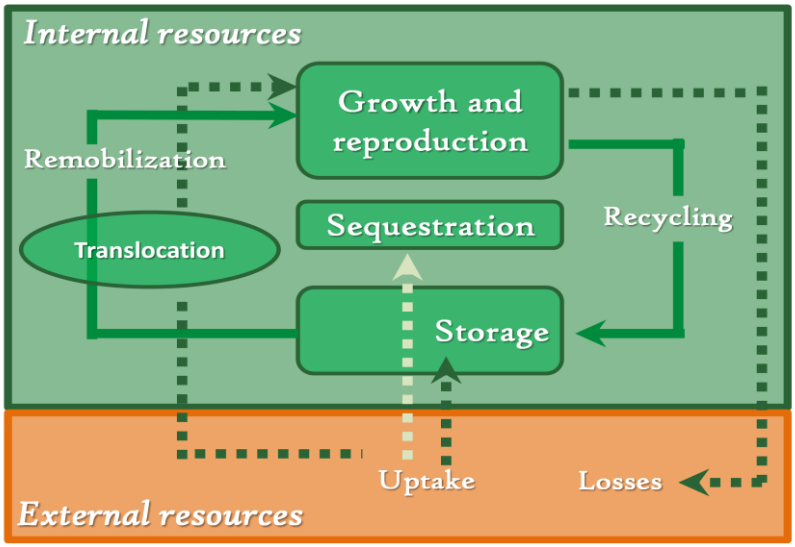


Figure 3. *Schematic representation of N and C acquisition and internal cycling in trees.* Exchange of resources between external sources and internal pools in the tree are shown as dotted lines: for uptake directly into storage, sequestration or use in growth, reproduction or other metabolism; for losses via senescence and abscission of leaves and roots. Internal cycling of resources is shown in solid lines, as seasonal remobilization from storage pools via translocation to other tissues for growth, reproduction or other metabolism. Recycling occurs when resources are withdrawn from tissues (usually during senescence, prior to abscission) for storage. Source Millard and Grelet (2010).

(absorción radical y foliar de N o fotosíntesis, respectivamente) o de la removilización de las reservas internas (Cerasoli *et al.* 2004; Millard and Grelet 2010). En las plantas leñosas, una buena parte de la demanda de C y N de los órganos en crecimiento en primavera se cubre con las reservas internas (Nambiar and Fife 1991; Millard and Grelet 2010).

La removilización de C es un proceso dirigido por la intensidad de la demanda de C del tejido en crecimiento (Millard and Grelet 2010). El C almacenado en los árboles presenta los valores mínimos durante la primavera como consecuencia de las pérdidas por respiración durante el invierno y del uso de reservas para la brotación, el crecimiento secundario y la floración. Sin embargo, en especies perennes la asimilación de C en invierno contribuye fuertemente a las reservas de C (Hansen *et al.* 1996; Körner 2003; Cerasoli *et al.* 2004; Kuptz *et al.* 2011). Por ello, la reducción de C durante el invierno y la primavera es menos intensa que en especies caducifolias. Durante el verano el contenido de C se incrementa como consecuencia de una ganancia neta por fotosíntesis, llegando a presentar un máximo en otoño.

La removilización de N está estacionalmente programada y, a diferencia del C, depende de la cantidad de N almacenado (proceso dirigido por el tamaño o intensidad de la fuente), y no por la demanda del nuevo crecimiento (Millett *et al.* 2005; Millard and Grelet 2010). Las reservas de N presentan los mínimos valores al final de la primavera, debido a su utilización para el crecimiento, y se incrementan cuando la absorción desde el suelo aumenta, generalmente en otoño. La absorción de N continúa durante el invierno hasta alcanzar un máximo en primavera (Loescher *et al.* 1990; Silla and Escudero 2003; Ueda and Tokuchi 2012).

Los nutrientes se almacenan en distintas partes de la planta, dependiendo del tipo del hábito foliar, de la especie y del nutriente. El C se acumula en toda la planta: tallos, corteza, y raíces (Spann *et al.* 2008), y en especies perennes también en hojas (Nambiar and Fife 1987; Rapp *et al.* 1992; Cerasoli *et al.* 2004; Palacio *et al.* 2007b). Como las plantas leñosas caducifolias pierden las hojas durante una parte del año, almacenan proporcionalmente más carbohidratos de reserva en tejidos leñosos que las perennifolias (Chapin III *et al.* 1990). Los lugares de almacenamiento de N en los árboles están restringidos a partes específicas dependiendo de la estrategia funcional de la especie (Millard 1996). Las plantas leñosas caducifolias tienden a almacenar el N en tejidos perennes como la madera y las raíces o la corteza (Millard and Proe 1991; Millard 1996; Cooke and Weih 2005), por el contrario, las plantas perennifolias almacenan el N principalmente en hojas, especialmente en las más jóvenes (Nambiar and

Fife 1991; Neilsen *et al.* 2001; Salifu and Timmer 2003; Silla and Escudero 2003). Aparte del hábito foliar, la capacidad de rebrote también determina el patrón de distribución de C y N, de forma que las especies rebrotadoras generalmente distribuyen más biomasa, C y N en las raíces que las especies no rebrotadoras (Palacio *et al.* 2007a).

La contribución de las reservas al crecimiento anual a escala de planta completa se ha estudiado principalmente en plantas leñosas caducifolias y en coníferas perennifolias. Sin embargo, apenas existe información sobre frondosas perennifolias, como las de los ecosistemas mediterráneos (ver Cerasoli *et al.* 2004; El Omari *et al.* 2003). Se han realizado numerosos estudios en especies mediterráneas sobre la removilización y reabsorción de carbohidratos y reservas de N a escala de rama o de hojas (Escudero *et al.* 1992; Pugnaire and Chapin 1993; Cherbuy *et al.* 2001; Milla *et al.* 2005). Sin embargo, dichos estudios proporcionan una visión muy parcial del reciclado de nutrientes al no incluir órganos potencialmente importantes para el almacenamiento de nutrientes, como las raíces, ni permiten establecer la contribución del C y N removilizado en la construcción de nuevos órganos.

La contribución de las reservas al crecimiento anual varía entre especies caducifolias y perennifolias. Sin embargo, entre especies del mismo hábito foliar pueden existir importantes diferencias, aparentemente sin ningún patrón claro (Chapin III *et al.* 1990; Millard and Grelet 2010). El nuevo crecimiento primaveral en árboles caducifolios depende completamente de los carbohidratos de reserva (Vizoso *et al.* 2008). Sin embargo, a medida que avanza la estación de crecimiento y las hojas nuevas adquieren plena capacidad fotosintética, las reservas de C pierden importancia para apoyar nuevos crecimientos (Dickson *et al.* 1990; Sloan and Jacobs 2008; Keel and Schädel 2010). Por el contrario, en las coníferas perennifolias la principal fuente de C para el nuevo crecimiento primaveral es, habitualmente, la fotosíntesis del momento, mientras que los carbohidratos de reserva tienen un papel menor (van den Driessche 1987; van den Driessche 1991; Rose 1992). Si bien para algunas coníferas los carbohidratos de reserva juegan un papel importante cuando la fotosíntesis está inhibida (Philipson 1988; Bollmark *et al.* 1999). Por otra parte, para las frondosas de hábito perennifolio, apenas existe información. En *Quercus suber*, las reservas de C tienen un papel pequeño en el crecimiento en primavera (Cerasoli *et al.* 2004). Con respecto al N, la contribución de las reservas de N varía desde una completa dependencia (Vizoso *et al.* 2008) hasta una pequeña contribución (menos del 20%) (Millard and Proe 1993; Millard and Grelet 2010).

La cantidad N en el suelo afecta al patrón general de asignación de biomasa y al uso de nutrientes en las plantas, si bien no parece afectar la removilización de N (Millard and Grelet 2010). La proporción de fuentes de N en el suelo también puede afectar el crecimiento y patrón de distribución de biomasa (Guo *et al.* 2002). Mientras algunas especies presentan mayor crecimiento cuando la fuente de N es NH_4^+ (Cruz *et al.* 1993; Yao *et al.* 2011), otras muestran mejor desarrollo con NO_3^- (Atkin and Cummins 1994) o con mezclas de NH_4^+ y NO_3^- (Öhlund and Näsholm 2001; Nicodemus *et al.* 2008).

Aplicaciones del conocimiento del uso del N en plantas

Desde un punto de vista aplicado, el conocimiento de los procesos fisiológicos y ecológicos relacionados con el N y sus distintas formas químicas, puede ser relevante para el ámbito de la restauración forestal y, en particular, para el cultivo de especies forestales. En este contexto, dado que la fuente de N aplicada condiciona los atributos funcionales de las plantas, conocer la proporción óptima entre fuentes de N es importante para producir plantas de calidad. Además, la fertilización nitrogenada influye en la cantidad de reservas de N, la capacidad fotosintética y la tolerancia a factores de estrés de los plantones (Vilagrosa *et al.* 2006; Andivia *et al.* 2011; Villar-Salvador *et al.* 2013). Una fertilización alta de N en vivero favorece el desarrollo en campo (Villar-Salvador *et al.* 2012). Así, controlando la proporción entre fuentes y las concentraciones de N, se puede maximizar la calidad de planta y la eficacia y eficiencia de la fertilización, minimizando, por tanto, la contaminación ambiental. La nutrición mineral de las especies mediterráneas es mucho menos conocida si se compara con la de especies de otros biomas (Oliet *et al.* 2004). Además el conocimiento sobre la respuesta morfo-fisiológica ante distintas fuentes de N en especies forestales mediterráneas se reduce, hasta donde hemos podido observar, a dos especies, *Ceratonia siliqua* L. y *Pinus pinaster* Ait. (Cruz *et al.* 1993; Cruz *et la.* 1997; Warren and Adams 2002, respectivamente). Por otro lado, el uso de la fertilización foliar en el cultivo de especies forestales es prácticamente desconocida, aunque potencialmente es una herramienta interesante a integrar en los programas de fertilización en viveros. Para ello, es necesario determinar la capacidad de adquisición de nutrientes por vía foliar en especies forestales, concretamente especies mediterráneas, y la fuente de N más efectiva para la sobrecarga de N.

Por último, la supervivencia de las plantas en repoblaciones en ambientes mediterráneos depende de que las plantas generen un amplio sistema radical con el que asegurarse el abastecimiento hídrico durante la sequía estival (Padilla

and Pugnaire 2007; Villar-Salvador *et al.* 2012). Por ello conocer si las especies de plantas dependen de las reservas o del C y N recientemente asimilado para la construcción de los nuevos órganos es importante para definir los protocolos de cultivo en vivero que potencien dichas capacidades.

Objetivos, hipótesis general y estructura de la tesis

El **objetivo general** de la Tesis Doctoral es comparar la capacidad de absorción de N y sus distintas formas químicas, distribuir y removilizar N y C, así como la respuesta morfológica y fisiológica ante distintas fuentes de N en plantas forestales mediterráneas. La investigación presentada en esta memoria se justifica por la necesidad de comprender una serie de procesos eco-fisiológicos relacionados con el uso del N que son poco conocidos en plantas mediterráneas y que considero que son relevantes para entender su ecología.

La **hipótesis general** de la Tesis es que el N juega un papel central en la ecología de las especies forestales mediterráneas, ya que éstas difieren en su capacidad de adquisición, distribución, removilización y respuesta al N dependiendo de sus características funcionales. Para ello, se han estudiado diversos procesos fisiológicos muy relevantes del ciclo de uso del N por las plantas: adquisición, almacenamiento y removilización y respuesta funcional.

Objetivos específicos

Los objetivos específicos de la tesis se han organizado en tres categorías según el principal proceso: adquisición de N, almacenamiento y removilización de las reservas de N y C y la respuesta funcional ante las distintas fuentes de N.

I. Los dos primeros objetivos específicos se centran en la **adquisición de N,** tanto por vía radical como por vía foliar, en un grupo ecológicamente heterogéneo de plantas forestales mediterráneas:

Objetivo específico 1. Comparar la capacidad de absorción total de N y las preferencias de absorción por las raíces y las hojas de formas inorgánicas (NO_3^- y NH_4^+) y orgánicas (aminoácidos y/o urea) de N, así como los mecanismos y las bases ecológicas de dicho proceso.

Objetivo específico 2. Estudiar la capacidad de las plantas mediterráneas de absorber aminoácidos de forma intacta.

Las principales cuestiones que me he planteado en relación con estos objetivos son:

- ¿Difieren las especies mediterráneas en su capacidad de adquisición de N?
- ¿Existe alguna relación entre las características ecológicas y funcionales de las especies y su capacidad de absorción de N y sus formas químicas?
- ¿Existe una segregación de nichos ecológicos en base a un uso diferencial de las fuentes de N?
- ¿Son las especies mediterráneas capaces de absorber aminoácidos intactos?
- ¿Influye el nivel de micorrización en la capacidad de absorción de aminoácidos?

II. El segundo proceso analizado es el **almacenamiento y removilización de las reservas de C y N** en cuatro especies leñosas perennifolias mediterráneas, siendo los objetivos abordados:

Objetivo específico 3. Comparar el patrón de almacenamiento del C durante el otoño-invierno y determinar la importancia de los distintos órganos de las plantas en la removilización de reservas de C y N durante la primavera.

Objetivo específico 4. Comparar la contribución relativa del C y N removilizado a partir de las reservas con la adquirida en el momento (absorción edáfica del N y fotosíntesis del momento) en la construcción de los órganos nuevos en primavera.

Las principales cuestiones que me he planteado son:

- ¿Qué partes de las plantas son utilizadas para el almacenamiento del C asimilado en condiciones invernales?
- ¿Existen partes de la planta que se priorizan para dicho almacenamiento?
- En especies leñosas mediterráneas perennifolias, ¿varía el uso de las reservas de C y N para la construcción de las nuevos órganos según la fase de desarrollo ontogénico durante la primavera?
- ¿Existen diferencias interespecíficas en la cantidad de C y N removilizado y el órgano que suministra dichas reservas?

III. Por último, dado que las distintas fuentes de N pueden tener consecuencias sobre el funcionamiento de la planta, se plantean dos objetivos específicos sobre la **respuesta funcional** de *Quercus ilex* y *Pinus halepensis*:

Objetivo específico 5. Analizar el efecto de la proporción y concentración de NH_4^+ y el NO_3^- sobre el crecimiento y fisiología de las plántulas de *Q. ilex* y *P. halepensis*, así como los mecanismos subyacentes en la respuesta de las plantas.

Objetivo específico 6. Estudiar el papel de la fertilización foliar con aminoácidos sobre el desarrollo de la planta.

Las preguntas que se han formulado son:

- ¿Depende la respuesta fisiológica y morfológica de las especies a la proporción y concentración de NH_4^+ y el NO_3^- en el fertilizante?
- ¿Condicionan las características ecológicas de las especies la respuesta a la proporción y concentración de NH_4^+ y el NO_3^-?
- ¿Puede la fertilización foliar con aminoácidos promover el desarrollo de las especies forestales?

Estructura de la tesis

Para abordar estos objetivos específicos, la Tesis Doctoral se ha estructurado en siete capítulos. Después del presente capítulo introductorio (Capítulo 1), se desarrollan los Capítulos 2 a 5 en los que se presentan los resultados de cuatro experimentos que analizan distintos procesos del ciclo de uso del N en plantas (Figura 4). Estos capítulos se corresponden con artículos científicos enviados a revistas científicas internacionales o en fase de preparación para su envío a una revista. Están escritos en inglés con sus correspondientes apartados de Resumen, Introducción, Material y Métodos, Resultados, Discusión y Bibliografía. Incluyen además un Resumen en español. Su estructura y contenidos se han mantenido lo más parecido posible a los manuscritos enviados. Esta estructura puede llevar a cierta repetición en la información aportada, pero facilita la comprensión de los capítulos de forma totalmente independiente.

En el **Capítulo 2** "*Mediterranean forest species have different uptake capacity and preference for nitrogen forms: evidence for an N-based fundamental niche differentiation*" se compara la capacidad y preferencia de absorción de distintas fuentes de N (glicina, NH_4^+ y NO_3^-) en nueve especies forestales mediterráneas (*Objetivo específico 1*) y se relacionan con su ecología y atributos funcionales. También, se estudia la capacidad de absorber por las raíces aminoácidos intactos (*Objetivo específico 2*). Las especies seleccionadas son: *Quercus ilex, Q. faginea,*

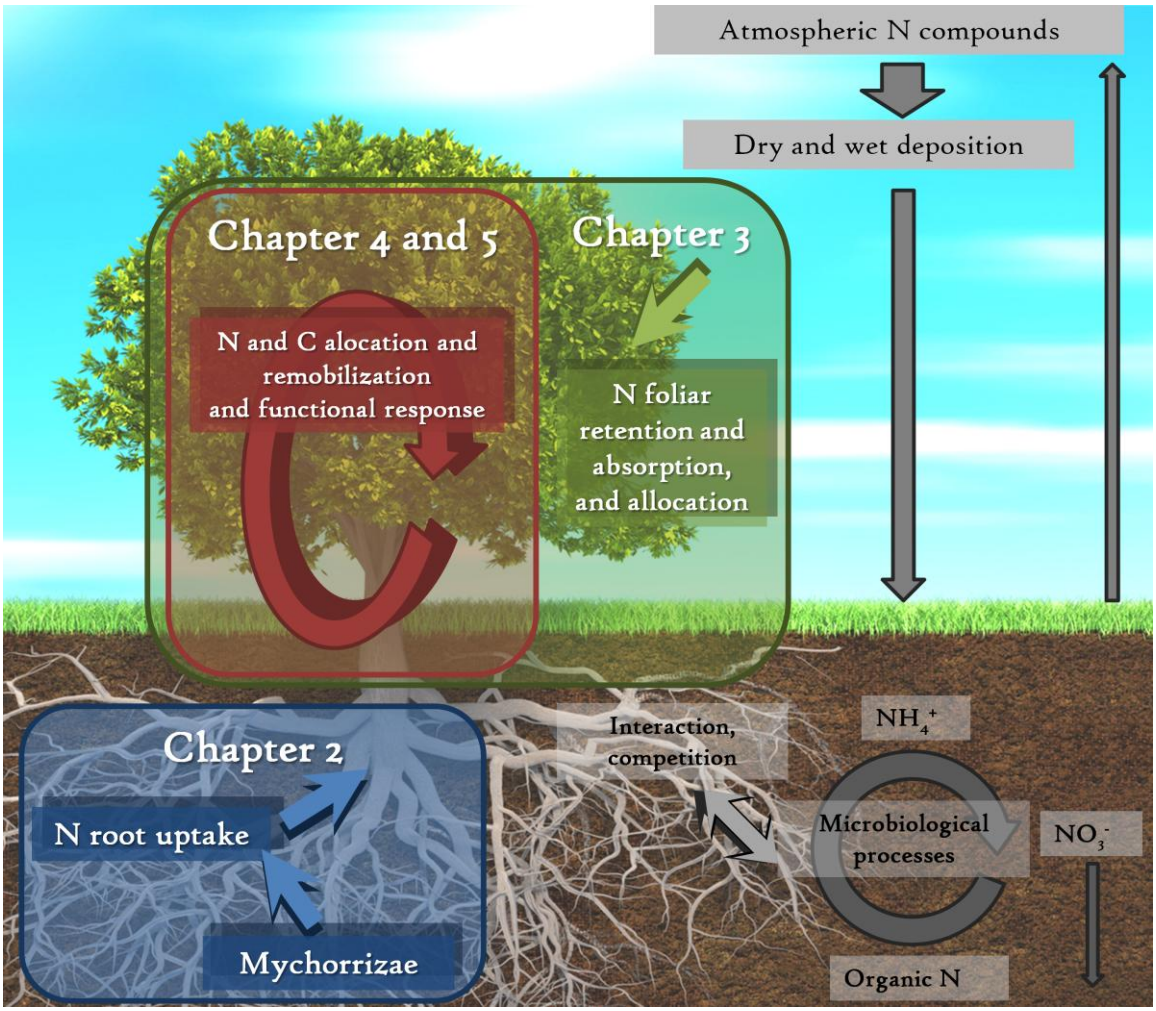


Figure 4. *Schematic structure of the Thesis.* Chapter 1 compares root uptake of N sources (glycine, NH_4^+ and NO_3^-) in nine Mediterranean ecologically and functionally distinct forest species, which dominante different stages of the ecological succession. Chapter 2 analyzes foliar absorption of N forms (glycine, urea, NH_4^+, and NO_3^-) in two Mediterranean trees and translocation to roots. Chapter 3 compares the storage of winter assimilated C and the contribution of remobilized C and N for new growth in four Mediterranean woody species. Finally, Chapter 4 analyzes the effect of N forms on the mid term morphophysiological responses in two Mediterranean trees.

Pinus nigra, *Juniperus thurifera*, *Rhamnus alaternus*, *Rosa canina*, *Thymus vulgaris*, *Santolina chamaecyparissus* y *Brachypodium retusum*. Este conjunto comprende especies de distinto hábito foliar y forma de crecimiento, desde herbáceas (*B. retusum*) y caméfitos (*T. vulgaris* y *S. chamaecyparissus*), hasta arbustos (*R. alaternus* y *R. canina*) y árboles (restantes), que ocupan distintas etapas en la sucesión ecológica. La amplia diversidad de especies estudiadas permite testar si, en especies mediterráneas, las preferencias por las fuentes de N varían a lo largo de la sucesión, y si el consumo de diferentes fuentes de N puede ser un

factor que explique la segregación de especies en función de sus nichos ecológicos fundamentales.

En el **Capítulo 3** "*Foliar absorption and root translocation of nitrogen sources in seedlings of two Mediterranean trees*" se estudia la capacidad de adquirir N orgánico intacto por las hojas y la absorción foliar de distintas fuentes de N (NH_4^+, NO_3^-, glicina y urea) (*Objetivo específicos 1 y 2*) y su translocación a las raíces como indicador de la capacidad de movilizar N desde las hojas a otros puntos de demanda. El estudio incluye la medida de la conductancia cuticular como indicador de la permeabilidad a través de la cutícula. Por último, se analiza la influencia de las fuentes de N en la sobrecarga de N de las plantas, un aspecto que tiene una importante aplicación para el cultivo de ambas especies en el vivero. El estudio se ha llevado a cabo con plantones de *Quercus ilex* y *Pinus halepensis*, puesto que son especies dominantes en muchos ecosistemas forestales mediterráneos, presentan estrategias ecológicas y funcionales contrastadas y son las especies más utilizadas en repoblaciones forestales de zonas mediterráneas.

En el **Capítulo 4** "*Growth capacity determines the contribution of carbon and nitrogen remobilization to seedling growth in Mediterranean evergreen woody plants*" se analiza en qué órganos se produce el almacenamiento del C asimilado durante el invierno, así como la contribución del C y N removilizado en el crecimiento de las nuevas raíces y nuevos brotes en primavera (Objetivos específicos 3 y 4) en cuatro especies leñosas mediterráneas perennifolias: *Quercus ilex, Q. coccifera, Olea europaea* y *Pinus hapelensis*. También se determina cuáles son los principales órganos que proporciona el C y N removilizado. Al igual que en capítulos precedentes, las especies seleccionadas son especies ampliamente distribuidas en el ámbito mediterráneo y representan estrategias ecológicas y grupos filogenéticos muy distintos.

En el **Capítulo 5** "*Nitrogen form and concentration interactively affect the performance of two ecologically distinct Mediterranean forest trees*" se estudian los efectos morfo-fisiológicos de la absorción radical de fuentes inorgánicas de N, NH_4^+ y NO_3^- a dos concentraciones contrastadas (*Objetivo específico 5*), así como la fertilización foliar con aminoácidos (*Objetivo específico 6*) en *Q. ilex* y *P. halepensis*. Además, se analizan los mecanismos subyacentes que explican la respuesta de las especies estudiadas mediante la medición de propiedades del medio de cultivo, intercambio gaseoso y pigmentos fotosintéticos. Con ello, se espera poder establecer cómo afectan las fuentes de N a distintas funciones fisiológicas, a la distribución de biomasa en la planta, y las proporciones entre fuentes de N o la fuente de N más adecuada para el cultivo en vivero de estas

especies. Además se analiza la eficacia de la fertilización foliar con aminoácidos en el cultivo de estas especies forestales.

En el penúltimo capítulo (**Capítulo 6**) se realiza una discusión general que integra los resultados del conjunto de la tesis. Por último, la memoria finaliza con las principales conclusiones de la Tesis Doctoral (**Capítulo 7**).

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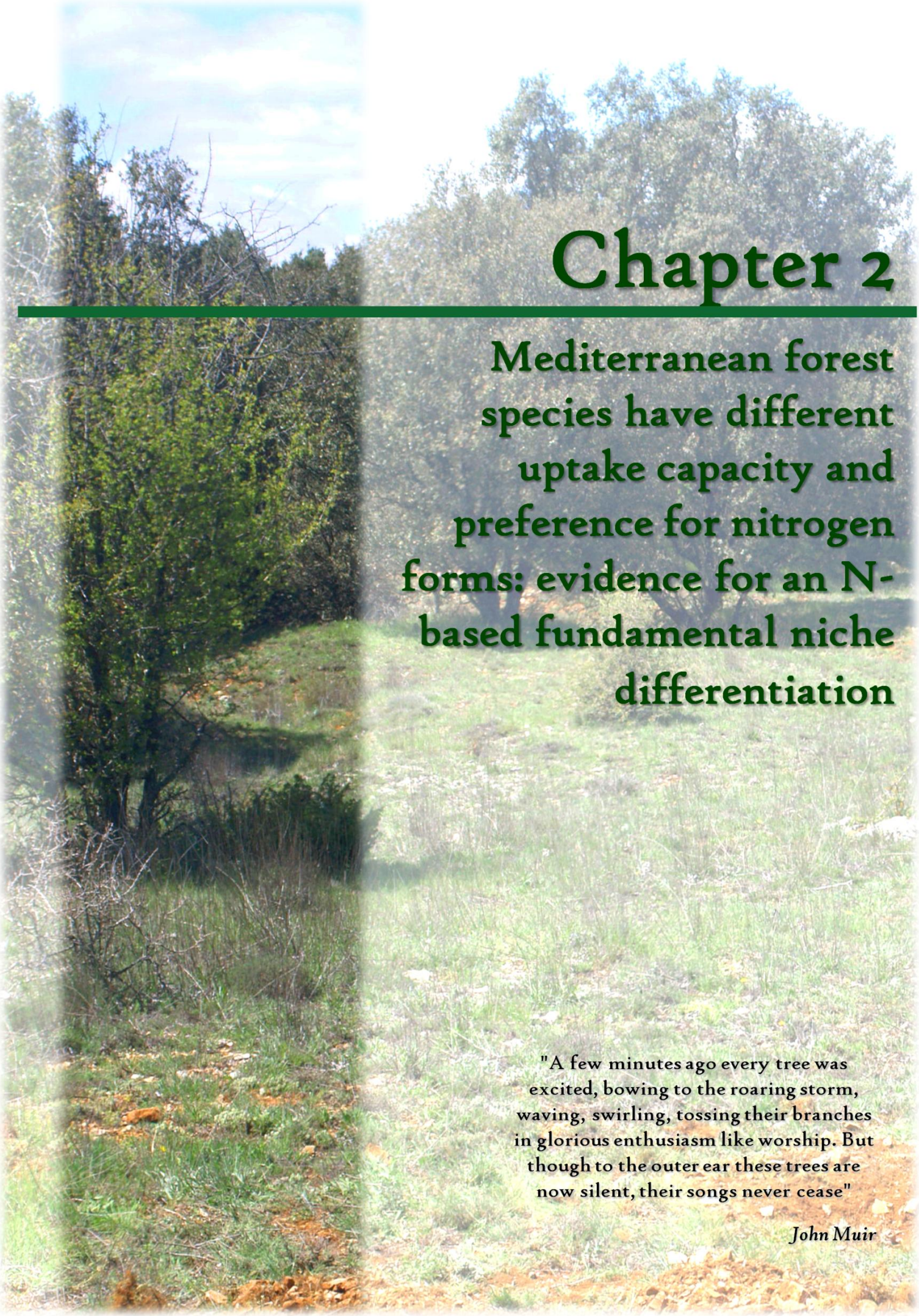

Chapter 2

Mediterranean forest species have different uptake capacity and preference for nitrogen forms: evidence for an N-based fundamental niche differentiation

"A few minutes ago every tree was excited, bowing to the roaring storm, waving, swirling, tossing their branches in glorious enthusiasm like worship. But though to the outer ear these trees are now silent, their songs never cease"

John Muir

Este capítulo reproduce el texto del siguiente manuscrito:

This chapter reproduces the text of the following manuscript:

Uscola M., Villar-Salvador P., Oliet Palá J.A. & Warren. C.R. 2013. Mediterranean forest species have different uptake capacity and preference for nitrogen forms: evidence for an N-based fundamental niche differentiation (In preparation).

Fotography: Mediterranean oak forest in Mandayona (Guadalajara)

By: M. Uscola

Las especies Mediterráneas forestales tienen distinta capacidad de absorción y distinta preferencia entre fuentes de nitrógeno: evidencias en la diferenciación de nicho funcional en base al uso del nitrógeno.

Resumen

Se considera que el agua y la luz son los recursos que más limitan la vida de las plantas en los ecosistemas forestales mediterráneos. Sin embargo, la coexistencia de especies con una estrategia similar de uso de agua y luz podría facilitarse si se diese una complementariedad de nichos ecológicos debida a diferencias en la adquisición de distintas formas químicas de N. En este experimento se analiza la capacidad de absorción de N y la preferencia por distintas fuentes de N en nueve especies forestales mediterráneas que representan un amplio rango de formas de crecimiento y ocupan distintas etapas sucesionales. La absorción de glicina doblemente marcada (^{13}C y ^{15}N) se comparó a la absorción de nitrato ($^{15}NO_3^-$) y amonio ($^{15}NH_4^+$) marcados en un experimento en invernadero en plantas de un año de edad que crecieron en macetas con suelo natural procedente de un bosque de *Quercus ilex* L.. La concentración de aminoácidos libres e intercambiables en el suelo, extraídos con 0.5 M K_2SO_4, fue de la misma magnitud que la concentración de N inorgánico, mientras que la concentración de NO_3^- fue generalmente superior a la del NH_4^+. Todas las especies presentaron la capacidad de absorber glicina intacta, y la simbiosis con micorrizas vesículo arbusculares (VAM) estuvo positivamente relacionada con la capacidad de absorción de glicina. Sin embargo, las VAM no tuvieron efecto en la absorción de N inorgánico. La colonización de ectomicorrizas fue demasiado baja y no se detectó relación con la absorción de ninguna forma de N. La herbácea y los caméfitos tuvieron mayor capacidad de absorción de N que los arbustos y los árboles. En todas las especies, la tasa de absorción de N procedente del NH_4^+ fue superior a la de glicina o del NO_3^-. Entre especies, la absorción de N estuvo positivamente relacionada con la longitud específica de las raíces y la actividad nitrato reductasa, pero no con la tasa de crecimiento relativo (RGR) o la concentración de N en hojas. Todas las especies utilizaron preferentemente NH_4^+ a expensas de NO_3^-, a pesar de que su abundancia en el suelo fue menor que la de NO_3^-. La absorción de glicina relativa a la disponibilidad en suelos no mostró ninguna tendencia clara entre especies. La similitud (S_i) entre el N absorbido de cada fuente y la disponibilidad de fuentes de N en el suelo, estuvo negativamente relacionada

con la preferencia por el NH_4^+. Además, RGR estuvo positivamente relacionada con la preferencia por el NH_4^+. Así, las especies de rápido crecimiento presentaron una alta preferencia por NH_4^+ y baja S_i, mientras que las especies de lento crecimiento presentaron baja o nula preferencia por el NH_4^+ y utilizaron la fuente de N más abundante en el suelo, es decir, baja S*i*. Las especies pioneras tendieron a tener menor S_i y mayor preferencia por el NH_4^+ que especies de etapas más maduras de la sucesión. Las especies que co-existen en la misma etapa sucesional presentaron diferente capacidad de absorción de N y distintas preferencias por las fuentes de N. Entre las pioneras, se encontraron diferencias tanto en la tasa de absorción como en la preferencia por el NH_4^+. Entre las más tardías, mientras los árboles dominantes utilizaron principalmente el NH_4^+, los arbustos subordinados prefirieron el NO_3^- o la glicina. Este estudio evidencia que las especies forestales mediterráneas muestran diferentes nichos fundamentales en base al N y que la preferencia por el NH_4^+ puede ser un determinante de la velocidad de crecimiento de las plantas. Se concluye que el N puede ser un importante vertebrador de la estructura de las comunidades forestales en ecosistemas mediterráneos, al igual que el agua y la luz.

Palabras clave: Aminoácido; amonio; crecimiento; glicina; micorriza; nicho ecológico; nitrato; sucesión ecológica.

Mediterranean forest species have different uptake capacity and preference for nitrogen forms: evidence for an N-based fundamental niche differentiation

Abstract

Water and light are considered the main resources constraining plant life in Mediterranean forest ecosystems. However, coexistence of species that compete for water and light could be facilitated by niche complementary due to uptake of different forms of nitrogen (N). We examined the N uptake capacity and preference for different N forms in nine Mediterranean forests species representing a range of growth forms and occupying different successional stages. Uptake of dual-labeled (^{13}C and ^{15}N) glycine was compared to labeled nitrate ($^{15}NO_3^-$) and ammonium ($^{15}NH_4^+$) in a greenhouse experiment using one-year-old seedlings growing in pots with natural field soil collected from a *Quercus ilex* L. forest. The pool of free and exchangeable amino acids, in 0.5 M K_2SO_4 extracts of soil, was as big as the total inorganic N pool, while the NO_3^- pool was generally bigger than NH_4^+ pool. All the species were able to take up intact glycine and vesicular arbuscular mycorrhyza (VAM) was positively related to glycine uptake. However VAM had no effect on inorganic N uptake by plants. Ectomycorrhiza colonization was too low and no effect was detected on N form uptake by plants. Chamaephytes and herb species had higher total N uptake than shrub and tree species. Overall, the N uptake rate of NH_4^+ was higher than for glycine and NO_3^-. Across species, N uptake was positively related to specific root length and plant nitrate reductase activity, but unrelated to relative growth rate (RGR) or the leaf N concentration. All species preferred NH_4^+ at the expense of NO_3^-, despite NH_4^+ being less abundant than NO_3^- soil. Glycine uptake relative to soil N availability did not show any clear trend among species. Similarity (S_i) between the proportion of N forms taken up and the proportion of N forms in soil was negatively related to species NH_4^+ preference. Species RGR was linked to NH_4^+ preference, with fast growing species having high NH_4^+ preference and low S_i, while slow growing had low or any NH_4^+ preference and used the N form most available in soil, i.e. high S_i. Pioneer species tended to have lower S_i and higher NH_4^+ preference than mid to late successional species. Co-occurring species within a successional stage showed different N uptake capacity and N forms preference. Pioneer species differed in N uptake capacity and NH_4^+ preference intensity among them. Among the late successional species we found that dominant trees mainly used

NH_4^+, while the subordinate shrubs preferred NO_3^- or glycine. This study provides evidence for N-based fundamental niche segregation in Mediterranean forest species and that NH_4^+ preference can be a determinant of plant growth capacity. Therefore we conclude that N is a driver of the structure of forest communities in Mediterranean ecosystems, such as water and light.

Keywords: Amino acid; ammonium; ecological niche; ecological succession; glycine; growth; mycorrhiza; nitrate.

Introduction

Mediterranean ecosystem have higher taxonomic and functional diversity than other temperate biomes (Cowling *et al.* 1996; Myers *et al.* 2000). Herbs, chamaephytes, and deciduous and evergreen shrubs and trees typically co-occur in Mediterranean ecosystems. Co-existence of multiple species may be possible if spatial and temporal variations in resource acquisition among plant species lead to resource partitioning (Tilman 1982; Kraft *et al.* 2008). Water and light are considered the main resources constraining plant life in Mediterranean forest ecosystems. Mediterranean plants exhibit significant differences in performance in response to these resources, which potentially enables niche segregation and, consequently, species coexistence (Zavala *et al.* 2000; Sánchez-Gómez *et al.* 2006; Gómez-Aparicio *et al.* 2006). However, coexistence of species, that have a similar use pattern of water and light, could also be possible through niche complementary due to different use of soil resources other than water (Davies *et al.* 1998; Kahmen *et al.* 2006).

Nitrogen (N) is an essential resource for plants, which strongly affects plant performance and distribution (LeBauer and Treseder 2008). Niche segregation among plants according to N can occur through differences in total N uptake rate and differences in preference for different chemical forms of N (Weigelt *et al.* 2005; Pfautsch *et al.* 2009; Ashton *et al.* 2010; Boudsocq *et al.* 2012). Plant growth is frequently limited by soil N availability in Mediterranean ecosystem (Delgado-Baquerizo *et al.* 2011). Therefore, differences in N uptake capacity and N chemical form preference could be axes in species ecological niche and drive species segregation in Mediterranean communities (Filella and Peñuelas 2003; Kahmen *et al.* 2006; Terradas *et al.* 2009). Whether species-specific partitioning of soil N pool occurs in Mediterranean ecosystems is almost unknown.

The classic paradigm of N cycle considers that inorganic N is the only N source absorbed by plants. However, an increasing body of evidences shows that organic N is also a potential N source for plants (Näsholm *et al.* 2000; Harrison *et al.* 2007). For instance, plants are able to acquire amino acids and small proteins as intact molecules (Bardgett *et al.* 2003; Paungfoo-Lonhienne *et al.* 2008; Näsholm *et al.* 2009). This implies that plants and microbes may compete not only for mineralized inorganic N, but also for organic N, which enables plants to short-circuit the mineralization cycle (Schimel and Bennett 2004). The capacity of plants to take up intact amino acids is widespread and has been demonstrated for plants from cold-climate ecosystems, such as in the arctic tundra, taiga and alpine communities (Kielland 1994; Näsholm *et al.* 1998; McKane *et al.* 2002; Xu *et al.* 2010), temperate ecosystems (Templer and Dawson 2004; Warren 2006; Kahmen *et al.* 2009; Gallet-Budynek *et al.* 2009; Schulz *et al.* 2011; Scott and Rothstein 2011), and also in crop plants (Näsholm *et al.* 2000; Ge *et al.* 2009; Jämtgård *et al.* 2010). However, relatively little is known about the N nutrition and metabolism or the ability to absorb organic N of Mediterranean plants. Although soil organic N concentration in Mediterranean ecosystems is 2-3 times lower than in cold, temperate or subtropical communities, organic N concentration is frequently higher than inorganic N in Mediterranean soils (Delgado-Baquerizo *et al.* 2011). Both organic and inorganic N show high seasonal variation in Mediterranean soils and organic N can co-dominate with either NO_3^- in communities dominated by herbs and chamaephytes or NH_4^+ in forest communities during the wet and mild season (Delgado-Baquerizo *et al.* 2011).

Plant species differ in their capacity to acquire N and also in each N form uptake rate. Uptake rate of each N form depends on the interaction between the external and internal levels of the N forms (Britto *et al.* 2001). The morpho-physiological characteristics that determine N acquisition rate are well known (Lambers 2008). Shoot N demand and growth rate are particularly important for determining root N uptake rate (Lambers 2008). Thus, slow-growing plants usually exhibit low tissue N concentration and N absorption rate relative to fast-growing plants. Additionally, N absorption of slow-growing plants responds weakly to increases in soil N concentration (Chapin III 1980; Lambers 2008). Nitrogen for tree growth can also be obtained through the remobilization of internal reserves (Cerasoli *et al.* 2004; Millard and Grelet 2010). N is usually stored in specific plant parts, which vary depending upon leaf habit. Leaves, especially the youngest ones, are major N storage sites in evergreen species (Wendler et al. 1995; Silla and Escudero 2003; Uscola et al.

2013). Thus, N in leaves can contribute to meet N demands decreasing N uptake dependence in the high demands moments.

In contrast, little is known about the functional and ecological basis of preferences for different N form (McKane *et al.* 2002; Weigelt *et al.* 2005). In one of the few studies to contrast fast with slow-growing species it was found that fast-growing herbs preferred inorganic over organic N, while slow-growing herbs had no preference for any N form (Weigelt *et al.* 2005). This relationship between growth capacity and N form preferences in fast growing species, however, has not always been detected (Dunn *et al.* 2006).

The most general explanation for species differences in N form preference is that it reflects species adaptation to the form of N that is most abundant in "natural" habitats (Kronzucker *et al.* 1997; Warren 2006; Kahmen *et al.* 2006). Following this logic, uptake of organic N is competitively advantageous for plants in arctic, alpine and boreal ecosystems (Schmidt and Stewart 1999) because soil microbial activity and N mineralization are low (Kielland 1994; Näsholm *et al.* 1998; McKane *et al.* 2002). In contrast, it is considered that use of organic N is relatively less important in plants from temperate ecosystems than in plants from cold-climate ecosystems due to higher N mineralization and availability of inorganic N (Owen and Jones 2001; Bardgett *et al.* 2003; Schimel and Bennett 2004). For example, organic N seems to have small relevance as a N source in tropical forests (Houlton *et al.* 2007). Disturbance and succession add an extra layer of complexity on top of general differences among biomes. In degraded ecosystems and early stages of ecological succession mineralization rates are high and nitrate (NO_3^-) is the main N form (Rice and Pancholy 1972; Wolt 1994; Kronzucker *et al.* 1997). Pioneer plant species are reputedly high NO_3^- assimilators, due to high nitrate reductase activity (NRa), which predominantly reduce NO_3^- in the shoot, and have a limited amino acid uptake capacity (Stewart *et al.* 1988; Nordin *et al.* 2001; Aidar *et al.* 2003). As ecological succession proceeds in forest ecosystems, mineralization decreases and ammonium (NH_4^+) and amino acids become the main N forms (Rice and Pancholy 1972; Wolt 1994; Kronzucker *et al.* 1997). Late successional plant have an overall preference for NH_4^+, have much higher capacity for amino acid uptake and mainly concentrate N assimilation in the roots (Stewart *et al.* 1988; Nordin *et al.* 2001; Aidar *et al.* 2003).

Preference for N forms can also vary among species living in the same habitat. Differences among plants in N form preference has been reported for temperate grasslands (Weigelt *et al.* 2003), arctic tundra (McKane *et al.* 2002) and alpine communities (Miller and Bowman 2003). This suggests that species

differences in N form preference may lead to segregation of ecological niches by partitioning use of limited soil N, which reduces competition for N and promotes species co-existence (McKane *et al.* 2002; Weigelt *et al.* 2005; Boudsocq *et al.* 2012). For example, the most dominant and productive species in the Alaskan tussock tundra ecosystem use the most available soil N form while the subordinate and least productive species use the less available soil N form (McKane *et al.* 2002). Similarly, differences between species in the take up capacity of inorganic N forms has been invoked to explain competitive exclusion, niche complementary and invasivity (Kronzucker *et al.* 2003; Pfautsch *et al.* 2009; Fraterrigo *et al.* 2011; Boudsocq *et al.* 2012).

Mycorrhizal associations may increase access to N, and thus mycorrhizal plants may have advantage over non-mycorrhizal plants (Näsholm *et al.* 1998, 2000; Andresen *et al.* 2008; Talbot and Treseder 2010). Vesicular arbuscular mycorrhiza (VAM) dominate among herbaceous and woody plant communities on mineral soils, while ectomycorrhizal species (ECM) predominate in forest ecosystems with surface litter accumulation (Read 1991). In comparison with VAM, ECM fungi generally have a more widely distributed network of external hyphae (Simard and Durall 2004; Talbot and Treseder 2010). The ability to access different N forms differs among mycorrhiza, VAM have lower capacity to mobilize organic N than ECM and, overall, have greater preference for NO_3^- than for NH_4^+ or organic N due to its lower protease activity (Read 1991; Talbot and Treseder 2010). This would provide ECM a competitive advantage over plants forming VAM and both mycorrhizal types would have a competitive advantage over non-mycorrhizal plants.

The aim of this study was to examine how Mediterranean forests plants belonging to different functional groups vary in their N uptake capacity and N form preference. The studied species are widely distributed plants in Mediterranean continental woodlands located at mid altitude (700-1100 m a.s.l.) and on limestone soils. Selected species are (1) three dominant mid– to late–succesional trees, *Pinus nigra* susbp. *salzmanii*, *Quercus ilex* subsp. *ballota* and *Q. faginea*; (2) two shrubs, *Rosa canina* and *Rhamnus alaternus,* which appear as subordinated species in mature forests dominated by the previous tree species, but can also colonize recently abandoned croplands; and (3) four early successional species, a tree, *Junipherus thurifera*, two chamaephytes, *Thymus vulgaris* and *Santolina chamaecyparissus*, and a grass, *Brachypodium retusum.* Also species, which differ in growth form and successional stage, have different functional traits such as growth rate (Cornelissen *et al.* 1996; Antúnez *et al.*

2001). To address our objective we performed an experiment in a greenhouse where plants of each species were cultivated individually in mineral soil collected from a Q. *ilex* woodland. We believe that use of field soil provides a more realistic picture of N form uptake and, consequently, potential differences in fundamental niche than using hydroponics (Warren 2009b). After twelve weeks of cultivation, plants were presented with an equimolar mixture of three labeled N sources (^{15}N-glycine, $^{15}NO_3^-$ and $^{15}NH_4^+$, each compound at 0.3 mM). Dual labeled glycine (^{13}C, ^{15}N) was used to determine whether the molecule is taken up intact (Nasholm and Persson 2001). N concentration in leaves, N leaf resorption and nitrate reductase activity were measured as surrogates of plant N demand, recycling and assimilation capacity, respectively. Finally, mycorrhizal type and colonization intensity was measured to take in account its effects in the different N forms uptake by plants.

We tested four hypotheses: (1) Rates of N uptake will be faster in species that are fast growing, which also will have N-rich tissues and high N assimilation capacity than species with the opposite traits; (2) Mediterranean species will be able to acquire intact amino acids; (3) early successional species will have fast N uptake rate and mainly use NO_3^-, while late successional species will have slow N uptake rate and mainly absorb NH_4^+ and the amino acid; and (4) N uptake capacity and N form preference will depend on new root mycorrhizal colonization.

Material and methods

Plant cultivation and experimental design

Seedlings of inland Spain provenances were obtained from a commercial nursery (Fuenteamarga S.A., Cabezón del Pisuerga, Valladolid, Spain) and the nursery of the Centro Nacional de Mejora Genética Forestal "El Serranillo", MAGRAMA. Seedlings were cultivated for eleven months in 300 mL containers (190/300-45, Plasnor, Gipuzkoa, Spain) containing an 1:1 mixture of blonde and black fertilized peat. The seedlings were cultivated according to standard cultivation protocols for Mediterranean nurseries (Villar-Salvador *et al.* 2004).

In early May 2010, before bud break, seedlings were transplanted into trays with 15 cavities of 1800 mL (high of the pot 290mm, 290/2000-15 Plasnor, Guipuzkoa-Spain) and filled with a mixture of soil and vermiculite. Soil was collected at the end of April in a Q. *ilex* forest located in Mandayona

(Guadalajara, Centre of Spain, 40°54′50.4′′N, 2°44′22.56′′W at 1061 m a.s.l.) (Figure 1). The bedrock was limestone and soil was obtained from two microsites: a wide forest gap and under the canopy of a closed *Q. ilex* stand. Vegetation cover in the gap was around 70% and was mainly composed by chamaephytes and graminoids such as *Thymus vulgaris, Thymus zygis*, *Teucrium gnapahalodes*, *Helianthemum spp.*, *Genista scorpius*, *Salvia lavandulifolia*, *Festuca hystrix* and several saplings of *Q. ilex, Rosa sp.* and *Juniperus oxycedrus*. Vegetation cover under the *Q. ilex* canopy soil was <5% and consisted in some graminoids, and individuals of *Asparagus acutifolius* and *Lonicera xylosteum*. Soil samples were taken from the first 15 cm and were sieved to 4 mm to remove large stones and stored in darkness at low temperature for no more than 1 week before being used.

Tray cavities were filled with a 1:1:1 (v/v/v) mixture of vermiculite (granulometry < 3 mm, Projar, Valencia, Spain) and soil from the gap and under the canopy of a *Q. ilex* stand. Vermiculite was added to increase soil aeration. Seedlings were grown in a greenhouse of the Juan Carlos I Royal Botanical Garden, Universidad de Alcalá until early August 2010. Light transmission of the greenhouse was 60% and air temperature varied from 15.3 to 32.0 °C. Pots were randomly arranged in space and their position was rotated every 15 days to minimize positional and edge effect. Seedlings were watered by hand individually with tap water twice a week until two weeks before the labeling experiment. Seedlings did not receive any external nutrients.

The experimental design consisted in a combination of nine species x

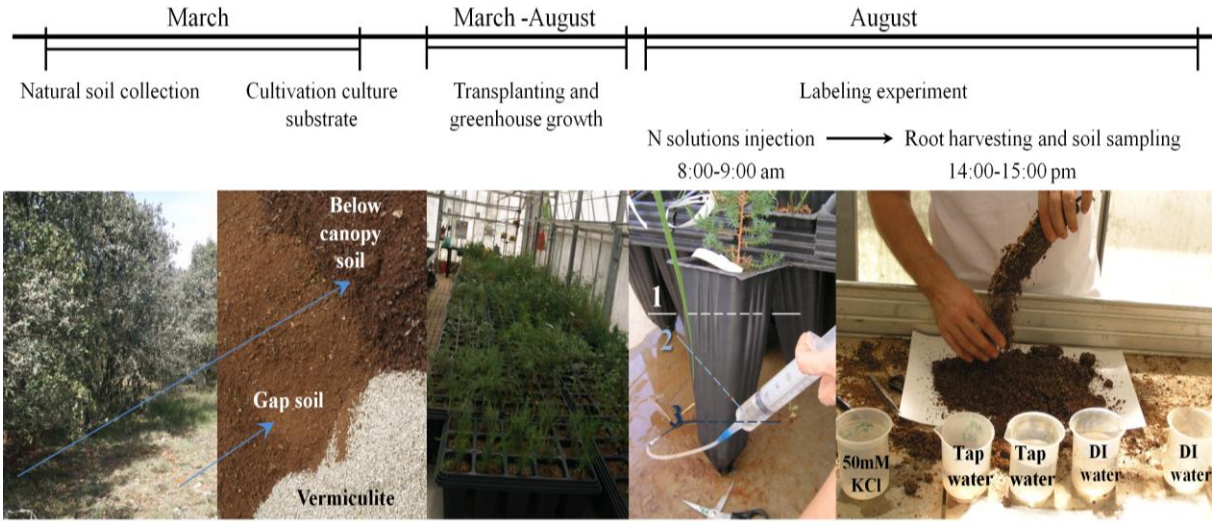

Figure 1. Flow diagram of the key events through the experiment.

three N forms, and for each treatment we had 25 replicates. The N uptake experiment was performed between 1st to 14th August, when all species had already produced abundant new roots.

^{15}N injection into the soil

To acclimate plants to N in the soil solution, plants were watered twice a week for two weeks with 200 mL of a solution containing 0.9 mM total N concentration as an equimolar mixture of ammonium (NH_4^+), nitrate (NO_3^-) and glycine. The last watering was done 2 days before labeling to enable the soil to dry out below field capacity, so that the solutions could be injected without drainage. To determine uptake of N-forms, plants were simultaneously supplied with the three N sources in equimolar proportions, at 0.9 mM total N concentration (*i.e.* 0.3 mM each N form). Three uptake solutions were prepared differing only in which N source was labeled (60 atom% $^{15}NO_3^-$, 60 atom% $^{15}NH_4^+$; ISOTEC Inc., Miamisburg, USA and Glycine 2-^{13}C, 99 atom%; ^{15}N, 98 atom %; Cambridge Isotope laboratory, England). Another solution was prepared with all the N forms in natural abundance.

Fifteen seedlings per specie were applied independent with one of each one of N form labeled solutions (labeled seedlings) and another 5 seedlings per species with the unlabeled solution (unlabeled seedlings). Solutions were injected into the soil between 07:00 and 08:00 h (solar time). Three holes were bored in the ridges of each tray cavity rotating the ridge in each injection. The holes were done at 5, 10, and 15 cm from the top of the container, while the bottom of the container was sealed to prevent fertilizer drainage (Figure 1). Labeled and unlabeled solutions was injected into the soil distributed in three aliquots of 60, 40, and 20 ml, which were injected horizontally from ridge to opposite ridge, in the top, middle and bottom hole, respectively using a 115 mm long perforated plastic needle. At the end only 0.557 mg N kg^{-1} dry soil of each N form was added. This external input of N is low enough to not alter N form proportions in natural soils (see below results soil N pools). The needle had 1 mm holes drilled in spiral every 5 mm. Solutions were horizontally injected to ensure that the fertilizer was evenly spread throughout the soil. Pots were randomly arranged in the greenhouse and plants were harvested 5 to 7 h after solution injection. The greenhouse was well ventilated to prevent assimilation of respired $^{13}CO_2$.

Plant sampling and determination of seedling growth

Ten seedlings per species were randomly sampled before the plants were transplanted to the trays in early May (t_0), and immediately before the labeling experiment (t_1). Shoots were cut at the cotyledon insertion point and separated into leaves and stems. Plugs were carefully washed to eliminate peat from roots. After seedling harvesting (t_0, t_1 seedlings), new and old roots were separated. Roots that protruded out the plug into the growing media were excised and considered as new roots. In most cases, new roots were not seen inside the plug and we assumed that most roots in the plug had grown during the nursery cultivation period. Then all seedling parts were washed with tap water, rinsed in distilled water and dried in a ventilated oven at 65 °C for 48 h to measure their mass. At t_1, seedlings were smaller than pots, thus root growth was not limited by pots size, and seedling mass (mean ± 1 SE) was: *B. retusum* 9.5±0.9, *T. vulgaris* 3.6±0.2, *S. chamaecyparissus* 2.8±0.2, *R. canina* 6.9±0.4, *R. alaternus* 8.7±0.2, *J. thurifera* 4.7±0.3, *P. nigra* 6.2±0.4, *Q. faginea* 6.7±0.6, *Q. ilex* 6.1±0.3 g.

Relative growth rate (RGR) was calculated as:

$$RGR = \frac{\ln(DM_1) - \ln(DM_0)}{(t_1 - t_0)} \qquad (mg\ g^{-1}\ day^{-1}) \qquad (1)$$

where DM_0 and DM_1 are the dry mass of whole seedlings before transplanting (t_0) and at the end of the experiment (t_1), respectively, and (t_1-t_0) is the number of days between harvests.

New roots of seedlings which received the unlabeled solution were used to measure natural ^{13}C and ^{15}N abundance (control seedlings or unlabeled seedlings). New roots (from unlabeled and labeled seedlings) were gently removed from the soil and washed once with 50 mM KCl for 1 min, twice with tap water and twice with deionized water to remove the tracer adsorbed to root surfaces. Roots were stored at -30 °C, except a fraction of new roots (approximately 20% of fresh weight) that were stored in moist paper at 4 °C for no longer than five days until mycorrhizal colonization analysis (see below).

New roots from unlabeled seedlings were also used for specific root length (SRL) measurement. Total root length of a sub-sample of new roots from each seedling was measured by the gridline method described in (Marsh 1971). Then, roots were dried in an oven at 60 °C for 48 h and weighed to measure their dry mass. SRL was determined as the ratio between the length

and mass of roots. Total root length of labeled seedlings was estimated by multiplying new root mass and SRL. The new root growth rate (RG) was determined as the new root mass between t_0 and t_1.

Mycorrhizal colonization

Mycorrhization was quantified only in new roots of each labeled seedling. Approximately, 20% of total new root mass was used. Only ectomycorrhiza (ECM) was detected in *Quercus* species and in *P. nigra*, while only vesicular-arbuscular mycorrhiza (VAM) was observed in the remaining species. Ectomycorrhizal colonization (M_{ECM}) was quantified as the proportion of root tips sheathed by hyphae following methodology in (Brundett *et al.* 1996). The number of observed root tips was 252, 903 and 250 for *P. nigra*, *Q. faginea* and *Q. ilex*, respectively. VAM was quantified by microscopy (at a 40x magnification) after root staining using the trypan blue method (Brundett *et al.* 1996). Mycorrhizal colonization intensity (M_{VAM}) was quantified as described by (Trouvelot *et al.* 1986). The root length examined for M_{VAM} varied depending on the SRL of the specie, from 100 cm in *J. thurifera* to 280 cm in *B. retusum*.

Nitrate reductase activity and nitrogen resorption efficiency

Nitrate reductase activity (NRa) was measured in leaves and roots. Two samples of 0.1-0.5 g of fresh leaf and two samples of 0.25-0.5 g fresh roots were collected from 10 seedlings per species and measured as described by (Stewart 1993). Samples were assayed colorimetrically for nitrite using modified Griess-Ilosvvay reaction. The color was quantified at 540 nm in a spetrophotometer (UV-1800, Shimadzu corporation, Japan). Blanks were prepared using reaction mixture (after incubation) in the same conditions as samples. Results of two duplicate samples per plant part were averaged.

For leaf N resorption efficiency (NRE) determinations, 5-10 leaves were collected from each of 10 seedlings per species at t_0. Two to six months later depending on the species, we sampled another set of 5-10 senescent leaves from each seedling. Leaf area of each sample was determined in a digital image analyzer (Delta-T Image Analysis System, 1.12, Delta-T Devices LTD, UK). The samples were oven-dried at 60 °C for 48 h and ground in a planetary ball mill (PM100, Retsch Haan, Germany). Composite samples were obtained by random arranging samples from three individuals. Nitrogen concentrations in all samples were determined by the Kjeldahl method in an auto-analyzer by gas segmented continuous flow (SAN ++, Skalar, Holand). NRE was calculated as:

$$NRE = \frac{(N_{gl} - N_{sl})}{N_{gl}} \times 100 \qquad (\%) \qquad (2)$$

where N_{gl} and N_{sl} are the leaf N concentration on an area basis at t_0 (green leaves) and of senescent leaves, respectively.

Determination of N forms in soil (free and exchangeable N)

At t_1, just before soil N injection, soil from five seedlings per species was removed from pots and homogenized to break soil aggregates. Aliquots of 500 g of soil were stored at 4 °C for no more than 1 week before they were extracted. Soil was extracted with 0.5 M K2SO4 (1:10 m/v) by shaking end-to-end at 100 rpm for 90 min at room temperature, preserved overnight in a fridge (4 °C) and finally filtered with a Whatman #1 paper filter (Whatman, Kent, UK) in a Buchner´s funnel by vacuum and stored at -30 °C until analysis. Exchangeable and free amino acids in soil extracts were determined by Agilent protocols. HPLC separation of ortho-phthalaldehyde derivatives and subsequent fluorescence detection (Agilent Technologies, Santa Clara, USA for further information see Gratzfeld-huesgen (1998-1999)). HPLC was performed, with standards every four samples, using the following equipment: HPLC Agilent 1100 Series with automatic autosampler (Agilent Technologies, Santa Clara, USA). Separation was achieved using a column Hypersil AA-ODS (2,1 x 200 mm) 5µm (Agilent 79916AA-S72, Santa Clara, USA). The mobile phase A was made with sodium acetate tri-hydrate, and triethylamide, and acetronitrile for mobile phase B and the online derivatization was performed using ortho-phthalaldehyde (OPA, Fluka 79760) for primary amino acids and 9-Fluorenylmethyl chloroformate (FMOC, Fluka 23186) for secondary amino acids. Flow rate was 0.45 mL min^{-1} and gradient start with 100%A, at 17 min and finish at 0% at 25 min. Detection was via a G1321A scanning fluorescent detector (excitation at 390 nm and detection at 450 nm). Peak areas of chromatograms were analyzed and compared to AA S 18 standards (Fluka 090M8723, Sigma-Aldrich, St. Louis, United States).

Both NO_3^-, NH_4^+ were measured on the same K_2SO_4 extracts used for amino acid determination. NH_4^+ was measured by a modification of the tartrate–nitroprusside–hypochlorite method (Baethgen and Alley 1989), while NO_3^- was measured after reduction to nitrite with vanadium (III) and quantification with the Griess reagent (Miranda *et al.* 2001). Both analyses were performed on an auto-analyzer (CFA SAN++, Skalar, Breda, The Netherlands).

Plant ^{15}N analyses and calculations

New roots from labeled and unlabeled seedlings were ground in a planetary ball mill (PM100, Retsch Haan, Germany) and analyzed for N and C concentration and ^{15}N and ^{13}C abundance using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer (PDZ Europa 20-20, Sercon Ltd., Cheshire, UK) at the University of California Davis Stable Isotope Facility.

The amount of ^{15}N or ^{13}C taken up ($U_{labeled}$) was calculated as:

$$U_{labeled} = \frac{N_s(A_s - A_c)}{(A_1 - A_{lc})} \quad \text{(mg N)} \quad (3)$$

where N_s is the N (or C) content of the labeled sample, A_s and A_c are the ^{15}N (or ^{13}C) abundance in the labeled and unlabeled seedlings, respectively; while A_1 and A_{lc} are the ^{15}N (or ^{13}C) abundance of the labeled and unlabeled N (and C) in the fertilizer, respectively.

Equimolar amounts of NO_3^-, NH_4^+ and glycine were added but these were diluted by soil pools these forms. Because the size of these pools differed among species (see Table 1), isotope pool dilution differed among N forms and consequently affecting uptake of unlabeled N forms. Uptake of unlabeled N ($U_{unlabeled}$) was determined from pools of NO_3^-, NH_4^+ and glycine (see below) before the treatments were imposed:

$$U_{unlabeled} = U_{labeled} \frac{m_{unlabeled}}{m_{labeled}} \quad \text{(mg N)} \quad (4)$$

where $m_{unlabeled}$ is the mass of the unlabeled pool of NO_3^-, NH_4^+ or glycine, $m_{labeled}$ is the mass of the (added) labeled N pool. The total amount of N or C taken up was calculated as the sum of $U_{labeled}$ and $U_{unlabeled}$. To calculate N uptake rate (N_u), $U_{unlabeled}$ was standardized by the duration of the labeling period and new root mass. In order to standardized possible differences between species due to different soil availability of N forms, $N_{recovery}$ (%) for each N form was calculated as the total N form taken up relative to the soil content of the N form.

Preference (hPr) for a specific N form (h) was calculated as:

$$h\,\mathrm{Pr} = u_h - a_h \quad (\%) \quad (5)$$

where u_h is the take up percentage efficiency of a N form and was calculated as the proportion between uptake of a specific N form (h) and its concentration in soil; a_h is the percentage of soil N that is in a specific N form. If species do not show any preference for any N form, uptake pattern will mirror the N form availability pattern in soil and preference for each N form would be zero. Negative preference values mean that an N form is taken up in a lower proportion than its proportion in soil, while a positive preference value implies that the N form is proportionally taken up more than its proportion in soil.

We calculated the Similarity (S) index for a species (i) as the percentage similarity between uptake preference and N proportion in soil across all N forms, using equation described by McKane *et al.* (2002):

$$S_i = 100 - 0.5\Sigma_{h=1}^{T}\left|h \Pr\right| \qquad (\%) \qquad (6)$$

where h represents the preference of each N form, and S_i consider all the N forms analyzed (treatments, T=3). Similarity (S_i) measures the degree to which the absorption pattern of the N sources in a species is similar to the proportion of the N forms in the soil. Maximum similarity values indicate that N form absorption follows the same pattern of N form availability in soil, while low similarity values indicate that the uptake patterns of N sources in the species are very different from the existing proportions in the soils.

Because glycine can be decomposed by microbes before uptake, we followed two methods to assess whether glycine was taken up intact. The first method calculates the proportion of intact glycine absorbed comparing how much the slope of the regression line between $C_{labeled}$ against $N_{labeled}$ in plant deviates from the regression line of slope = 1 predicted from the stoichiometry of intact dual labeled glycine uptake (i.e., 1 moles of ^{13}C per mole of ^{15}N) (Näsholm *et al.* 1998). The second method analyzed the amount of ^{15}N-$2^{13}C$-glycine in a sample by gas chromatography-mass spectrometry (GC–MS). For a detailed description of the method see (Warren, 2009). ^{12}C,^{14}N-glycine was quantified from mass 246, and ^{13}C,^{15}N-glycine was quantified from mass 248 after subtracting the contribution to mass 248 from unlabeled samples.

Statistical analyses

N forms in soils (NO_3^-, NH_4^+, total N from amino acids) was analyzed by a two-way ANOVA. N preferences, S_i and total N_u were analyzed by one-way ANOVA with specie as independent variable. We checked if NO_3^- and glycine preference values differed from 0 by T-Test for single means. Soil amino acid composition was also analyzed by one-way ANOVA. To test if

mycorrhization affected N_u of N forms, data were analyzed by ANCOVA with mychorrhizal colonization as a covariate. Plant species mycorrhized with VAM and ECM were analyzed separately as root colonization was calculated using different methodology. If covariate was not significant, N_u was analyzed by two-way ANOVA with species and N form as independent variables. To analyze if species differ in their ranking for N forms preferences, interaction between species and N form was analyzed by ANOVA. Data homocedasticity was checked with Levene´s test. When ANOVA assumptions were not met, data were transformed. However, some variables remained heterocedastic and were analyzed by the Kruskal-Wallis non parametric test. Relationship between variables was analyzed using linear regression analysis. Statistical analyses were conducted with STATISTICA 7.0 software (StatSoft, Inc, Tulsa, USA).

Results

Soil N pools

Total amino acid concentration was two times higher than the concentration of $N\text{-}NO_3^-$ or $N\text{-}NH_4^+$ ($F_{2,126}$=197;P<0.001) (Table 1). However, differences between N forms depended on species (Interaction Species x N form $F_{16,126}$=11.9;P<0.001). $N\text{-}NH_4^+$ concentration was higher than $N\text{-}NO_3^-$ concentration in *R. canina* and *R. alaternus*, lower than $N\text{-}NO_3^-$ in *T. vulgaris*, *J. thurifera*, *P. nigra* and *Q. ilex*, and without differences in *B. retusum*, *S. chamacyparissus* and *Q. faginea*. N-glycine concentration was around six times lower than the concentration of each inorganic N forms. $N\text{-}NH_4^+$ ($F_{8,35}$=0.40; P=0.92), N-glycine ($F_{8,28}$=1.5; P=0.20) and total N-amino acid ($F_{8,35}$=2.02; P=0.07) concentration did not differ among species. However, $N\text{-}NO_3^-$ differed between species ($H_{8,43}$=31.4; P>0.001), with *J. thurifera, P. nigra* and *Q. ilex* having the highest $N\text{-}NO_3^-$ concentration and *R. canina* and *R. alaternus* the lowest.

We detected the 15 amino acids analyzed in soil but they showed very different concentration ($H_{13,588}$=4678; P<0.001). The most abundant amino acid was arginine, followed by glutamic acid, alanine and valine. These four amino acids accounted for 65% of total amino acids (Figure 2). Glycine was the fifth most abundant amino acid. Other amino acids, which proportion accounted for less than 4% of total N amino acid concentration, included isoleucine, tyrosine, phenolalanine, methionine and histidine.

Table 1. N concentration of different chemical N forms (NO_3^-, NH_4^+, glycine and total amino acids) from K_2SO_4 extracts of experimental substrate (1:1:1 v/v/v mixture of vermiculite and soil from a gap and under the canopy of a *Quercus ilex* forest) after 3-4 months with seedlings of nine Mediterranean forest species. Data are average (mg N kg dry $soil^{-1}$) ± 1 standard error (n=4-5). Nitrite (NO_2^-) was not included as concentrations were lower than 0.05 mg N kg dry $soil^{-1}$.

Specie	N-Glycine	N- total amino acids	N-NH_4^+	N-NO_3^-
Brachypodium retusum	3.3±1.0	66.8±6.4	28.7±0.3	30.4±2.6
Santolina chamaecyparissus	7.8±0.3	93.9±7.5	28.6±0.6	33.5±1.3
Thymus vulgaris	5.0±1.3	55.5±6.5	27.5±1.4	45.7±4.0
Rosa canina	5.2±0.7	66.9±7.6	28.5±0.5	13.2±3.7
Rhamnus alaternus	6.5±1.8	70.5±4.8	28.3±0.2	6.1±0.6
Juniperus thurifera	5.0±0.6	75.9±8.3	27.7±1.3	51.3±6.1
Pinus nigra	5.5±1.5	60.7±8.1	27.2±0.9	54.8±5.9
Quercus ilex	9.1±2.6	60.2±6.5	27.7±1.1	52.8±1.7
Quercus faginea	7.4±1.9	65.0±6.6	28.5±0.8	38.8±9.2

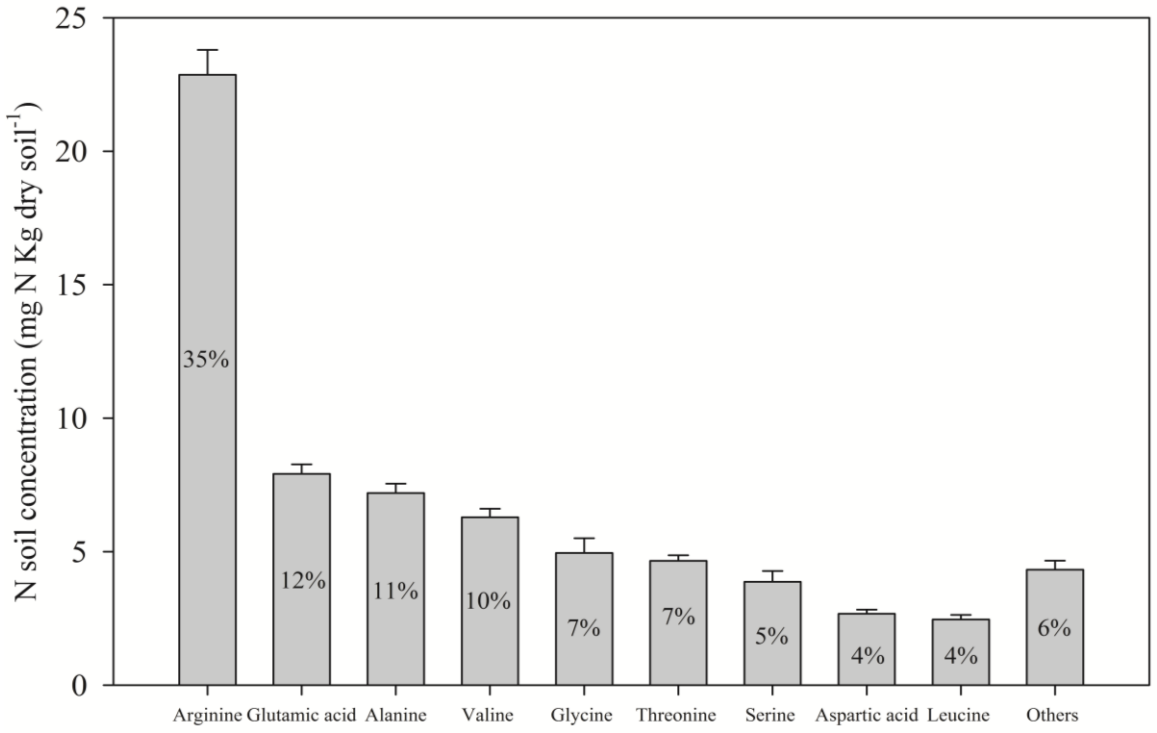

Figure 2. Concentration of free and exchangeable main amino acids in the soil from K_2SO_4 extracts used in the experiment. Values within the bars indicate the contribution of each amino acid to total amino acid concentration. Soil consisted in an 1:1:1 v/v/v mixture of vermiculite and soil collected in a gap and under the canopy of a *Q. ilex* stand. Soil was analyzed after cultivation of seedling of nine species for 3-4 months. Data are the mean of all soil samples analyzed (n=45; 9 species × 5 replicates) ± 1 standard error.

N uptake and plant functional attributes differences among species

^{15}N abundance in labeled roots was significantly higher than in roots of unlabeled seedlings (data not shown). Total N_u differed among species ($H_{8,468}$=3923; P<0.001), with the two chamaephytes and *J. thurifera* having the highest and lowest N_u values, respectively (Figure 3a). When N-forms were considered separately, NH_4^+ was taken up faster (46 to 66 % of total N_u), NO_3^--N_u had intermediate uptake rate (20 to 49 % of total N_u) and glycine had the slowest N_u (6 to 16% of total N_u; $F_{2,203}$=511; P<0.001). This general pattern varied among species (interaction N form x specie $F_{16,203}$=30.0; P<0.001). In *B. retusum* and *S. chamacyparissus* NH_4^+ N_u was highest, glycine N_u was lowest and NO_3^- N_u was intermediate, whilst in *T. vulgaris*, *J. thurifera*, *P. nigra*, *Q. ilex*, and *Q. faginea* NO_3^- and NH_4^+ N_u, were the highest and similar among them. Finally, in the two shrubs, *R. canina* and *R. alaternus*, NH_4^+ N_u was higher than the N_u of the other N forms, which were similar between them. Uptake rate of N forms were highly and positively correlated (data not shown).

N forms $N_{recovery}$, standardized N uptake rates by the N form soil availability, varied between 2 and 10% in NH_4^+, 2-6% in glycine and 1-8% in NO_3^- (Figure 3b). Species differed in their N forms $N_{recovery}$ ($F_{8,64}$=15.9, P<0.001; $F_{8,64}$=30.8, P<0.001 and $H_{8,64}$=58.5, P<0.001 for glycine, NO_3^- and NH_4^+, respectively), being highest for all N forms in chamaephytes (higher than 5%), followed by *R. canina* (5-7%), grass and *R. alaternus* species, and lowest in trees (3% or lower), with *J. thurifera* having the smallest $N_{recovery}$ values for all the N forms. NH_4^+ $N_{recovery}$ was highest in all the species, except in *R. alaternus* where $N_{recovery}$ of NO_3^- was the highest. Glycine $N_{recovery}$ did not shown differences with NO_3^- $N_{recovery}$ in most of species but it was higher than NO_3^- $N_{recovery}$ in *P. nigra*, *R. canina* and *Q. ilex*.

Brachypodium retusum had the highest RGR followed by the two conifers, while chamaephytes and *Q. faginea* had intermediate RGR values and *R. canina*, *R. alaternus* and *Q. ilex* had the lowest RGR (Table S1 in Supplementary material). Chamaephytes and the grass species had the highest SRL while both conifers had the lowest SRL. NRE generally followed the trend: grass > chamaephytes > shrubs > *Quercus* species > conifer species. Plant NRa was highest in chamaephytes, followed by shrubs, conifers, grass and lowest in *Quercus* species. N concentration in leaves was highest in both conifers, followed by chamaephyts, *Quercus* species, *R. alaternus*, *B. retusum* and finally *R. canina*.

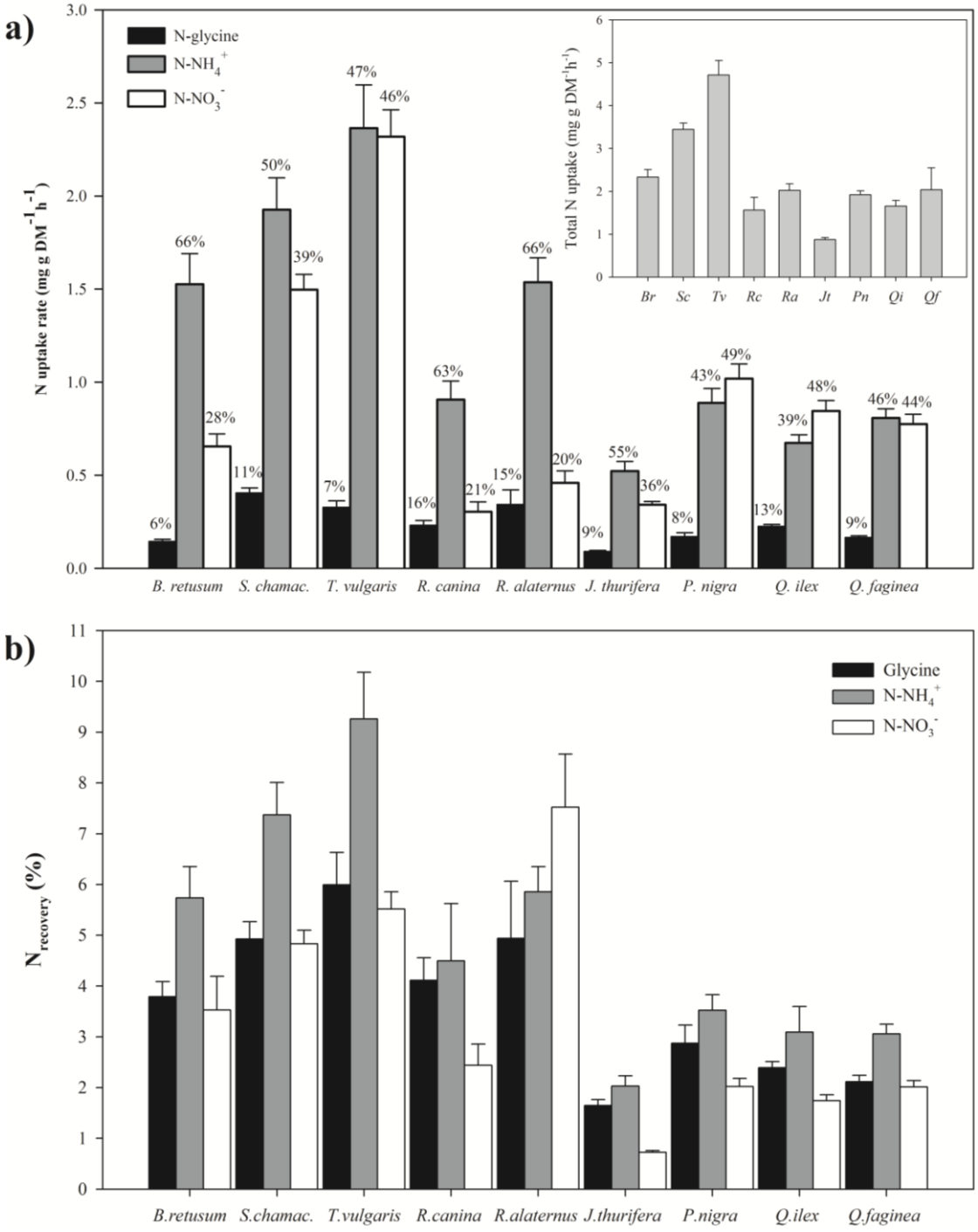

Figure 3. N uptake rates (a) and $N_{recovery}$ (b) of three N forms (glycine, NO_3^- and NH_4^+) by intact roots of one-year-old seedlings of nine Mediterranean forest species. The rate of uptake was determined by incubating seedlings for 6-8 h in experimental substrate (1:1:1 v/v/v mixture of vermiculite and soil from a gap and under the canopy of a *Q. ilex* forest). The uptake rate was measured using an equimolar mixture of three N forms labeled with ^{15}N at 0.9 mM total N concentration and adjusted considering the dilution with the N forms availability in the substrate. Data are mean of eight replicates per species. Error bars are one standard error. In figure (a) numbers indicate the relative contribution of each N form to total N uptake. Inserted figure indicates the total N uptake rate.

Across species, total N_u was not related to neither RGR (Figure 4a) nor N_{gl} and NRE (Figure S1a and b Supplementary material). However, total N_u was highly and positively related to SRL (Figure 4b), plant NRa (Figure 4c) and leaves / root NRa ratio (Figure S1c). RGR was highly and positively related to N_{gl} and NRE (Figure S1d and S1e in Supplementary material). N_{gl} and NRE were positively and significantly related (Figure S1f). SLR was highly and positively related to total length of new root (Figure S2a in Supplementary material) but negatively to RG (Figure S2b).

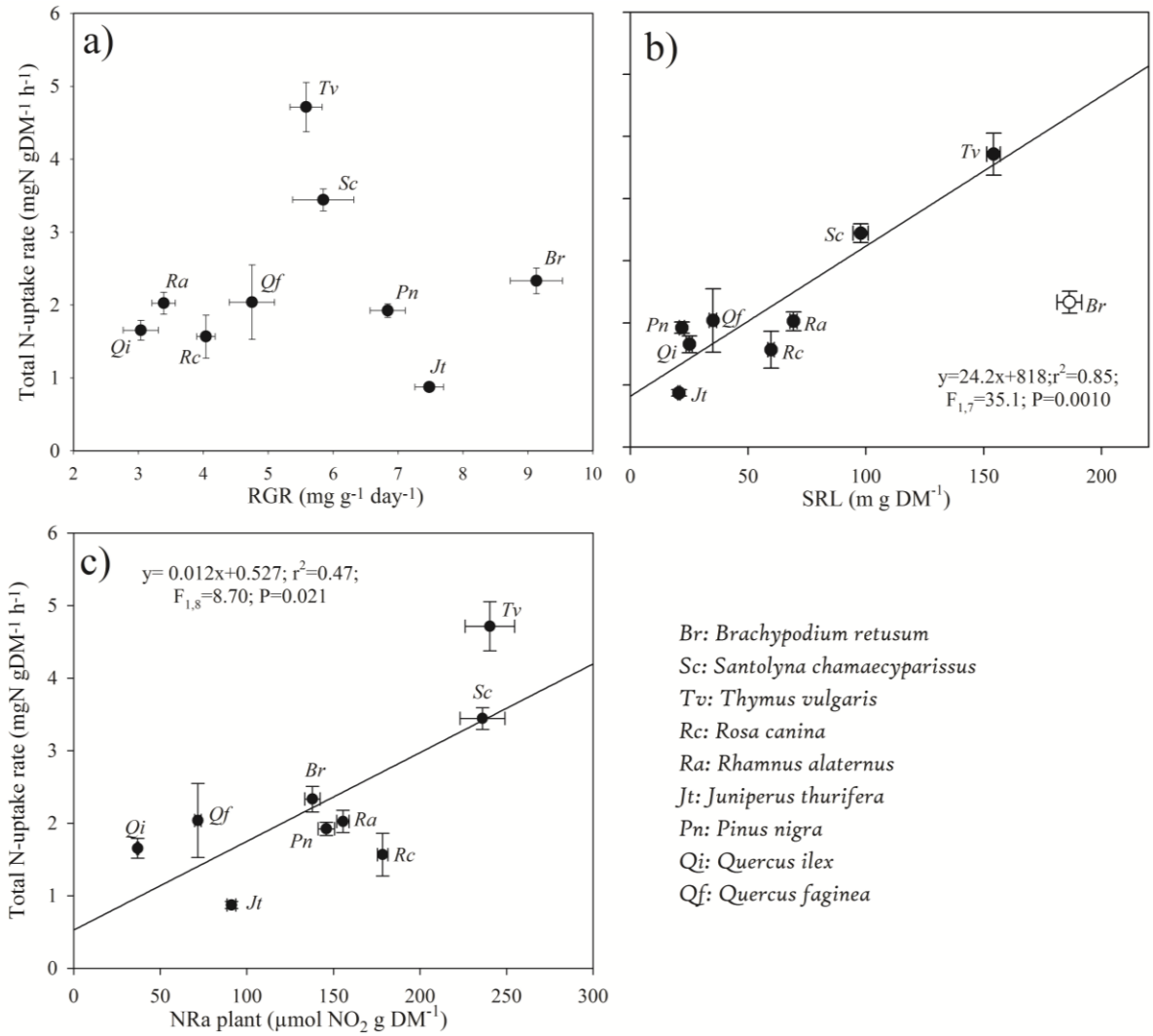

Figure 4. Relations of total N uptake rate with the relative growth rate (RGR) (a), the specific root length (SRL) (b) and the plant nitrate reductase activity (plant NRa) (c) across species. Each point represents a species mean and error bars are one standard error (n=8). In figure (b), *B. retusum*, which is shown in an open circle, was excluded from the regression analysis.

Mycorrhization and its influence on N uptake

Among the plant species that were only mycorrhized by ECM, M_{ECM} values were small and not all seedlings showed new roots with mycorrhized tips. However, M_{ECM} showed species differences (Table S1 in Supplementary material). *Pinus nigra* had the highest M_{ECM} value and high proportion of individuals with ECM colonization (58% of seedlings) followed by *Q. faginea*, which showed 71% of plants having ECM colonization. Finally *Q. ilex* had the lowest M_{ECM} and proportion of individuals with ECM colonization. Among the plant species that were mycorrhized with VAM, all seedlings had VAM colonization and arbuscules in new roots. M_{VAM} showed species differences. *Rosa canina* had the highest M_{VAM} and *T. vulgaris* the species with lowest M_{VAM}.

M_{ECM} was not a significant covariate for any N-form uptake rate. M_{VAM} was a significant covariate for glycine uptake rate (see below) but not for NH_4^+ and NO_3^- uptake rate.

N forms preferences

Species differed in their N forms ranking preferences (Interaction N form × Species $F_{16,226}=5.74$; $P<0.001$) (Figure 5a). All species had preference for NH_4^+ at the expense of NO_3^- ($F_{8,72}=41.29$; $P<0.001$), except *R. alaternus*, which preferred NO_3^- at the expense of glycine and had a NH_4^+ uptake rate similar to its proportion in the soil. *Juniperus thurifera* and *B. retusum* had the highest NH_4^+ preference and *R. canina* and *Q. ilex* the lowest NH_4^+ preference, while the remaining species had intermediate NH_4^+ preference values. NO_3^- preference approximately followed the inverse pattern of NH_4^+ preference ($F_{8,72}=65.89$; $P<0.001$). NO_3^- preference in *R. alaternus* was not different from zero (t-value=0.07; P=0.93). Glycine preference also showed differences among species ($F_{8,72}=36.0$; $P<0.001$); *Rosa canina*, *J. thurifera*, *P. nigra* and *Q. ilex* had positive preference values for glycine, with highest values for *R. canina* and *J. thurifera*. In both chamaephytes, in the shrub *R. alaternus* and in the tree *Q. faginea*, glycine preference was negative ,while in *B. retusum* glycine preference was not different from cero (t-value=0.76; P=0.47).

The similarity index (S_i) between the pattern of N form uptake and N form availability in soil showed significant difference among species ($F_{8,72}=48.9$; $P<0.001$) (Figure 5b). The conifers and the grass species had the lowest S_i, while *Quercus* species and the shrubs had the highest S_i, with values > 90%. The chamaephytes exhibited intermediate values. Lower S_i values across species

were negatively associated to higher NH_4^+ preference (r^2=0.91; P<0.001) and positively associated to lower NO_3^- preferences (r^2=0.90; P<0.001). Across species, RGR was highly and negatively related to Similarity index (Figure 5b) and highly and positively related to NH_4^+ preference (Figure 5c). *Rhamnus alaternus* and *Q. ilex* showed the highest S_i values and the lowest RGR and NH_4^+ preference. On the contrary, *B. retusum* and *J. thurifera*, which had the lowest S_i values, were the fastest growing species and had the highest NH_4^+ preference.

Intact glycine uptake

M_{VAM} but not M_{ECM} was a significant covariate in glycine-N_u ANCOVA ($F_{1,53}$=13.45; P<0.001). An increase in M_{VAM} enhance glycine N uptake, as indicated by the positive relationship between both variables (data not shown). After taking into account the M_{VAM} effect by adjusted means at the same M_{VAM} value, species differed in glycine N_u ($F_{5,53}$=13,45, P<0.001). *Thymus vulgaris* had the highest glycine N_u, followed by *J. thurifera*, *B. retusum*, *S. chamaecyparissus* and *R. alaternus,* which had intermediate N_u values. Finally, *R. canina* had the lowest N_u (Table 2).

$N_{labeled}$ and $C_{labeled}$ were highly and positively related in all studied species (Table 2) except in *P. nigra*, which showed a marginal relationship. If all glycine would have been taken up intact, $N_{labeled}$ and $C_{labeled}$ would be equivalent and slopes of the linear regressions between $N_{labeled}$ and $C_{labeled}$ would be one. However, slopes of the fitted lines were different than 1. The slope in *R. canina* was higher than one so we could not calculate the proportion of intact glycine by the ^{13}C- ^{15}N molar ratio method. Slope differences among species indicate that *Quercus* species had similar proportion of intact glycine uptake, which were close to 70%. Estimated proportion of intact glycine uptake in *J. thurifera*, T. *vulgaris*, *B. retusum* was slightly above 50%, while in *R. alaternus* it was around 40%. Finally, *P. nigra* had the lowest intact glycine uptake, with only 30% of labeled ^{15}N recovered in roots being intact glycine.

The GC-MS results indicated that the roots of all species contained small amounts of intact ^{13}C-^{15}N-glycine molecules with significant differences among species (Table 2). However, the amount of ^{13}C-^{15}N-glycine molecules was 10^4 times lower than the amount of N from intact glycine estimated by ^{13}C-^{15}N molar ratio method. Both M_{ECM} and M_{VAM} were not significant covariates in ANCOVA of intact ^{13}C-^{15}N-glycine uptake rate. Intact ^{13}C-^{15}N-glycine was detected only in some of the sampled seedlings. A low proportion of *Quercus* species seedlings had dual labeled glycine, while this proportion was high in *B.*

retusum, *S. chamaecyparissus*, and *R. alaternus* and intermediate in the remaining species.

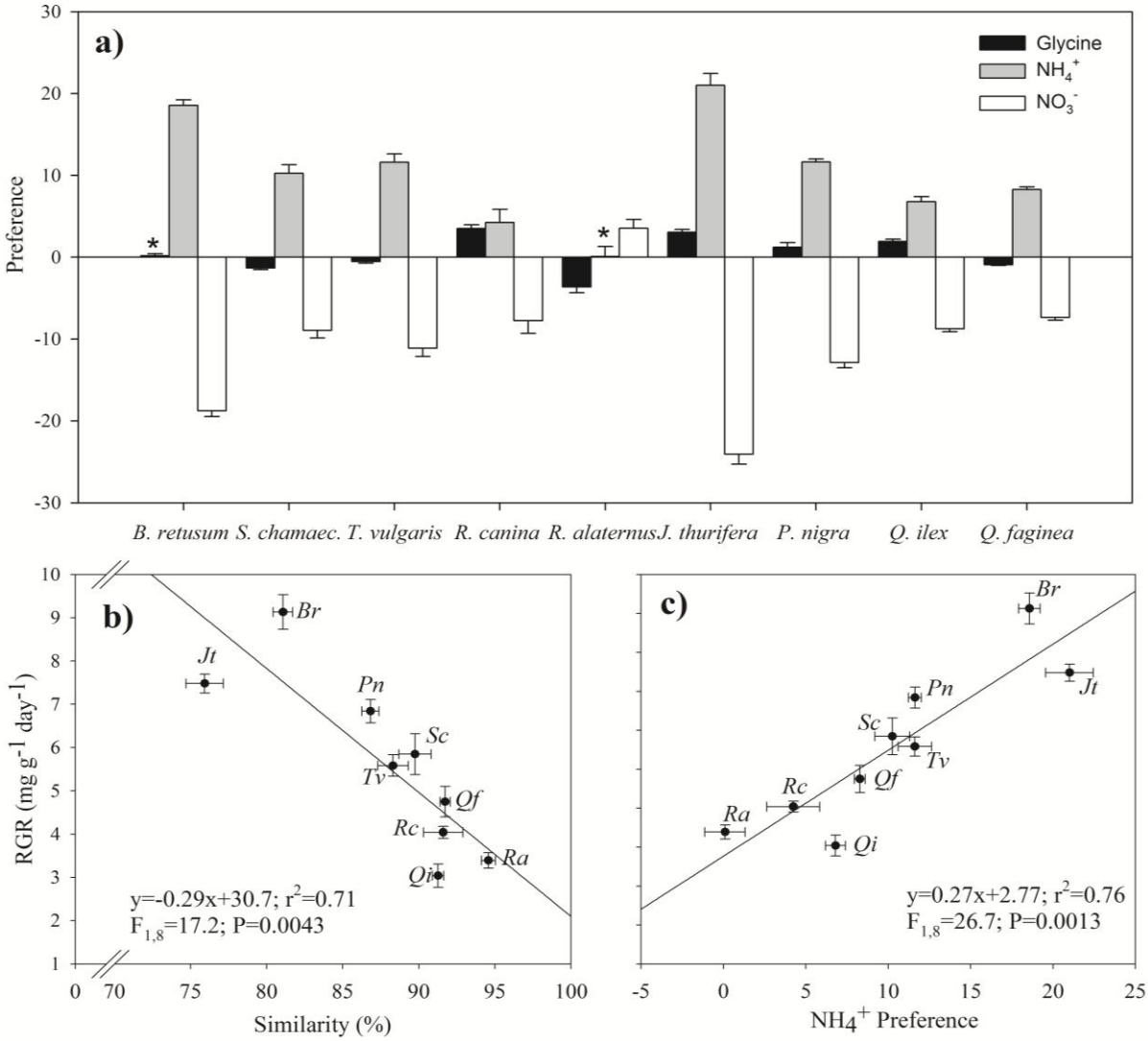

Figure 5. Preferences of three N forms (glycine, NO_3^- and NH_4^+) by intact roots of one-year-old seedlings of nine Mediterranean species (a) incubated for 6-8 h with an equimolar mixture of three N forms labeled with ^{15}N at 0.9 mM total N concentration and adjusted considering the dilution with the N forms availability in the substrate. Experimental substrate was a mixture of vermiculite and soil from a gap and under the canopy of a *Q. ilex* forest (1:1:1 v/v/v). Data are the mean of eight replicates per species. Error bars are one standard error. Each point represents a species mean and bars are one standard error. * indicates preferences values not statistically different from zero. Figures b and c show the relations of relative growth rate (RGR) with the similarity index (S_i) and with the NH_4^+ preference across studied species, respectively. *Br: Brachypodium retusum, Sc: Santolina chamaecyparissus; Tv: Thymus vulgaris; Rc: Rosa canina; Ra: Rhamnus alaternus; Jt: Juniperus thurifera; Pn: Pinus nigra; Qi: Quercus ilex; and Qf: Quercus faginea.*

Table 2. Total N-glycine uptake not accounting and accounting for the mycorrhization intensity (M_{VAM}) (μg N gDM^{-1} h^{-1}). For total N-glycine after accounting for MVAM colonization results are least square means calculated at M_{VAM}=22.43. Total N-glycine represents the N uptaken from both labeled and unlabeled glycine pools in soil. IRMS $N_{labeled}$ and $C_{labeled}$ (μg N gDM^{-1} h^{-1}) represents the N and C uptaken from lable glycine in new roots. Number of seedlings out of 10 with dual labeled glycine (N) and amount of dual labeled glycine detected by GC-MS (ng N gDM^{-1} h^{-1}). Note differences in units between variables. Data are means ± one SE. n.s.= P>0.05; *= P<0.05; **=P<0.01; ***= P<0.001.

	Total glycine uptake	Total glycine uptake after accounting for M_{VAM}	IRMS			GC-MS	
			$C_{labeled}$ *vs.* $N_{labeled}$ relationship	$N_{labeled}$	$C_{labeled}$	N	Glycine uptake rate
Brachypodium retusum	145±11	129±2	y=0.54x+0.099; r^2=0.81 **	20.5±1.4	12.9±2.7	7	6.6±2.3
Santolina chamaecyparissus	404±28	180±1	y=0.752x-0.314; r^2=0.88 ***	11.4±0.6	7.0±0.6	9	13.8±3.8
Thymus vulgaris	328±35	16±2	y=0.561x-0.139; r^2=0.52 *	12.9±0.6	10.0±1.9	5	4.6±1.8
Rosa canina	232±25	207±1	y=1.043x-0.955; r^2=0.94 ***	8.9±0.5	3.2±0.4	4	8.2±3.7
Rhamnus alaternus	343±78	59±1	y=0.414x-0.033; r^2=0.74 **	15.5±1.7	8.3±1.3	10	143.6±18.1
Juniperus thurifera	90±6	361±2	y=0.561x-0.136; r^2=0.58 *	26.7±5.4	9.8±2.4	4	4.0±1.7
Pinus nigra	171±21		y=0.561x-0.139; r^2=0.52 *	22.2±2.1	9.5±2.4	6	8.1±2.3
Quercus ilex	225±11		y=0.774x-0.022; r^2=0.71 **	26.6±1.6	7.5±1.5	1	0.2±0.2
Quercus faginea	165±10		y=0.663x-0.048; r^2=0.66 *	32.4±3.0	13.0±1.5	3	12.0±6.5
Species	$H_{8,64}$=58 ***	$F_{5,53}$=17,4 ***	$F_{8,58}$=2.7 *	$F_{8,72}$=11.3 ***	$F_{8,72}$=2.8 **		$F_{8,90}$=55.4 ***
M_{VAM}		$F_{1,53}$=13.4 ***					$F_{1,53}$=1.11 ns
M_{ECM}		$F_{1,22}$=0.20 ns					$F_{1,25}$=0.39 ns

Discussion

Plant species representing an ample range of ecological strategies commonly found in Mediterranean ecosystem showed notable differences in N form preference and uptake capacity. Moreover, the relative growth rate, a central functional trait for the ecology of plants, is linked to preference for NH_4^+. These results indicate that N uptake plays a central role in the functioning of Mediterranean forest communities.

Soil composition in N chemical forms

The amino acid pool in our experimental soil was as big as the inorganic N pool, supporting the idea that organic N is an abundant N source in Mediterranean-ecosystem soils (Delgado-Baquerizo *et al.* 2011), as has previously been reported for cold-climate ecosystems (Kielland 1994; Näsholm *et al.* 1998; McKane *et al.* 2002).

The N concentration due to free and exchangeable amino acids across species was 4-6 nmol N g soil DM^{-1}, which is twice the concentration reported in a Mediterranean grassland of *Stipa tenacisima* measured in the same weak salt solutions (Delgado-Baquerizo *et al.* 2013). Organic N availability widely varies among vegetation types and seasons in Mediterranean ecosystems (Delgado-Baquerizo *et al.* 2011), which can explain soil amino acid concentration differences between studies. Soil amino acid concentration in our study was around ten times lower than that reported for the taiga (30-350 nmol g^{-1}, Werdin-Pfisterer *et al.*, 2009), arctic tundra (114-592 nmol g^{-1}, Kielland, 1995), and an alpine dry meadow (21-210 nmol g^{-1}, Lipson *et al.*, 1999) (extracted in water or weak salt solutions). In dry temperate communities, such as Mediterranean type-ecosystems, soil organic N (and consequently amino acid) availability is lower than in cold climate ecosystems due to higher mineralization rates and lower productivity (Carreira *et al.* 1994; Gallardo *et al.* 2000; Schimel and Bennett 2004; Delgado-Baquerizo *et al.* 2011). The dominant amino acids in our mixture of forest soils were arginine, glutamic acid, alanine, valine and glycine, which are also the most usual amino acids in soils of other biomes (Yu *et al.* 2002; Andresen *et al.* 2008).

The proportion of inorganic N sources and amino acids in soil differed among species suggesting that plant species affected the proportion of N forms in soil. While NH_4^+, total amino acid and glycine levels remained more or less constant, NO_3^- widely varied among species. These differences among species

in the proportion of N forms could reflect both a depletion of a N form in soil as a results of distinct N uptake preferences among species and changes in microbe community and activity, driven by plant species (Lata *et al.* 1999; Gallardo *et al.* 2000; Hawkes *et al.* 2005).

Differences in N uptake capacity among species

Rates of N uptake differed among species and were related to species growth form. The chamaephytes and the herb species had higher uptake rate than the shrubs and trees, yet with differences among them. Similar to our findings, Osone and Tateno (2005) found that herbs had higher N uptake than trees. Among four European trees, Schulz *et al.* (2011) found higher N uptake rates in *P. sylvestris* than *Q. petraea.* Here all tree species had similar N uptake rates, except *J. thurifera,* which had the lowest N uptake among our tree species.

Differences among species in N uptake rate were related to root structural traits and N assimilation capacity. However, contrary to our first hypothesis, N uptake was not related to either RGR or leaf N concentration, which are surrogates of plant N demand (Poorter 1990; Cornelissen *et al.* 1996). Several studies have reported positive relationships between RGR or tissue N concentration and N uptake (Weigelt *et al.* 2005; Osone and Tateno 2005; Schulz *et al.* 2011), but others also have found weak or no relationship between RGR and N uptake (Harrison *et al.* 2008) or negative relationship between N uptake rate and leaf N concentration (Kahmen *et al.* 2006). Our results, therefore, do not support a N-related syndrome between N-exploitative species (characterized by high RGR, leaf N concentration and uptake rate) and N conservative species (opposite traits) (Reich *et al.* 1998; Osone and Tateno 2005; Maire *et al.* 2009). Specifically, conifers in our study showed high RGR and leaf N concentration but low N uptake, which points out that these species have other mechanisms, independent of N uptake, to maintain high RGR and leaf N. This mechanism can be remobilization of N reserves. Two line of evidences support this idea. On the one hand, RGR was positively related to leaf N resorption. Leaves are the main source of N for remobilization in evergreen woody species (Palacio *et al.* 2007; Millard and Grelet 2010; Uscola *et al.* 2013) and the conifers of our study had the highest leaf N resorption efficiency. On the other hand, fast growing species use a greater amount of remobilized N for new growth than slow growing species (Uscola *et al.* 2013).

N uptake was positively correlated with specific root length, almost certainly because higher SRL increases the surface area of roots in contact with soil and explored soil volume (Figure S2 in Supplementary material) (Hodge *et*

al. 2009; Maire *et al*. 2009). Our results also support the existence of a tradeoff between root mass allocation and absorption efficiency (Maire *et al*. 2009). The herb species and the chamaephytes had a high fibrosity root system, where a low investment in root mass resulted in a high SRL and total root length. On the contrary, shrubs and trees had lower SRL and root length, in spite of having much more higher root mass than the chamaephytes and the herb species. A greater investment in root mass at the expense of root N uptake among growth forms, probably reflects a balance between promoting nutrient uptake on upper soil layers and plant stability or access to deep soil layers to avoid drought stress and take up mineral nutrients concentrated deep in the soil (McCulley *et al*. 2004; Terradas *et al*. 2009). By developing different root architecture, woody plants can minimize competition by having access to resources in different parts of the soil (Jumpponen *et al*. 2002; Filella and Peñuelas 2003).

Nitrogen metabolism can affect N uptake by increasing sink strength (Seco *et al*. 2008). Consistent with this idea, N uptake was greater in species with higher NRa activity and with this activity concentrated in the foliage. High NRa in leaves is more effective for NO_3^- assimilation than in roots as NO_3^- is reduced in presence of light and hence is less energetic costly than if it assimilated in the root (Lambers 2008). As NO_3^- and NH_4^+ uptake rate were highly and positively correlated it is likely that plants that had high NRa also had high NH_4^+ metabolization capacity.

N form preference

Species differed not only in total N uptake but also in preference for the chemical forms of N. All of the studied species were able to take up all N chemical forms including intact glycine. In most cases, NH_4^+ was taken up faster than NO_3^- and glycine. In contrast, Schulz *et al*. (2011) found higher NO_3^- uptake rates than NH_4^+ in four European trees (including one oak and one pine). However differences between experiments could be attributable to methodological differences. Contrary to Maire *et al*. (2009) study, which concluded an NH_4^+ *vs*. NO_3^- uptake trade-off among herb species, we did not detect any uptake tradeoff between N forms. In fact the species with high total N uptake ability also had high N uptake of all the individual N forms.

Glycine was absorbed slower than NH_4^+ and NO_3^-, in accordance with other studies (Kielland 1994; Lipson *et al*. 1999a; Andresen *et al*. 2008). However, this difference was mainly attributed to the lower glycine concentration in soil but not to lower intrinsic uptake efficacy (Table 1). For example, *R. canina* had similar N efficiency ($N_{recovery}$) for glycine and NH_4^+, and glycine efficiency was

higher than NO_3^- in several species. Therefore our results point out that amino acids might be a significant N source for plants in Mediterranean ecosystems if all the soil amino acid pool is considered. Other organic N forms, not considered here, can contribute to enhance the contribution of organic N to plant nutrition, such as combined amino acids, peptides or proteins (Yu *et al*. 2002; Paungfoo-Lonhienne *et al*. 2008).

In accordance with our second hypothesis, all species were able to absorb intact glycine. Uptake of intact glycine was confirmed by GC-MS results and enrichment of root tissues in ^{15}N and ^{13}C, which were strongly correlated in most species (Näsholm *et al*. 1998). This is, as far as we know, the first study documenting that Mediterranean plants can take up intact amino acids as an N source and we demonstrate it for a broad number of ecologically distinct species. Unfortunately, we could not have a precise estimation of the amount of intact glycine taken up in each species because the used methods have some limitations due to post-uptake metabolism (Jones *et al*. 2005; Warren 2012). In the IRMS analysis, a similar ^{13}C-^{15}N molar ratio to the reference stoichiometry of intact dual-labeled glycine can also be achieved if glycine is decomposed in the soil and ^{13}C and ^{15}N are subsequently taken up independently (Jones *et al*. 2005). Additionally, underestimation of intact glycine in roots due to differential transfer of ^{15}N and/or ^{13}C to other plant organs is plausible (Warren 2009c, 2012). Finally, loss of $^{13}CO_2$ due to respiration after glycine metabolism can also alter organ ^{13}C- ^{15}N molar ratio (Warren 2012). These limitations are evidenced by the weak ^{13}C- ^{15}N relationship in *P. nigra* and higher slope than expected in *R. canina*. The GC-MS method, which measures the exact amount of intact glycine inside the plant, indicated small amounts of dual labeled glycine in all the species, especially in *Q. ilex*. Glycine is very quickly metabolized (Warren 2012) and our experiment lasted 6 h, which might explain why only a small proportion of intact glycine was detected inside the root.

Differences among species in preferences for N forms were also indicated by differences in Similarity index (S_i). High S_i values, such as those in *R. alaternus*, indicate that uptake of N forms was proportional to their availability in soil (*i.e.* no preference for any N form). In contrast, low S_i values, such as those in *J. thurifera*, indicate a strong asymmetry between the proportion in N form uptaken and the proportion of N forms in the soil. Reduction in S_i across species was mainly due to higher NH_4^+ preference at the expense of NO_3^- (Figure 5) in all species except *R. alaternus*. Furthermore, S_i was strongly and negatively related to preference for NH_4^+. Several studies have shown that species preference are mainly for the most available N form in soil (McKane *et al*. 2002; Warren 2006; Houlton *et al*. 2007). In tropical forest

communities, species showed preference for inorganic N over organic N, and switched their preference for either NH_4^+ or NO_3^- depending on their abundance (Houlton *et al.* 2007). Notably, NH_4^+ in our study was not, in general, the most abundant chemical form of N in soil (Table 1), reinforcing, thereby, the hypothesis that Mediterranean forest species have a general inherent preference for NH_4^+.

Do mycorrhizae matter for N uptake?

We found that VAM colonization was a significant covariate for glycine N uptake. Both variables were positively related, supporting the importance of considering mycorrhization in comparative studies on amino acid uptake. The fastest glycine uptake measured by both IRMS and GC-MS methods was found in *R. canina*, which also had the highest M_{VAM}. In contrast *B. retusum* or *J. thurifera*, which had low M_{VAM}, also had the lowest glycine uptake. However, neither ECM nor VAM influenced inorganic N uptake, providing only partial support to our fourth hypothesis. Previous studies have also reported positive relationships between N uptake rate and mycorrhizal symbiosis (Rains and Bledsoe 2007; Talbot and Treseder 2010; Whiteside *et al.* 2012). Mycorrhizae increase the volume of soil influenced by rhizospehere and can take up intact amino acids more efficiently than free-living soil microbes (Chalot and Brun 1998; Leake *et al.* 2004; Talbot and Treseder 2010).

The lack of any effect of ECM on N uptake might be explained by low ECM colonization. As mycorrhizal colonization was measured in new roots and colonization was small, it seems that ECM colonization was at an initial phase. Moreover, ECM require more time than VAM to be effective for nutrient uptake (Frank and Groffman 2009; Talbot and Treseder 2010). During early colonization phases, fungi invest more resources for growth rather than transferring resources to plant host (Treseder 2005; Talbot and Treseder 2010).

Ecological consequences of differential N form uptake

Differences in the rate of N uptake and preferences for N forms among species support the idea that Mediterranean species have different N-based fundamental niches. Moreover, differences in S_i and, consequently, NH_4^+ preference were linked to differences in RGR (Figure 5), which is an essential attribute for plant fitness influencing the competitive ability of plants and, consequently, on community structure (Grime and Hunt 1975; Tilman 1982). Thus, fast growing species exhibited low S_i and high NH_4^+ preference, while slow growing species had inverse pattern, high S_i and low NH_4^+ preference.

Using NH_4^+ as a N source has two advantages. First, uptake and metabolization of NH_4^+ is comparatively less energetically costly than NO_3^- uptake and metabolization (Bloom *et al.* 1992). Consequently, plants using NH_4^+ might proportionally invest more energy and resources for growth (Maire *et al.* 2009; Boudsocq *et al.* 2012). Second, NH_4^+ is an abundant source of N in Mediterranean soils (Bonilla and Rodá 1992; Gallardo *et al.* 2005; Delgado-Baquerizo *et al.* 2011). Among grassland species, the fast growing species preferred inorganic N forms, while slow growing species did not show N form preferences (Weigelt *et al.* 2005). Unfortunately, this study did not differentiate between inorganic N forms.

Using a ecosystem functioning model that included NH_4^+ *vs.* NO_3^- preference, Boudsocq *et al.* (2012) concluded that community biomass and primary productivity are maximized at a slightly higher values of NH_4^+ preference. In other ecosystems, while the most dominant and productive species tend to use the most abundant N form in soil, the less dominant species have low S_i and use the less available N forms (McKane *et al.* 2002; Kahmen *et al.* 2006). In our study, among the mid to late successional species, the dominant trees (*Quercus species* and *Pinus nigra*), which account for most biomass in woodlands clearly preferred NH_4^+, not necessarily the most abundant N source, while the subordinate shrubs preferred NO_3^- (*R. alaternus*) or glycine (*R. canina*).

Pioneer species usually are considered to have higher N uptake rate and tend to mainly use NO_3^- as N source, while late succesional species have lower uptake rate and prefer NH_4^+ and organic N (Stewart *et al.* 1988; Nordin *et al.* 2001; Aidar *et al.* 2003; Kronzucker *et al.* 2003). The herb and the chamaephytes, which are pioneer species, had higher N uptake rate than mid-late succesional species. However, the pioneer tree *J. thurifera* had the lowest uptake of all species. Similarly, our pioneer species had an overall preference for NH_4^+. Moreover, the herb and the chamaephytes had negative preference for organic N, but *J. thurifera* had higher or similar preference for glycine than mid-late succesional trees. However, pioneer species had higher NH_4^+ preference than mid-late succesional species, and higher RGR.

Co-occurring species within the same succesional stage showed different N uptake capacity and N preferences, suggesting N-based fundamental niche segregation. Thus, among pioneer species *J. thurifera* had very low N uptake while *T. vulgaris* had very high N uptake. Similarly, though all pioneer species preferred NH_4^+ over NO_3^-, NH_4^+ preferences intensity differed among them, with higher NH_4^+ preference in *B. retusum* and *J. thurifera*. Co-occurring species

in late succesional stages have similar N uptake rates, but important differences in N forms preference. While the trees used NH_4^+ the shrubs preferred NO_3^-. Among the late succesional trees *Quercus* species had lower NH_4^+ preference than *P. nigra*.

Future research should also consider other functional attributes and ecological processes that might contribute N-based niche segregation in Mediterranean species. For instance, root distribution in the soil profile and phenology, and potential changes in N form preference due to competition with other plants or due to changes in the availability of N forms (Jumpponen *et al.* 2002; Filella and Peñuelas 2003; Xu *et al.* 2006; Houlton *et al.* 2007; Andresen *et al.* 2008; Aanderud and Bledsoe 2009; Ashton *et al.* 2010).

Conclusions

Our data show that a variety of ecologically distinct Mediterranean forest species that frequently coexist show important differences in N uptake rate and preference for N sources. All the studied species had the ability to take up glycine as an intact molecule, and VAM was associated to this capacity. However, VAM had no effect on inorganic N. ECM were too low, and no effect was detected in none N form. Rates of N uptake were positively correlated with specific root length and nitrate reductase activity, but unrelated to relative growth rate or leaf N concentration. N uptake rates differed among growth forms, with chamaephytes and herb having higher N uptake rates than shrubs and trees. However, higher RGR was relative to lower similarity, specially due to NH_4^+ specialization. Thus, fast growing species exhibited higher NH_4^+ preferences while slow growing species had no preferences and use the N forms as a function of N forms soil availability. N forms preferences were not linked to successional stage, as most of the species shown higher NH_4^+ preference. Functional niche segregation due to differential use of N was found between species co-occurring in a successional stage. Pioneer species differed in N uptake rates, but also in the intensity of NH_4^+ preference as a function of their RGR. Among mid–late successional stage, the dominant trees preferred NH_4^+, while subordinate shrubs used glycine or NO_3^-. This results suggests that the studied Mediterranean species have fundamental niches based on N and its chemical forms, thereby reducing competition and facilitating co-existence. Therefore, water and light would not be the only abiotic environmental drivers of community structure in Mediterranean ecosystems, and N would play a central role in the functioning of Mediterranean forest communities.

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Table S1. Relative growth rate (RGR), new root mass, specific root lenght (SRL), root growth (RG), total length of new root (L_{NR}), endo-mycorrhizal colonization intensity (M_{VAM}), percentage of root tips with ecto-mycorrhizae (M_{ECM}), total plant nitrate reductase activity (NRa plant), N concentration in green leaves at the beginning of the experiment t_o (N_{gl}) and N resorption efficiency (NRE) in one-year-old seedlings of nine Mediterranean forest species. The last column are the p-values of one-way ANOVA or Kruskal wallis test, were n.s.= P>0.05; *= P<0.05; **=P<0.01; ***= P<0.001.

	B. retusum	*S. chamaec.*	*T. vulgaris*	*R. canina*	*R. alaternus*	*J. thurifera*	*P. nigra*	*Q. ilex*	*Q. faginea*	Species
RGR ($mg\ g^{-1}\ day^{-1}$)	9.13±0.40	5.85±0.47	5.84±0.25	4.04±0.14	3.39±0.18	7.48±0.22	6.84±0.27	3.04±0.27	4.75±0.35	$H_{8,860}$=206; ***
New roots mass (g)	0.22±0.02	0.13±0.01	0.17±0.01	0.28±0.01	0.43±0.02	0.41±0.03	0.34±0.02	0.25±0.02	0.50±0.03	$H_{8,319}$=177; ***
SRL ($m\ g\ DM^{-1}$)	186.3±5.3	97.8±3.2	154.2±2.9	59.7±1.4	69.3±1.5	20.6±0.5	21.9±1.1	25.0±1.3	35.1±1.5	$F_{1,80}$=740; ***
RG ($g\ day^{-1}$)	1.94±0.17	1.23±0.12	1.42±0.10	2.62±0.10	3.67±0.19	4.41±0.30	3.43±0.16	2.83±0.18	4.48±0.21	$H_{8,319}$=232; ***
L_{NR} (cm)	41.3±1.2	12.8±0.4	25.6±0.5	16.5±0.4	29.8±0.7	8.5±0.2	7.4±0.4	6.1±0.3	17.5±0.8	$H_{8,150}$=102 ***
M_{VAM} (%)	10.6±1.3	25.8±1.8	3.1±0.9	61.1±1.8	17.1±1.7	14.9±1.2				$H_{5,154}$=112; ***
M_{ECM} (%)							7.72±2.44	1.70±0.68	2.80±0.70	$H_{2,78}$=8.33; ***
NRa plant ($\mu mol\ NO_2\ g\ DM^{-1}$)	138±4	236±13	240±14	178±3	156±44	91±3	146±5	37±1	72±2	$H_{8,3049}$=1538; ***
N_{gl} ($mg\ N\ m^{-2}$)	1212±53	2551±202	3704±363	1004±29	1273±33	7029±396	5624±217	2297±103	2519±55	$F_{1,37}$=170; ***
NRE (%)	30.1±2.3	65.1±1.7	80.3±0.9	53.6±1.5	44.6±2.1	88.7±0.4	92.3±0.2	22.0±2.4	61.7±1.2	$H_{8,251}$=220; ***

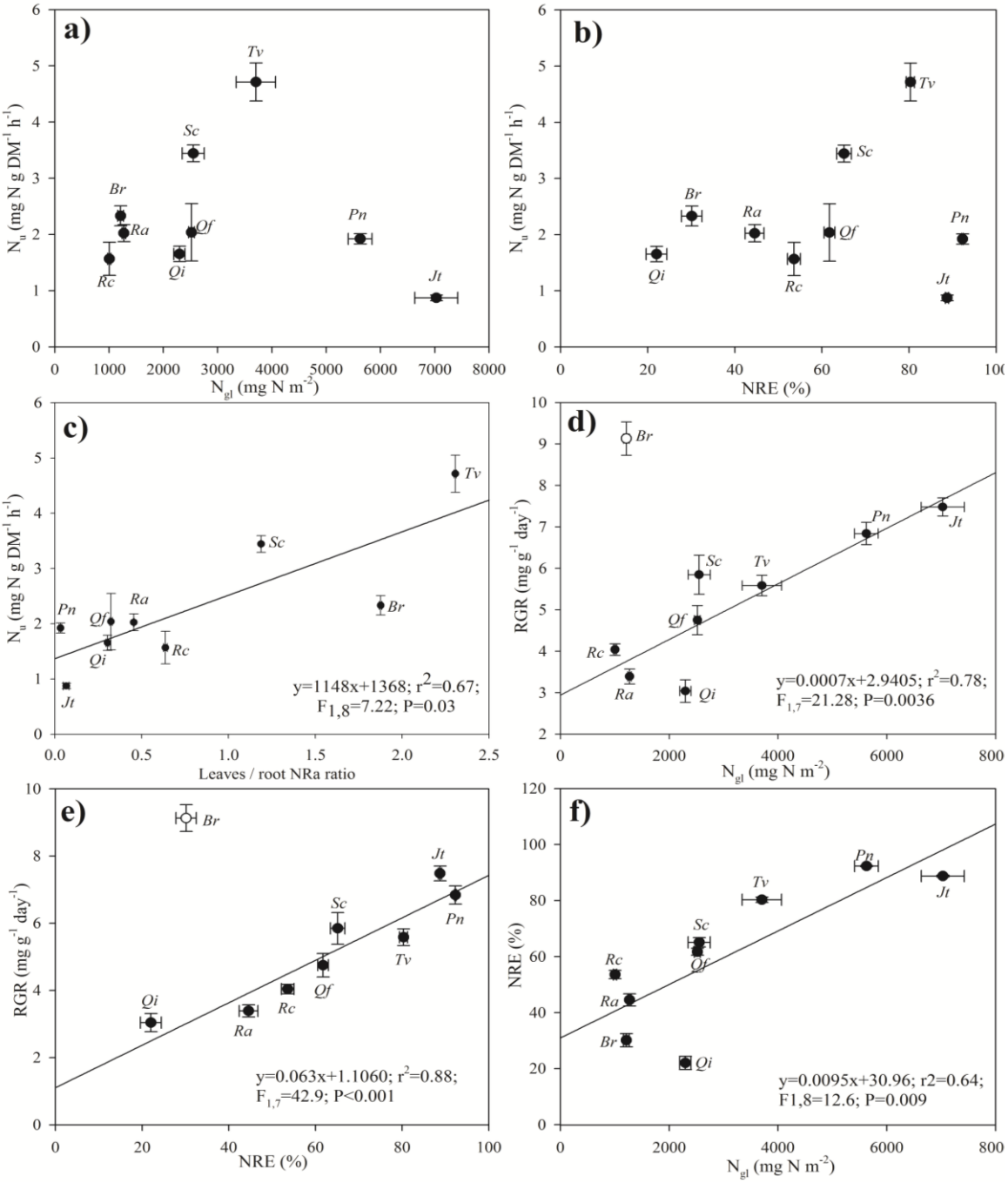


Figure S1. Relations of N uptake rate (N_u) with N concentration in green leaves at the beginning of the experiment (N_{gl}) (a), the N resoption efficiency (NRE) (b), and with leaves/root NRa ratio (c). Relationship between relative growth rate (RGR) with N_{gl} (d), NRE (e) and relationship between NRE and N_{gl} (f). Each point represents a species mean and bars are one standard error. *Brachypodium retusum* was excluded from the analyses in subfigures (c) and (d), and its values are indicated as an open circle.*Br: Brachypodium retusum, Sc: Santolina chamaecyparissus; Tv: Thymus vulgaris; Rc: Rosa canina; Ra: Rhamnus alaternus; Jt: Juniperus thurifera; Pn: Pinus nigra; Qi: Quercus ilex; and Qf: Quercus faginea.*

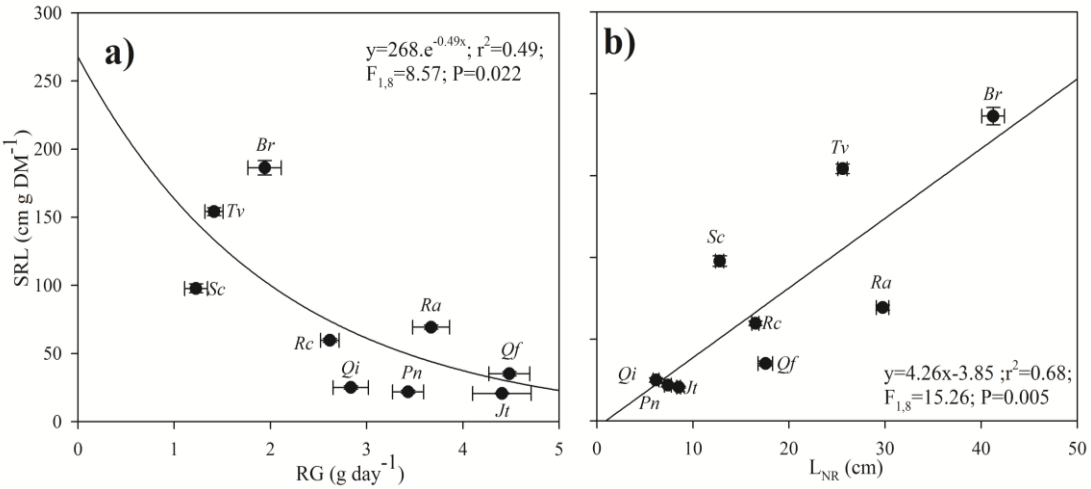
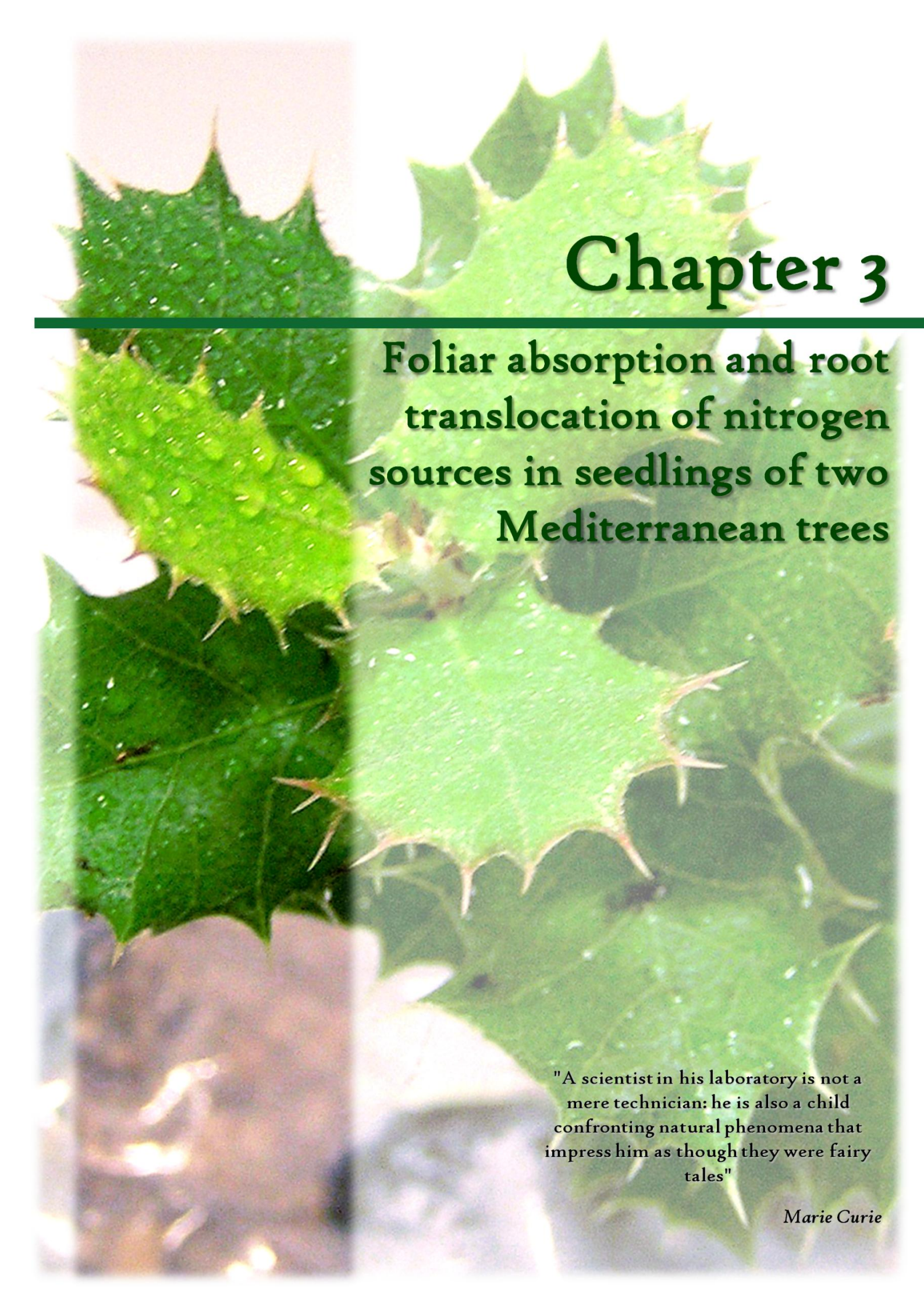


Figure S2. Relationships of specific root length (SRL) with root growth (RG) (a) and with total length of new root (L_{NR}) (b). Each point represents a species mean and bars are one standard error. *Br: Brachypodium retusum, Sc: Santolina chamaecyparissus; Tv: Thymus vulgaris; Rc: Rosa canina; Ra: Rhamnus alaternus; Jt: Juniperus thurifera; Pn: Pinus nigra; Qi: Quercus ilex; and Qf: Quercus faginea.*



Chapter 3

Foliar absorption and root translocation of nitrogen sources in seedlings of two Mediterranean trees

"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales"

Marie Curie

Este capítulo reproduce el texto del siguiente manuscrito:

This chapter reproduces the text of the following manuscript:

Uscola M., Villar-Salvador P., Oliet Palá J.A. & Warren. C.R. 2013. Foliar absorption and root dynamics translocation of nitrogen sources in seedlings of two Mediterranean trees (Submitted to **Tree Physiology**).

Fotography: Leaves of *Quercus ilex* seedlings after foliar fertilization.

By: M. Uscola

Absorción foliar y translocación a raíces de distintas fuentes de nitrógeno en plantones de dos especies de árboles mediterráneos

Resumen

La mayor parte del N que demandan las plantas es absorbido por las raíces. Sin embargo, el N depositado en el dosel de las plantas puede ser absorbido a través de las hojas y contribuir a la nutrición de la planta. La mayoría de los estudios de absorción foliar de nutrientes se han realizado con una única especie, y en especies agrícolas o en especies forestales de ecosistemas templados húmedos o boreales. Apenas existe información sobre la capacidad de absorber compuestos de N a través de las hojas en especies forestales de ecosistemas mediterráneos, donde, habitualmente, el N es un recurso limitado. Este estudio compara la absorción foliar de nitrato (NO_3^-), amonio (NH_4^+), urea y glicina en dos especies perennifolias mediterráneas, *Quercus ilex* y *Pinus halepensis*. Se prepararon soluciones de cada fuente de N a una concentración 40 mM N, con las que se fumigaron grupos independientes de plantones de un año de edad tres veces al día durante 48 h. Las fuentes de N estuvieron enriquecidas en ^{15}N, y la glicina doblemente marcada con ^{13}C y ^{15}N. Se utilizó la conductancia cuticular al vapor de agua (g_c) como un indicador de la permeabilidad de la cutícula al agua. *Quercus ilex* tuvo mayor absorción foliar de N que *P. hapelensis*. Ni g_c ni el área foliar explicaron las diferencias de absorción foliar de N entre especies, que probablemente estuvieron relacionadas con las diferencias en densidad estomática y la presencia de tricomas en Q. *ilex*. Las fuentes de N tuvieron distintas tasas de absorción, y consecuentemente diferente porcentaje de recuperación, conforme el siguiente orden para ambas especies: urea > NH_4^+ ≥ glicina ≥ NO_3^-. La alta correlación entre ^{15}N y ^{13}C absorbido y los resultados de cromatografía de gases-espectrometría de masas demuestran que una alta fracción de la glicina se absorbió intacta, siendo mayor en *P. halepensis* que en Q. *ilex*. En ambas especies, un incremento en g_c se relacionó con una mayor absorción de todas las fuentes de N, excepto en NO_3^- cuya absorción fue independiente de variaciones de g_c. Cambios en g_c afectaron más a la absorción de urea y NH_4^+ que a la de glicina, y el efecto fue más intenso en la encina que en el pino. Este resultado sugiere que la variación de la permeabilidad de la cutícula al agua está controlado por distintos mecanismos en cada especie, y que a su vez afecta de forma diferente a las formas de N. La fertilización foliar incrementó el contenido de N de parte aérea y radical, siendo este incremento mayor en las fuentes de N más absorbidas. Sin embargo, sólo un 40% del incremento de N se pudo explicar por la absorción foliar. El N absorbido por el follaje fue rápidamente transportado hacia las raíces, siendo más translocadas las fuentes de N inorgánicas que las orgánicas. Este estudio demuestra que dos importantes especies forestales mediterráneas pueden absorber por vía foliar cantidades significativas de fuentes de N, tanto inorgánicas como orgánicas, y que esta vía de penetración puede jugar un papel importante en la nutrición de N de los plantones.

Palabras clave: amonio; conductancia cuticular; fertilización foliar; glicina; nitrato; *Pinus halepensis*; nutrición vegetal; *Quercus ilex*; urea.

Foliar absorption and root translocation of nitrogen sources in seedlings of two Mediterranean trees

Abstract

Most nitrogen N taken up by plants is absorbed by roots. However, N deposited on plant canopies can also be absorbed through leaves and affect plant nutrition. Most studies on nutrient foliar absorption have been performed in crop and wet temperate and boreal forest plants. However, there is almost no information about the capacity to absorb N compounds through foliage in forest tree species from Mediterranean-type ecosystems, which are usually N-limited. This study compares the absorption of nitrate, ammonium, urea and glycine by foliage of two Mediterranean evergreen tree species, *Quercus ilex* and *Pinus halepensis*. One-year-old seedlings were sprayed three times per day for 48 h with 40 mM of each N source, which were labeled with ^{15}N. Glycine had a dual ^{13}C and ^{15}N labeling. Cuticular conductance (g_c) was used as a surrogate of cuticle permeability to water. *Quercus ilex* had higher foliar N absorption than *P. hapelensis*. Neither g_c nor total plant leaf area explained differences between species in absorption rates, which were likely linked to differences in stomatal density and trichome presence. N sources differed in foliar absorption rate and, consequently, in N recovery according to the following ranking for both species: urea > NH_4^+ ≥ glycine ≥ NO_3^-. The strong positive relationship between ^{15}N and ^{13}C uptake together with gas chromatography-mass spectrometry results, indicate that a significant fraction of glycine had penetrated intact in seedlings, with *P. halepensis* having higher intact glycine uptake rate than Q. *ilex*. In both species, higher g_c was associated with faster absorption of all N sources, except NO_3^-, which was absorbed independently of g_c. Changes in shoots g_c affected more urea and NH_4^+ than glycine absorption, and the effect was more intense in oak than in pine, suggesting that variation in cuticle permeability in both species are determined by different mechanisms and each N form were differently affected. Foliar fertilization led to increase shoot and root N content with largest increments for those N forms taken up most quickly. However, only around 40% of the increment in seedling N content was explained by foliar N uptake. Absorbed N was rapidly translocated to roots, with the proportion translocated to roots being larger for inorganic than organic N sources. This study demonstrates that two important Mediterranean forest tree species can absorb significant amounts of inorganic and organic N sources through leaves and that this penetration pathway can play an important role for seedling N nutrition.

Key words: ammonium; cuticule conductance, foliar fertilization; glycine; nitrate; *Pinus halepensis*; plant nutrition, *Quercus ilex*; urea.

Introduction

Nitrogen (N) is a nutrient limiting primary productivity in natural and managed terrestrial ecosystems (LeBauer and Treseder, 2008). Nitrogen demand for tree growth can be met either from external sources or by remobilization of internal stores (Millard and Grelet, 2010). Along with root uptake, plants can also absorb N through leaves (Eberhardt and Pritchett, 1971; Fageria *et al.*, 2009), which is a less effective nutrient pathway than roots due to the cuticle covering the leaf epidermis. Although foliar N absorption is lower than root N uptake, its importance for plant nutrition and function cannot be neglected (Sanz *et al.*, 2002). To meet their N demand, plants can take up either inorganic N compounds, such as nitrate (NO_3^-) and ammonium (NH_4^+), or organic N compounds, such as urea, amino acids, peptides, and proteins (Inselsbacher *et al.*, 2007; Paungfoo-Lonhienne *et al.*, 2008; Näsholm *et al.*, 2009).

Nutrients can penetrate leaves through several pathways. Although the main role of plant cuticle is to prevent uncontrolled water loss from plants to atmosphere, cuticle is partially permeable to gases, water, and several water and oil soluble compounds (Wilson, 1992; Bondada *et al.*, 2006). Absorption of dissolved compounds is assumed to occur mainly via the cuticle (Peuke *et al.*, 1998). Cuticle thickness and composition varies among species, while within plant species properties of the cuticle may vary depending on growth conditions (Hull *et al.*, 1975). Variation in cuticle thickness and composition may explain differences among plants in the rates at which compounds are absorbed (Baur *et al.*, 1997). Leaf cuticular conductance (g_c) to water vapor is strongly determined by cuticle structure and chemical properties and, consequently, it can be a proxy of cuticle permeability to several water soluble compounds (Niederl *et al.*, 1998; Burghardt and Riederer, 2003; Schreiber, 2005). In addition to absorption via the cuticle, nutrients may be also absorbed through stomata and trichomes (Hull *et al.*, 1975; Benzing *et al.*, 1976; Schreiber, 2005; Bondada *et al.*, 2006; Eichert and Goldbach, 2008). The relative importance of cuticle, stomata or trichome pathways for nutrient uptake differs among plant species (Haynes and Goh, 1977).

Nitrogen reaches leaf surface via dry and wet deposition both from natural and anthropogenic sources (Wilson, 1992; Rennenberg and Gessler, 1999). Man has dramatically increased N deposition in Europe during the last decades (EMEP 2000). N deposition in *Quercus ilex* L. (holm oak) and *Pinus halepensis* Mill. (Aleppo pine) woodlands, two common forest systems in the

Mediterranean basin, can reach up to 15 and 38 kg ha^{-1} yr^{-1}, respectively (Avila *et al.*, 2002; Michopoulos *et al.*, 2004) and forest canopies can retain up to 70% of N deposition (Adriaenssens *et al.*, 2010). N deposition in the Mediterranean basin is chiefly composed of inorganic N (mainly NO_x and NH_4^+), while organic forms of N, such as amino acids and urea, can reach up to 25% of total N deposition (Cornell, 2011). Most deposited N in semi-arid regions enters as dry deposition and in seasonal climates, such as Mediterranean ecosystems, dry N deposition mainly is concentrated during the dry season (Raison and Stottlemyer, 1991; Millán. *et al.*, 2002; Ochoa-Hueso *et al.*, 2011). This may result in highly concentrated solutions on leaf surfaces after small rain events or dew deposition in early fall. Thus, use of high N concentrations in studies on N deposition are ecologically realistic (Burkhardt, 2010) and may be useful for disentangling the negative versus positive effects of deposition. For example, high N deposition can have important negative ecosystem effects (Rennenberg and Gessler, 1999); whereas nutrient absorption through the canopy may also stimulate tree growth, especially in N-limited ecosystems (Sanz *et al.*, 2002).

Nitrogen fertilization in the nursery strongly influences the functional performance and field survival and growth of planted seedlings (Villar-Salvador *et al.*, 2012). Foliar fertilization is used to supply nutrients in crop species, especially when nutrients cannot be easily taken up from soil, for instance, P and Fe in limestone soils (Zohlen and Tyler, 2000) and/or during high nutrient-demand periods (Dong *et al.*, 2002). Nitrogen absorbed through foliage is directly incorporated to metabolism with lower microbial competition and nutrient leaching (Rennenberg and Gessler, 1999; Dong *et al.*, 2002). Foliar fertilization is rarely used in forest nurseries and plantations but it has potential for nutrient loading of seedlings without stimulating new growth or delaying dormancy during autumn (Bi and Scagel, 2008) and for improving nutrition of seedlings planted in very poor soils (Navarro, 2012). Urea, NH_4^+, and NO_3^- are typical N forms used for foliar fertilization. Urea is widely used because it is quickly absorbed, has lower toxicity than inorganic N forms, is highly soluble in water and oil and it is cheap (Bondada *et al.*, 2006; Stiegler *et al.*, 2009). Most studies on foliar absorption of N have been made with inorganic sources or urea independently (Bowman and Paul, 1992; Dong *et al.*, 2002; Bondada *et al.*, 2006). Few studies have compared the absorption of amino acids with other N sources (but see Eberhardt and Pritchett, 1971; Stiegler *et al.*, 2009), which complicates comparisons of N forms. Additionally, there is little information about the effect of amino acid foliar fertilization in forest tree species (Maini, 2006). Selecting appropriate N sources for foliar sprays is not only important for

uptake efficiency or plant performance but also to minimize foliage damage (Fageria *et al.*, 2009).

The objective of our study is to compare short term foliar absorption and subsequent translocation to roots of four N sources (urea, glycine, NH_4^+, and NO_3^-) in two Mediterranean evergreen trees, *P. halepensis* and *Q. ilex* ssp. *ballota* (Desf) Samp.. Moreover, we assessed if both trees can take up intact amino acids. Translocation to roots will be an indicator of the ability to transport N absorbed by leaves to other plant organs. The species have contrasting ecological and morpho-physiological characteristics and are widely distributed in the Mediterranean basin and used for afforestation. Pine is a fast growing pioneer tree (Zavala *et al.*, 2000), which has lower stomatal conductance and density than the oak, lacks trichomes, and wax on needle surface forms clusters of tubules (Mohammad Suleiman, 1986; Boddi *et al.*, 2002; Baquedano and Castillo, 2006). By contrast, oak is a slow growing late-succesional species (Zavala *et al.*, 2000), whose leaves have a dense layer of trichomes on the adaxial side and smooth wax cuticle layer (Bussotti and Grossoni, 1997; Paoletti *et al.*, 1998). We hypothesized that *Q. ilex* will have higher rates of N foliar absorption than *P. halepensis*, due to its smooth structure of cuticle waxes, higher stomata density, and trichomes presence. To perform this study we sprayed ^{15}N-^{13}C labeled solutions of the four N sources and assessed label abundance in shoots and roots after 48h.

Material and methods

Foliar treatments, experimental design and ^{15}N-^{13}C labeling

Ninety-six seedlings per specie of *P. halepensis* and *Q. ilex* subsp. *ballota* (thereafter *Q. ilex*) were cultivated outdoors at the nursery of the Centro Nacional de Mejora Genética Forestal "El Serranillo" (MAGRAMA) using seeds from inland Iberia Peninsula provenances. Seedlings were grown for 14 months in individual 305 mL pots (Super-Leach™, Bardi S.A.L., Navarra, Spain) filled with fertilized peat (Kekkilä F6, Kekkilä Oy, Finland) following standard cultivation protocols in Mediterranean nurseries (Villar-Salvador *et al.*, 2004a). The N sources tested were: ammonium (NH_4^+), nitrate (NO_3^-), urea ($CO(NH_2)_2$), and glycine (NH_2CH_2COOH). NO_3^- was supplied as KNO_3, and NH_4^+ as $SO_4(NH_4)_2$. N concentration of each N source was 40 mM. For each N source we prepared two solutions. The first solution contained a N source at natural abundance (0.3664 atom% ^{15}N and 1.082 atom% ^{13}C), while the second solution contained the same N source but enriched in ^{15}N (labeled N source) (60

atom% $^{15}NO_3^-$, 60 atom% $^{15}NH_4^+$, 98 atom% urea-$^{15}N_2$, Sigma Aldrich Co, Milwaukee, USA) or 2-$^{13}C^{15}N$ labeled glycine (98-99 atom% ^{15}N-^{13}C glycine, Cambridge Isotope Laboratories, London, UK). Each labeled solution was applied to different groups of 7-14 seedlings per species (labeled seedlings), while the unlabeled solution was applied to different groups of six seedlings (unlabeled seedlings).

Application method and quantification of the solution retained in shoots.

Pots were sealed with a plastic sheet to avoid substrate contamination during foliar fertilization (Figure 1). Shoots were sprayed both laterally and from above to ensure complete impregnation of the shoot, three times per day (7:00, 12:00 and 17:00 h solar time) for two days inside a laboratory. Photosynthetic photon flux density was < 200 µmol m^{-2} s^{-1} and temperature and relative humidity during the experiment were 22.5±0.5 °C and 42.9±1.6 % (mean ± 1 SE), respectively. The solution volume needed to saturate the shoot without dripping was previously determined. The initial and final weight of the sprayer was recorded to quantify the solution applied to each seedling. To quantify the amount of solution intercepted by the canopy we surrounded shoots with a cone of filter paper, which was inserted in a plastic bag to prevent humidity loss and weighed before and immediately after spraying. The amount of solution retained by each plant (RV) was calculated as the difference between the total

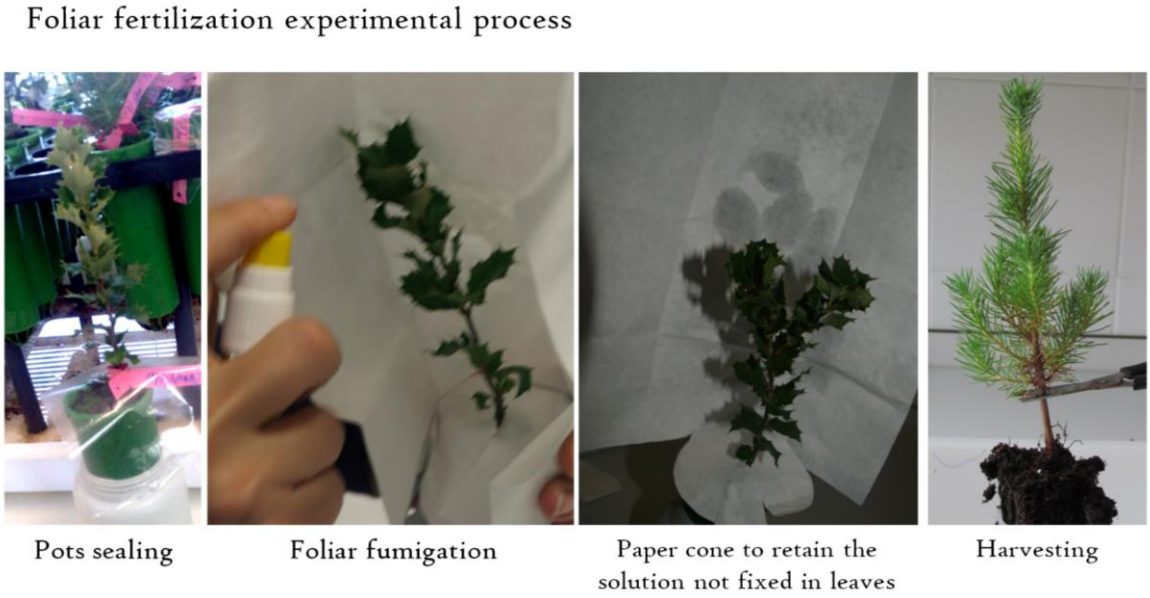


Figure 1. Flow diagram of the key events through the experiment.

solution applied and the solution retained in the filter paper:

$$RV=[P_{fs}-P_{is}]-[P_{fp}-P_{ip}] \quad \text{(mL)} \quad (1)$$

where; P_{fs}, P_{fp} are final weight of the sprayer and filter paper, respectively; and P_{is}, P_{ip} are initial weight of the sprayer and filter paper, respectively. Density of solution was ~1 mg mL^{-1}. Total liquid amount sprayed per plant at the end of the experiment was 6.05 ± 0.06 mL. There was no differences in total amount of sprayed liquid between species ($F_{1,84}$=0.20; P=0.66) or treatments ($F_{3,84}$=0.64; P=0.59).

Plant material processing and cuticular conductance to water vapor determination.

Four plants per species were harvested at the beginning of the experiment (control plants). At the end of the experiment, both labeled and unlabeled plants were harvested, shoots were cut at the point where the plastic was sealed under the first leaf of the shoot, and roots were frozen until processed. For each shoot residual transpiration was measured following methodology in Villar-Salvador *et al.* (2004b), whereby shoots were weighed to the nearest 0.1 mg every 20 min for 8 h. By plotting shoot fresh weight against time, a curvilinear relationship was obtained, in which the linear portion represents water loss from plant surfaces after complete stomatal closure. After fresh weight measurement, leaf area was determined with an image analyzer (Delta-T Image Analysis System 1.12, Delta-T Devices LTD, UK) and stem diameter and height were used to determine the stem surface area assuming the stem to be a cone. Area of absorbing surface (AS) was calculated as leaf + stem area. Residual transpiration was calculated as the ratio of the slope of the linear portion of the time-weight plot and AS. Cuticular conductance to water vapor (g_c) was calculated from measured rates of residual transpiration using an Ohm's Law analogy (Nobel, 1983):

$$RT = \delta Cv \times g_c \quad \text{(mmol } m^{-2}\ s^{-1}\text{)} \quad (2)$$

where RT is the residual transpiration, δC_v is the mole fraction concentration gradient of water vapor between the leaf and the laboratory atmosphere (based on temperature and relative humidity measurements taken next to seedlings).

After measuring residual transpiration, leaves and stems were washed with soapy water and root plugs were carefully washed from the growing media. All plant material was rinsed in de-ionized water twice, oven-dried at 60

°C for 48 h and weighed to determine their mass. Plant mass was 5.1±0.2 and 2.04±0.05 g for *Q. ilex* and *P. halepensis*, respectively. Within species, seedling mass did not differ among treatments ($F_{3,112}$=0.52; P=0.67). Similarly, AS did not differ between species (78.1±1.8 cm^2; $F_{1,111}$=0.52; P=0.52), N sources ($F_{3,106}$=0.031; p=0.81) or species x N sources interaction ($F_{3,111}$=0.02; P=0.99).

Isotopic analyses and calculation of absorption and allocation to roots

Roots and shoots were separately ground in a ball mill (PM100, Retsch, Haan, Germany). N and C concentration and ^{15}N and ^{13}C abundance were determined by IRMS (CF-IRMS Isochrom, Micromass, UK) at the Stable Isotopes Laboratory of the University of California-Davis. The amount of labeled N ($N_{labeled}$) was calculated:

$$N_{labelled} = X_N \times [N_{organ}] \times DM \quad \text{(mg)} \quad (3)$$

where $[N_{organ}]$ is the organ (shoot or root) N concentration; DM is the organ mass, and X_N is the fraction of labeled N in the organ (Näsholm *et al.*, 1998):

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

where A_{LO} and A_{UO} are the ^{15}N abundance of the organ in labeled and unlabeled samples, respectively. A_{LF} and A_{UF} are the ^{15}N abundance of the labeled and unlabeled fertilizer, respectively. The amount of labeled C ($C_{labeled}$) was calculated using the same equations but substituting X_N, N_{organ} or ^{15}N abundance with X_C, C_{organ} and ^{13}C abundance, respectively.

N absorption rate of each labeled N source was calculated with the followings equations:

$$\text{Absorption} = \frac{N_{labelled}}{AS \times time} \quad (\mu g\ N_{labeled}\ cm^{-2}\ day^{-1}) \quad (5)$$

Absorption efficiency ($N_{recovery}$) of each N source was calculated as the percentage of absorbed N relative the total amount of N that was retained on the shoot.

Because glycine can be decomposed by leaf surface microbes, we followed two methods to assess whether glycine was taken up intact. The first method calculates the proportion of intact glycine absorbed comparing how much the slope of the regression line between $C_{labeled}$ against $N_{labeled}$ in plant deviates from the regression line of slope = 1 predicted from the stoichiometry

of intact dual labeled glycine uptake (i.e., 1 moles of ^{13}C per mole of ^{15}N) (Näsholm *et al.*, 1998). The second method analyzed the amount of ^{15}N-$2^{13}C$-glycine in a sample by gas chromatography-mass spectrometry (GC–MS). For a detailed description of the method see Warren (2009). ^{12}C,^{14}N-glycine was quantified from mass 246, and ^{13}C,^{15}N-glycine was quantified from mass 248 after subtracting the contribution to mass 248 from unlabeled samples.

Statistical analyses

The effect of N sources and species on absorption rate was analyzed by analysis of covariance (ANCOVA) with g_c as the covariate. As the parallelism hypothesis was not met, separate slopes ANCOVA were conducted. Differences between species or among N sources in the slope of the regressions between N source absorption *vs.* cuticular conductance were conducted by checking the significance of regression coefficient β_3 from the model:

$$\text{N absorption} = \beta_0 + \beta_1 \cdot g_c + \beta_2 \cdot Z + \beta_3 \cdot g_c \cdot Z + \varepsilon \qquad (4)$$

were β_i are the regression coefficients, Z is a categorical variable representing the species or the N sources and ε is the random term of the model (Doménech, 1999). Differences between species in absorption rate of intact and deaminated glycine were assessed by one-way ANCOVA with g_c as covariate. Species and N source effect on partitioning of labeled N into shoots and roots, $N_{recovery}$ and organ N concentration was analyzed by two-ways ANOVA. Data homocedasticity was checked according to Levenne´s test. When ANOVA assumptions were not met, data transformation was conducted. Significance level was α=0.05 for all analyses. Statistical analyses were realized with STATISTICA 7.0 (StatSoft, Tulsa,USA).

Results

Absorption of N sources and cuticular conductance

The total volume of solution retained by the seedlings at the end of the experiment (RV) differed between species, ($F_{1,96}$=120; P<0.001), with *Q. ilex* retaining more solution than *P. halepensis* (3.31±0.10 and 2.11±0.05 mL for oak and pine, respectively). RV was independent of fertilization treatments ($F_{3,96}$=2.2; P=0.098). RV in each application was positively related to AS in both species (RV=2.1·AS+177.5; r^2=0.32; $F_{1,56}$=25.6; P<0,001 for *P. halepensis* and RV=3.4·AS+283.2; r^2=0.53; $F_{1,58}$=19.3; P<0.001 for *Q. ilex*) and regression lines differed between species ($F_{1,96}$=5.9; P=0.017). The intercept of regression line,

which represents the amount of solution retained by aboveground parts other than leaves or main stems, such as short stems of twigs or leaf margin, was higher in *Q. ilex* than in *P. halepensis*. Similarly, RV per leaf area unit, as indicated by the slope of the regression line, was 62% higher in *Q. ilex* than in *P. halepensis* (3.4 and 2.1 μl cm^{-2}, respectively).

Oak had lower g_c than pine (4.9±0.4 *vs.* 13.1±1.5 mmol m^{-2} s^{-1}, respectively). In contrast, averaged across the different N sources, *Q. ilex* had higher absorption rate than *P. halepensis* (1.4±0.2 *vs.* 0.6±0.1 $\mu gN_{labeled}$ cm^{-2} day^{-1}). Absorption rate was strongly positively related to g_c after pooling N sources and species at plant level ($F_{8,74}$=5.5; P<0.001). Slopes regressions of *Quercus ilex* were significantly steeper than slopes of *P. halepensis* (Figure 2). Also, slopes of linear regressions between plant absorption rate and g_c differed among N sources in both species ($F_{3,26}$=17.1; P<0.001 and $F_{3,27}$=21.7; P<0.001 for *P. halepensis*

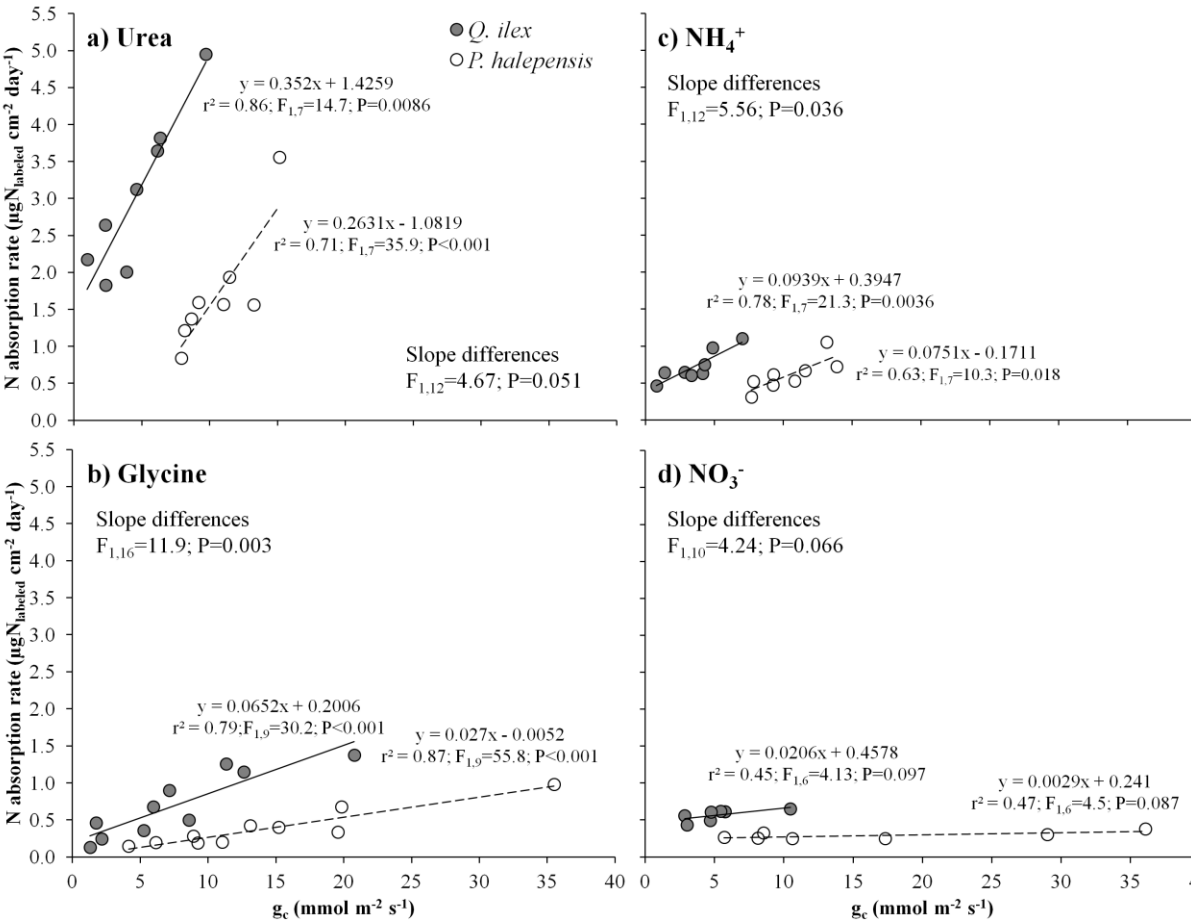


Figure 2. Linear regressions between plant absorption rate of different N sources: urea (a), NH_4^+ (b), glycine (c) and NO_3^- (d) and cuticular conductance (g_c) and in *Quercus ilex* and *Pinus halepensis* one-year-old seedlings, which were sprayed three times a day for two days with 40 mM N solutions. Each point represents an individual.

and Q. *ilex*, respectively). Slopes of regression lines were steepest for urea and lowest for glycine, whilst slopes for NH_4^+ had intermediate values in both species. Regressions between plant NO_3^- absorption rate and g_c were not statistically significant.

Species and N source affected foliar absorption at plant level. *Quercus ilex* had higher absorption rate than *P. halepensis* ($F_{1,74}$=8.0; P=0.007). Species × N source interaction was not significant ($F_{3,74}$=2.1; P=0.11). N source differed in absorption rates ($F_{3,74}$=7.5; P<0.001). The rate of urea absorption was approximately three times higher than the other N sources (Figure 3a). No statistical differences in absorption rates were found among the remaining N sources, although the general trend was that absorption rate decreased in the following order: NH_4^+ > glycine > NO_3^-.

Nitrogen absorption efficiency, translocation to roots and organ N content

Species ($F_{1,53}$=9.5; P=0.003) and N source ($F_{3,53}$=133; P<0.001) affected the proportion of N retained on foliage that was taken up ($N_{recovery}$). However, the effect N source on $N_{recovery}$ depended on species (Species × N source interaction, $F_{3,53}$=4.9; P=0.004). The recovery of urea was significantly higher than for the other N sources in both species (Figure 3b). NO_3^- had the lowest $N_{recovery}$ in Q. *ilex*, while NH_4^+ and glycine had intermediate values. $N_{recovery}$ in *P. halepensis* decreased in the following order: NH_4^+ > NO_3^- > glycine.

For all N sources most (> 90%) absorbed N was recovered in shoots (Figure 3c). Oak translocated higher amounts of N to roots than pine for all N sources (data not shown). Nitrogen source ($F_{3,45}$=47; P<0.001) and species ($F_{1,45}$=10.5; P=0.002) affected proportion of $N_{labeled}$ in roots, although interaction between both factors also was significant ($F_{3,45}$=13.1; P<0.001). Root partitioning of $N_{labeled}$ was smaller in oak than in pine for all N sources except for NO_3^-. Both organic N sources had lower proportion of $N_{labeled}$ in roots than inorganic N sources in Q. *ilex*, while this only occurred for urea in *P. halepensis*.

Seedlings were not N deficient prior the beginning of the experiment. Control plants in *P. halepensis* had higher shoot N concentration than controls in Q. *ilex* (16.3±0.5 and 14.9±0.2 mg g^{-1} for pine and oak, respectively; $F_{1,5}$=6.9; P=0.046). However, N content was higher in Q. *ilex* than in *P. halepensis* in both control and N fertilized seedlings ($F_{1,117}$=113; P<0.001 for shoots and $F_{1,106}$=894; P<0.001 for roots). N source affected shoot and root N content ($F_{4,117}$=3.7; P=0.007 and $F_{4,106}$=5.5; P<0.01 for shoot and root N content, respectively). Overall, control plants had lower shoot and root N content than foliar fertilized

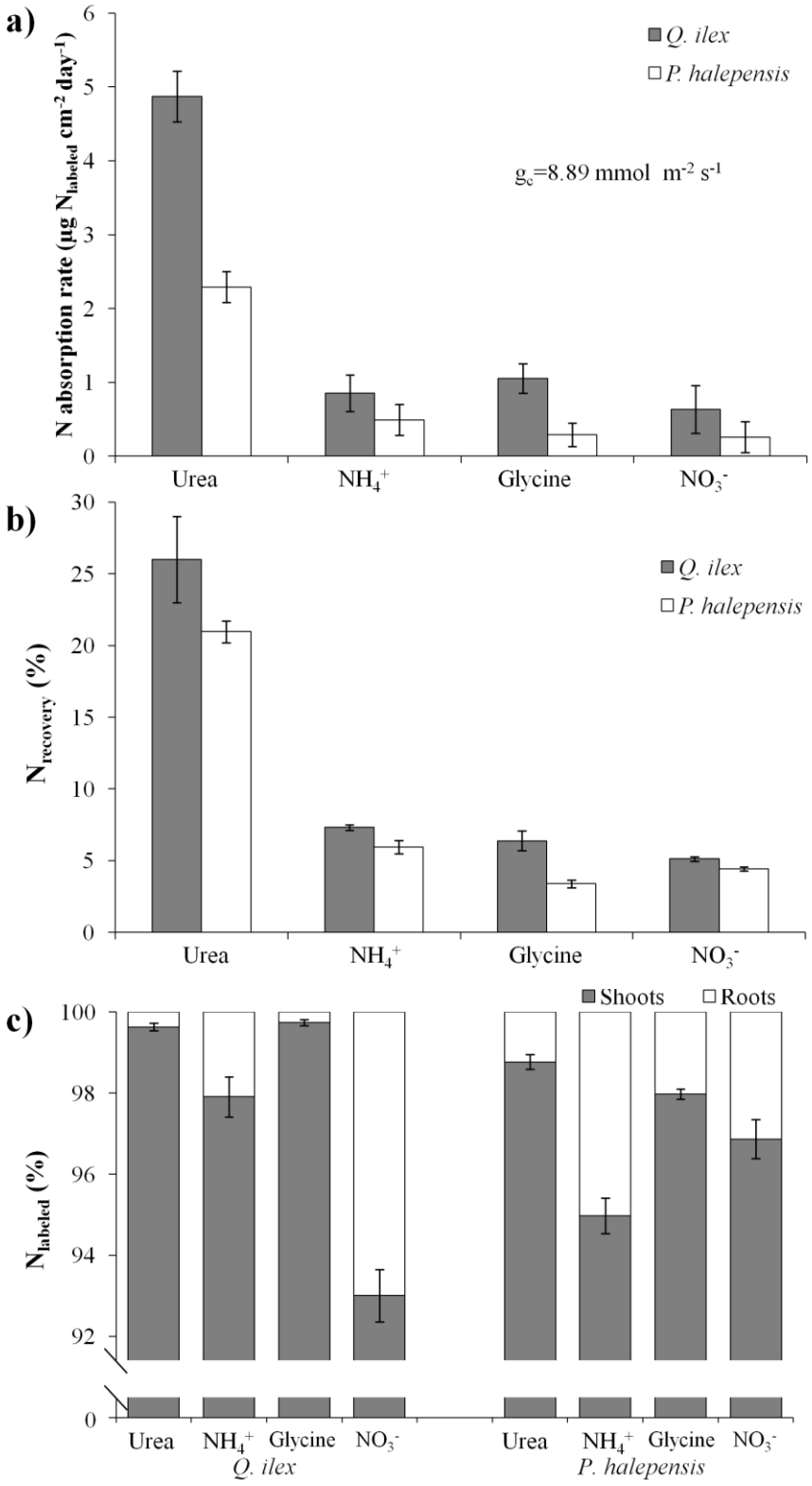


Figure 3. N Absorption rate at plant scale (a), N absorption efficiency ($N_{recovery}$) (b) and percentage of total $N_{labeled}$ recovered in shoots and roots (c) in urea, glycine, NH_4^+ and NO_3^- foliar fertilized *Quercus ilex* and *Pinus halepensis* one-year-old seedlings, which were sprayed three times a day for two days. Concentration of sprayed solutions was 40mM N. Data are means ± 1 SE, except for foliar absorption rate which results are adjusted least squares means computed at g_c=8.89 mmol s^{-1} m^{-2}.

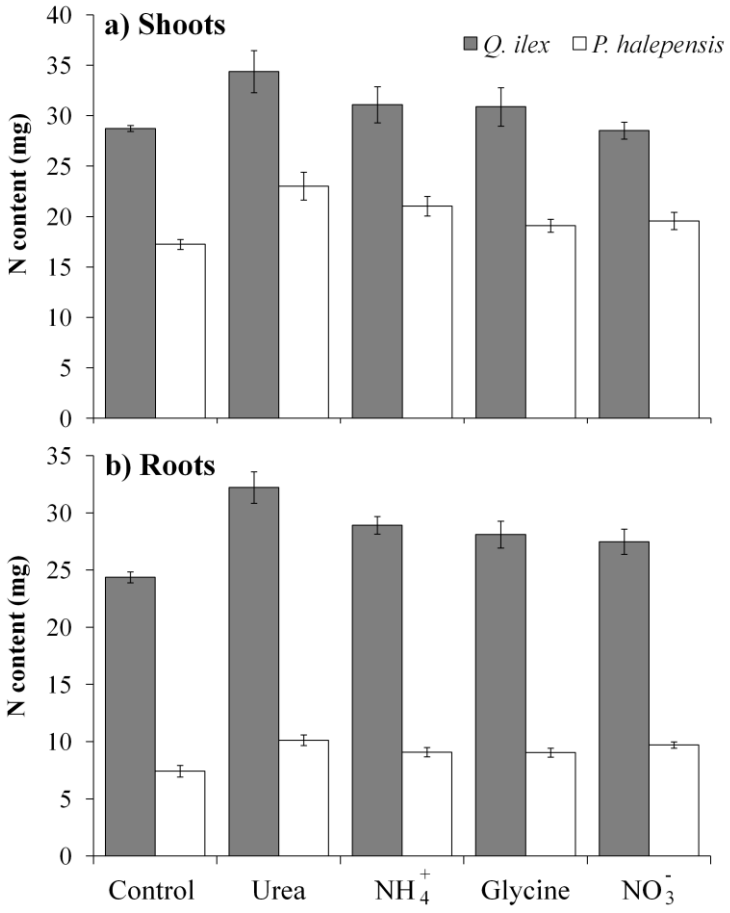


Figure 4. N content in shoots (a) and roots (b) of *Quercus ilex* and *Pinus halepensis* one-year-old seedlings that were sprayed three times a day for 2 days with 40 mM N solutions of urea, NH_4^+, glycine and NO_3^-. N concentration of plants immediately before foliar fertilization (control) is given. Data are means ± 1 SE.

seedlings (Figure 4). Shoot and root N content was higher in seedlings fertilized with urea than in seedlings fertilized with the remaining N sources. The general trend in both organs for the remaining N sources was that N content decreased in the following order: $NH_4^+ > NO_3^- \geq$ glycine.

Intact glycine absorption

The proportion of glycine absorbed as intact molecules was estimated by the slope of the linear regression between plant $C_{labeled}$ and $N_{labeled}$. $C_{labeled}$ was strongly linearly related to $N_{labeled}$ at plant level. Based on the slope of the regressions, the proportion of intact glycine absorbed was higher in pine (85%) than in oak (65%) (slope differences $F_{1,26}=26.7$; $P<0.001$) (Figure 5a). *Quercus ilex*

had higher intact glycine plant absorption rate than *P. halepensis* ($F_{1,18}$=27.5; P<0.001, 0.68±0.04, 0.25±0.04 $\mu gN_{labeled}$ m^{-2} day^{-1} for *Q. ilex* and *P. halepensis* respectively) and g_c was a significant covariate ($F_{1,17}$=10.8; P<0.001).

^{15}N enrichment was detected in shoots and roots of glycine-fertilized seedlings in both species. ^{13}C enrichment was detected in the shoots of both species, but only in the roots of pine (data not shown). $C_{labeled}$ was strongly linearly related to $N_{labeled}$ at shoot level. The proportion of intact glycine estimated in shoots, was high for both species as $C_{labeled}$ *vs.* $N_{labeled}$ linear

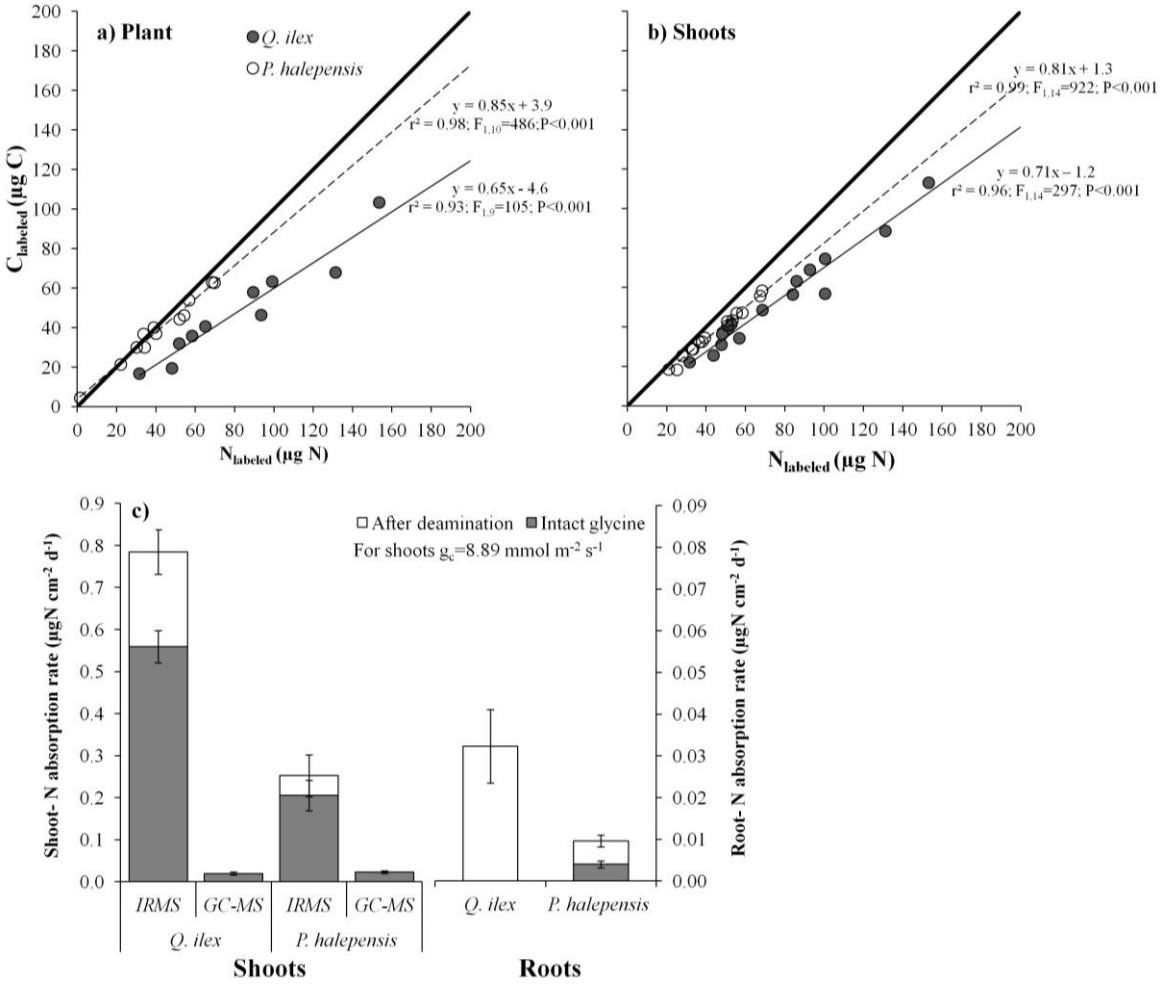


Figure 5. Regressions between $C_{labeled}$ and $N_{labeled}$ at plant (a) and shoot (b) level in glycine foliar-fertilized *Quercus ilex* and *Pinus halepensis* one-year-old seedlings that were sprayed three times a day for 2 days with 40 mM N solutions. Each point represents an individual. Solid thick line is the expected reference line with slope 1 according to ^{13}C and ^{15}N stoichiometry in intact dual labeled glycine. Subfigure (c) is total (intact + de-aminated glycine) and intact glycine absorption rate estimated by regression analysis in shoots and roots, and dual labeled glycine detected by GC-MS in shoots in both species (note the different scales of Y-axes for shoots and roots in (c)). Least square means ± 1 SE in shoots were computed for g_c =8.89 mmol m^{-2} s^{-1}.

regression slope indicate (Figure 5b). Intact glycine in shoots was also significantly higher in *P. halepensis* (81%) than *Q. ilex* (71%) (slope differences $F_{1,27}$=26.7; P<0.001). The amount of N from total glycine (intact + after deamination) estimated in shoots and from intact glycine was higher in *Q. ilex* than *P. halepensis* (Figure 5c) ($F_{1,29}$=29.6; P<0.001 for total N from glycine and $F_{1,29}$=28.54; P<0.001 for N from intact glycine). The g_c was also a significant covariate ($F_{1,29}$=8.86; P=0.0010 for total and $F_{1,29}$=10.0; P<0.001 for intact glycine, respectively). The uptake rate of intact dual labeled glycine detected by GC-MS in shoots was small compared with the uptake rate estimated by the ^{13}C-^{15}N molar ratio method, being higher in *P. halepensis* than in *Q. ilex* ($F_{1,23}$=6.46; P=0.018) (4.5 % and 13.2 % of total glycine uptake in *Q. ilex* and in *P. halepensis*, respectively). g_c was a significant covariate for intact dual labeled glycine detected by GC-MS ($F_{1,25}$=15.9; P<0.001). N taken up from glycine was mainly in shoots. *Pinus halepensis* roots were both ^{15}N and ^{13}C enriched but no significant relationship between $C_{labeled}$ and $N_{labeled}$ was observed (data not shown) and $C_{labeled}$ was lower than $N_{labeled}$ with $C_{labeled}$ / $N_{labeled}$ ratio being 0.39. The mean ^{13}C abundance in *Q. ilex* roots was similar to that in control plants, and no $C_{labeled}$ was found in roots in most seedlings (data not shown).

Discussion

Differences in foliar absorption between species and among N sources

This experiment demonstrates that both species are able to absorb through leaves all studied N sources. Despite the high concentration used (40 mM N) in foliar sprays, none of the N sources caused foliar necrosis or other adverse symptoms, indicating that the two species have high salinity and N toxicity tolerance. In accordance with our hypothesis absorption was higher in oak than in pine. Similar to our findings, three broadleaf trees had higher NH_4^+ and NO_3^- foliar uptake than *Pinus sylvestris* L. (Adriaenssens *et al.*, 2010). Our results suggest that N atmospheric deposition and foliar fertilization in the nursery will potentially have greater impact on nutrition of oak than on pine. Several explanations can be put forward for the species differences. First, *Q. ilex* leaves had higher liquid retention than *P. halepensis* needles. As both species had similar AS, we suggest that *Q. ilex* retains more liquid than *P. halepensis* because retention of liquid by *Q. ilex* is enhanced by possession of broad leaves inserted with a steep angle and a layer of fused-stellate trichomes that are specially abundant in the abaxial leaf side and in stems (Bussotti and Grossoni, 1997). In agreement with our findings, *Pinus sylvestris* also showed lower RV than three

deciduous broadleaf trees (Adriaenssens *et al.*, 2010). Second, as foliar absorption is a concentration gradient driven process (Brumme *et al.*, 1992) and shoot N concentration was higher in *P. halepensis* than in *Q. ilex*, thus, the potential for N diffusion in pine was probably lower than in oak. Third, N absorption can occur through stomata, and species differ in their ability to take up compounds through them (Eichert and Goldbach, 2008). Positive relationships between foliar absorption and the number and distribution of stomata have been observed in several species (Eichert and Goldbach, 2008; Fernández and Eichert, 2009). On this basis, we expected stomatal penetration of N compounds to be more difficult in *P. halepensis* than in *Q. ilex* because pine has lower stomatal density than oak (80 *vs.* 465 stomata mm^2 for *P. halepensis* and *Q. ilex*, respectively) (Mohammad Suleiman, 1986; Paoletti *et al.*, 1998; Boddi *et al.*, 2002). Furthermore, while stomata of *Q. ilex* are located at the leaf surface (Bussotti and Grossoni, 1997), stomata of *P. halepensis* are inside epistomatic chambers (Mohammad Suleiman, 1986), which frequently are partially sealed with a wax plug that are believed to act as filters limiting particle deposition (Burkhardt, 2010). Finally, the abaxial face of *Q. ilex* leaves are covered by a dense layer of fused-stellate trichomes (Bussotti and Grossoni, 1997). The base and branches of trichomes have been identified as penetration sites for solutes (Hull *et al.*, 1975; Benzing *et al.*, 1976; Schreiber, 2005) and residence time of solution in contact with the cuticle is increased by trichomes (Karabourniotis *et al.*, 1998; Morales *et al.*, 2002).

The greater N absorption in oak relative to pine cannot be explained by differences in cuticle water permeability. *Quercus ilex* shoot surface was less permeable to water (as indicated by g_c) but had higher N absorption than *P. halepensis* (Figure 2). However, the importance of the cuticle pathway in both species is supported by our finding of a strong relationship between g_c and absorption of all N sources except NO_3^- at an intraspecific level. Interestingly, the slope of the relationship between absorption rate and g_c differed between species, which suggests that the factors underpinning permeability to N differ between species and are not solely a function of g_c. A partial explanation may lie in the observation that composition and structure of cuticle waxes differs between the two species (Mohammad Suleiman, 1986; Panahi *et al.*, 2012) and differences in cuticle absorption are more related to the composition and structure of the cuticle waxes than to wax content or cuticle thickness (Santier and Chamel, 1998; Bondada *et al.*, 2006; Adriaenssens *et al.*, 2010). Also, in contrast to the smooth cuticle in *Q. ilex* (Bussotti and Grossoni, 1997), the cluster tubular wax cuticle structure in *P. halepensis* (Boddi *et al.*, 2002) may

reduce N absorption due to lower solution contact with the leaf surface (Adriaenssens *et al.*, 2010).

The relative rates of absorption of the different N sources were the same in the two species: urea > NH_4^+ > NO_3^- = glycine. Other studies also have found faster absorption of urea than other N sources (Eberhardt and Pritchett, 1971; Stiegler *et al.*, 2009) and NH_4^+ to be absorbed faster than NO_3^- (Brumme *et al.*, 1992; Peuke *et al.*, 1998). In contrast, the epiphytic tank Bromeliad *Vriesea gigantea* (Gaudich.) had higher uptake preference for NH_4^+ than for urea, NO_3^- or several amino acids (Inselsbacher *et al.*, 2007). In accordance with higher absorption of urea than the other N sources, $N_{recovery}$ of urea was the highest (25%) while the remaining N sources had $N_{recovery}$ values between 8 and 3 %, which are low when compared with $N_{recovery}$ values in other studies. For instance, Klein and Weinbaum (1985) found $N_{recovery}$ values > 60 % in *Olea europaea* and close to 94% in *Prunus dulcis* for urea, while Eberhardt and Pritchett (1971) found $N_{recovery}$ values of 71%, 45% for and 39% for urea, NO_3^-, and NH_4^+, respectively in *Pinus elliottii*. Also Stiegler *et al.* (2009) found $N_{recovery}$ greater than 40% for all N sources in *Agrostis stolonifera*. Differences with previous studies may be explained by either lack of surfactant in solutions or the study method and the duration of the experiment.

Differences among N forms in absorption rates may be related to their penetration mechanisms and their physicochemical properties. While ionic compounds can only use polar aqueous pores to penetrate cuticle, non-ionic molecules, such as organic N compounds, can penetrate cuticle via both the lipophilic pathway and polar aqueous pores (Riederer and Schreiber, 2001; Eichert and Goldbach, 2008). This probably explains the highest uptakes rates in urea. Although, glycine is also a non ionic compound its uptake rate was lower than in urea and can be explained by its high molecular weight and other physicochemical properties (that are discussed below) (Baur *et al.*, 1997). Moreover, since leaf surfaces are partially permeable to water, compounds that are highly soluble in water penetrate more easily through polar aqueous pores than less soluble compounds (Riederer and Schreiber, 2001). Thus, urea, the compound that was taken up fastest, had the highest solubility (108 g 100 ml^{-1} at 20 °C) and lowest hygroscopicity (10% deliquescence point at 25 °C), which allows deposits to lengthen the aqueous phase for an extended period of time (Bondada *et al.*, 2006). By contrast, NO_3^-, the slowest N form taken up, had the lowest solubility and highest hygroscopicity (38 g 100 ml^{-1} and 94%, respectively). Finally, NH_4^+ and glycine had intermediate values of solubility

(75 and 0.225 g 100 ml^{-1}, respectively), and hygroscopicity (46 and 94%, respectively) and had also intermediate uptake rates between urea and NO_3^-.

Nitrogen sources were differently affected by differences in permeability of the cuticle to water (g_c, Figure 2), with urea being more affected than the other N sources as indicate by steeper slope of absorption rate *vs.* g_c regressions. These differences probably also reflect distinct penetration mechanism of the different N compounds discussed previously. Notably, the lack of significant relationship between NO_3^- absorption and g_c in both species suggest that limitations imposed to anion permeability through the cuticle are unaffected by differences in cuticle water permeability and that leaf penetration of NO_3^- probably is more linked to stomata than to cuticular pathway, as shown in other studies (Eichert and Burkhardt, 2001; Eichert and Goldbach, 2008). Finally, independence of NO_3^- absorption and g_c indicates that faster N absorption in plants with faster g_c is unlikely due to incomplete stomatal closure.

Due to low metabolic cost, plants can benefit from foliar fertilization with amino acids if these are taken up intact. Our study demonstrates that only part of the glycine retained on shoot surface was taken up intact. However, methodology used to assess intact glycine uptake, ^{13}C-^{15}N molar ratios and GC-MS, have some limitations due to post-uptake metabolism. According to the ^{13}C-^{15}N molar ratio method, a high proportion of glycine was taken up intact (85% and 65 % for pine and oak, respectively) (Figure 5). As we quantified ^{15}N and ^{13}C at plant level, underestimation of intact glycine due to transfer of ^{15}N and/or ^{13}C to other molecules inside the plant (Warren, 2009a) cannot be supported. However, a ^{13}C-^{15}N molar ratio similar to the reference stoichiometry of intact dual-labeled glycine could also be achieved if glycine is mineralized on leaf surface and ^{13}C and ^{15}N are subsequently taken up independently (Jones *et al.*, 2005). Similarly, $^{13}CO_2$ respiration loss after glycine metabolism can also alter organ ^{13}C-^{15}N molar ratio (Warren, 2012). Thus, results obtained from the ^{13}C-^{15}N molar ratio method must be interpreted with caution and considered as an estimation of potential intact glycine uptake. On the contrary, GC-MS method measures the exact amount of intact glycine inside the plant. The amount of intact glycine detected by this method was low in both species and notably lower than intact glycine estimated by the ^{13}C- ^{15}N molar ratio method. Glycine is quickly metabolized (Warren, 2012) and our experiment lasted 2 days, which might explain why only a small proportion of intact glycine was detected inside the plant. Both methods of quantifying intact glycine evidenced that holm oak had lower intact glycine absorption than

Aleppo pine. It is possible that a proportion of glycine was decomposed before absorption by phyllosphere microbial community, which usually is C-limited and tends to immobilize C-rich sources on leaf surface (Mercier and Lindow, 2000; Lindow and Brandl, 2003). Presence of a dense trichome layer in *Q. ilex* leaves might promote phyllosphere microbe growth (Mercier and Lindow, 2000) and consequently higher potential glycine deamination rate.

N loading and translocation as a function of N source

Foliar application of all N sources led to increased N content in shoot and roots, with the increment in N content consistent with differences among N forms in rates of absorption. This result indicates that nurseries might be able to use foliar fertilization as a means of seedling nutrient loading that complements fertilization regimes based on root uptake (Landis *et al.*, 1989). Our data indicate that foliar absorption not only leads to a direct increment in N, but also an indirect increment possibly by stimulating root activity. The primary evidence for this comes from the observation that 42 and 37% of the increment in N content was not explained by foliar uptake in *P. halepensis* and *Q. ilex*, respectively. This suggests that foliar fertilization might have stimulated N uptake by roots, a process which has been observed in *Malus domestica* Borkh (Dong *et al.*, 2002).

Both species rapidly translocated a small proportion of shoot absorbed N to roots, with labeled N from all N sources being detected in roots after two days. *Quercus ilex* translocated higher amount of N to roots than pine but proportionally it translocated slightly lower N amount to the roots (3%) than *P. halepensis* (4%). The proportion of N translocated to roots in both species was lower than the 15% reported for *Lolium perenne* L. (Bowman and Paul, 1992) or 10% for *Olea europaea* L. (Klein and Weinbaum, 1985) two days after fertilizer application. Nevertheless the higher proportional partitioning in the species can be attributed to different relative sink strength of the tissues. Our experiment was carried out during spring when sink strength of shoots is usually higher than roots (Millard and Grelet, 2010); thus we expect higher translocation to roots when shoot but not root growth is arrested, such as occurs in the fall (Klein and Weinbaum, 1985; Dong *et al.*, 2002). The greater N translocation in all N sources and greater C translocation in glycine fertilized-seedlings in pine than in oak may be due to differences in the N sources metabolism or to a different root demand between species (Bi and Scagel, 2008; Oosterhuis and Weir, 2010). High NO_3^- translocation in both species could be explained by lower assimilation capacity in leaves than in roots (Rennenberg and Gessler,

1999). Concentration of nitrate reductase in both species is lower in leaves than in roots (8±2 and 825±70 μmol NO_2 g fresh weight^{-1} in leaves and roots respectively in *P. halepensis* and 28±3 and 146±12 μmol NO_2 g FW^{-1} in *Q. ilex* Uscola unpublished data). Conversion of urea to amino acids mainly occurs in leaves (Calanni *et al.*, 1999), which might explain the proportionally lower urea root translocation relative to reminder N sources in both species. Finally, glycine metabolism seems to differ between *Q. ilex* and *P. halepensis*. Results suggest that glycine is completely metabolized in oak shoots as neither intact glycine nor ^{13}C enrichment was detected in *Q. ilex* roots, while ^{13}C enrichment was found in *P. halepensis* roots.

Conclusions

This study demonstrates that both Mediterranean tree species are able to absorb all studied N sources through leaves. Absorption was species-dependent being higher in oak than in pine. Also, foliar absorption was different depending on N sources, following the pattern urea > NH_4^+ > glycine ≥ NO_3^-. A portion of applied glycine was taken up intact, which was higher in pine than in oak. Cuticle permeability to water did not explain interspecific differences in absorption rate, but within species cuticle permeability was positively related with absorption of all N sources except NO_3^-. A small proportion of the N absorbed by shoots was rapidly translocated to roots. Shoot fertilization increased organ N content, but only a part of this increase could be attributed to shoot N uptake with the remainder probably reflecting an increase in root uptake in foliar-fertilized plants. We show that leaf fertilization, especially with urea, can be an interesting N fertilization tool, for both nursery cultivation and nutrient improvement of outplanted seedlings.

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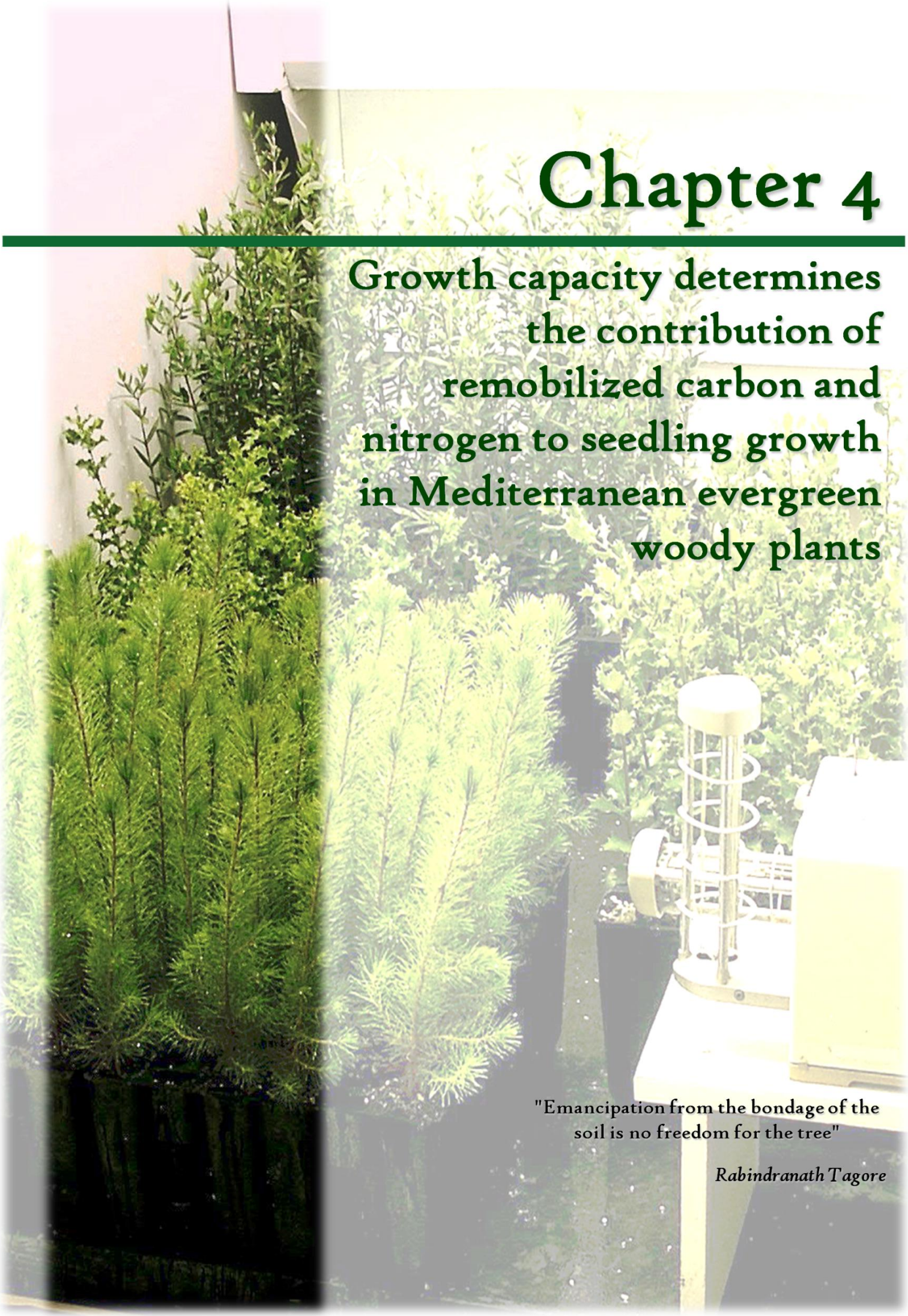

Chapter 4

Growth capacity determines the contribution of remobilized carbon and nitrogen to seedling growth in Mediterranean evergreen woody plants

"Emancipation from the bondage of the soil is no freedom for the tree"

Rabindranath Tagore

Este capítulo reproduce el texto del siguiente manuscrito:

This chapter reproduces the text of the following manuscript:

Uscola M., Villar-Salvador P., Gross P. & Maillard P. 2013. Growth capacity determines the contribution of carbon and nitrogen remobilization to seedling growth in Mediterranean evergreen woody plants (Submitted to **New Phytologist**).

Fotography: *P. halepensis*, *Quercus coccifera*, *Q. ilex* and *O. europaea* seedlings in a growing chamber in INRA –Nancy facilities.

By: M. Uscola

La capacidad de crecimiento condiciona la contribución del carbono y nitrógeno removilizado en el crecimiento de las plántulas de especies leñosas perennifolias mediterráneas.

Resumen

El carbono (C) y el nitrógeno (N) necesarios para el crecimiento de las plantas pueden provenir de fuentes externas, como el N edáfico y la fotosíntesis del momento, o de la removilización de recursos internos. La removilización de las reservas de C y N depende de la forma de vida y el hábito foliar. Mientras la mayoría de los estudios sobre removilización de C y N se han realizado en frondosas caducifolias y coníferas perennifolias, este estudio se centra en cuatro especies mediterráneas perennifolias: *Quercus ilex, Q. coccifera*, *Olea europaea* y *Pinus hapelensis*. Se han comparado (1) los patrones de distribución del C asimilado durante el invierno, (2) la contribución de las reservas removilizadas de C y N a la construcción de nuevos órganos en primavera y (3) la importancia relativa de los distintos órganos de la planta como fuentes de C y N. Se utilizó un doble marcaje con isótopos estables de ^{13}C y ^{15}N para distinguir la contribución del C y N viejo (almacenado) y nuevo (fotosíntesis del momento y N edáfico) al crecimiento de nuevas raíces y brotes. El C asimilado en condiciones invernales se distribuyó dentro de la planta en función del tamaño del órgano. Las quercíneas distribuyeron el C asimilado en invierno principalmente en raíces gruesas, mientras que *O. europaea* y *P. halepensis* lo hicieron en las hojas. Aún así, las hojas fueron el sitio prioritario de almacenamiento para todas las especies, excepto para *Q. coccifera,* ya que su contenido de C invernal fue mayor al esperado por su tamaño. Durante el crecimiento primaveral, los nuevos órganos estuvieron más enriquecidos en C y N nuevo que los órganos viejos. Poco después del transplante, las raíces nuevas se construyeron principalmente con reservas de N (> 74%) en todas las especies pero a mitad de la primavera, el suelo suministró la mayor parte del N (> 64%). El C asimilado por la fotosíntesis del momento aportó la mayor parte del C de las raíces nuevas (> 60%), excepto en *Q. coccifera*, en la que la contribución del C de la fotosíntesis de momento fue menor del 50%. Entre especies, la proporción de C y N removilizado utilizado en la construcción de los brotes se incrementó con la tasa de crecimiento relativa. Así, la construcción de nuevos brotes en las quercíneas, especies de crecimiento más lento, se construyeron principalmente con C y N nuevos, mientras que en *P. halepensis*, la especie de crecimiento más rápido, los brotes estaban construidos principalmente con C y N viejo. La

removilización de N estuvo, alta y positivamente, relacionada con la cantidad de reservas de N al principio de la primavera y con el crecimiento absoluto. Las hojas viejas fueron la principal fuente de recursos en todas las especies, ya que presentaron una gran reducción en el contenido de C y N y, además, esta reducción fue mayor a la esperada por su contenido inicial. Tanto los tallos como las raíces viejas también fueron importantes fuentes de recurso, excepto en *P. halepensis* cuyo follaje suministró casi todo el C y N utilizado. Concluimos que las plantones de las cuatro especies estudiadas tienen distinto patrón de almacenamiento y utilización de C y N, y que las diferencias de crecimiento entre las especies condicionan el papel del C y N removilizado en el crecimiento de los nuevos brotes.

Palabras clave: ^{13}C; marcaje; ^{15}N; *Olea europaea*; *Pinus halepensis*; *Quercus*; removilización; reservas.

Growth capacity determines the contribution of remobilized carbon and nitrogen to seedling growth in Mediterranean evergreen woody plants

Abstract

The carbon (C) and nitrogen (N) needed for plant growth can either come from external sources, *i.e.* soil N and current photosynthesis, or through remobilization of internally stored resources. Remobilization of stored C and N depends on plant life form and growth habit. While most studies to date on C and N remobilization have been carried out in deciduous broadleaf woody plants and evergreen conifers, our study focuses on four evergreen Mediterranean trees: *Quercus ilex, Q. coccifera*, *Olea europaea* and *Pinus hapelensis*. We compared the allocation pattern of C assimilated in winter, the contribution of remobilized N and C to the construction of new organs, and the relative importance of different plant compartments as sources of C and N in seedlings. We used dual ^{13}C and ^{15}N stable isotope labeling to disentangle the contribution of old (stored) and new C and N (currently assimilated C, and soil-derived N) to new growth. Carbon assimilated under winter conditions was partitioned throughout the plant depending upon the size of the plant organs. *Quercus* species allocated most winter C in coarse roots while *O. europaea* and *P. halepensis* allocated it in leaves. However, leaves were priority storage sites for all species except *Q. coccifera* because they contained more winter C than expected for their size. During spring growth, new organs were more enriched in new C and N than old organs. For all species, remobilization was the main N source (> 74%) for new fine root growth soon after transplanting in early spring but by mid spring, soil N supplied most of the N (> 64%) in new fine roots. Currently assimilated C supplied most of the C (> 60%) in new fine roots by mid spring, except in *Q. coccifera,* which was less than 50%. Across species, the proportion of remobilized C and N in new shoots increased with relative growth rate. *Quercus* species, the slowest growing of the four species, primarily used new C and N for shoot growth while in *P. halepensis*, the fastest growing species, shoots were mainly enriched by old C and N. Nitrogen remobilization was positively related to both stored N content at the beginning of spring and absolute growth rate. Old leaves were major sources of C and N in all species, showing the greatest reduction in C and N and, in most cases, the reduction was proportionally greater than expected from their size. Old stems and old roots also supplied high amounts of C and N in all species except in *P. halepensis*, which foliage supplied almost all stored

resources. We conclude that seedlings of the four Mediterranean evergreen woody species have different C and N storage and utilization patterns and that growth differences among species drive the utilization of remobilized C and N for new shoot growth.

Key words: ^{13}C; labeling; ^{15}N; *Olea europaea; Pinus halepensis; Quercus;* remobilization; reserves.

Introduction

Carbon (C) and nitrogen (N) for tree growth can be obtained through the remobilization of internal reserves and/or through current photosynthesis and soil N absorption (Millard 1996; Körner 2003; Cerasoli *et al.* 2004; Millard and Grelet 2010). Spring growth and reproduction in temperate and boreal trees consumes considerable amounts of C and N, which are to a great extent supplied through the remobilization of stored compounds (Chapin III *et al.* 1990; Nambiar and Fife 1991; Brüggemann *et al.* 2011). These reserves also support growth when available external resources are limited, they allow the plant to recover after disturbances and enhance nutrient use efficiency and nutrient residence time (Chapin III *et al.* 1990; Millard and Grelet 2010). Within species, the remobilization of C reserves is controlled by sink (growth) strength. In contrast, N remobilization is mainly a source-driven process that depends upon the amount of stored N (Neilsen *et al.* 2001; Millett *et al.* 2005; Millard and Grelet 2010).

Woody plants store C in all parts of the plant. However, evergreen woody plants allocate higher amounts of non-structural carbohydrates to the leaves than do deciduous plants (Chapin III *et al.* 1990; Cerasoli *et al.* 2004; Palacio *et al.* 2007b; Spann *et al.* 2008). Carbon assimilated in winter contributes to C stores in evergreen woody plants (Hansen *et al.* 1996; Cerasoli *et al.* 2004; Kuptz *et al.* 2011). Unlike C, N is usually stored in specific plant parts, which vary depending upon leaf habit. Deciduous species tend to store N in woody organs (Millard and Proe 1991; Millard 1996; Silla and Escudero 2003; Cooke and Weih 2005) while leaves, and especially the youngest leaves, are major N storage sites in evergreen species (Nambiar and Fife 1991; Wendler *et al.* 1995; Salifu and Timmer 2003; Silla and Escudero 2003). Resprouting ability also affects C and N storage patterns: sprouting species generally allocate more C and N to the roots than do non-sprouting species (Myers and Kitajima 2007; Palacio *et al.* 2007a).

Remobilization studies at a whole plant scale have mostly focused on deciduous broadleaf woody species and evergreen conifers (see review by Millard and Grelet 2010 and Brüggemann *et al.* 2011). However, little information exists for evergreen broadleaf species, even though they are major components of several forests ecosystems worldwide, such as the Mediterranean biome. Since remobilization of stored resources is often coupled to plant phenology (Hoch *et al.* 2003; Körner 2003; Milla *et al.* 2005), the contribution of remobilized C and N to new organ growth greatly differs among plant species. It also seems to depend upon plant age, life form, soil fertility, climatic conditions, competition and depredation (Chapin III *et al.* 1990; Maillard *et al.* 2001; Salifu and Timmer 2003; Millett *et al.* 2005). Nitrogen remobilization is usually triggered by important annual growth and reproductive events. Among individuals within species, the size of the N pool drives N remobilization and, consequently, plant growth (Dyckmans and Flessa 2001; Milla *et al.* 2005; Millard and Grelet 2010). With regard to C, deciduous woody plants greatly rely on C remobilization for early shoot and root growth, then this dependency decreases as the new leaves become photosynthetically active (Dickson *et al.* 1990; Sloan and Jacobs 2008; Vizoso *et al.* 2008; Keel and Schädel 2010). For conifers, however, current photosynthesis is usually the main C source that fuels early root and shoot growth and stored carbohydrates become more important when current photosynthesis is suppressed (Philipson 1988; van den Driessche 1991; Atzmon *et al.* 1994; Hansen *et al.* 1996; Brüggemann *et al.* 2011). New growth has also shown high dependence on current photosynthesis in the Mediterranean evergreen broadleaf *Quercus suber* (Cerasoli *et al.* 2004). However, Brüggemann *et al.* (2011) concluded that differences in C remobilization patterns between evergreen and deciduous species may be smaller than initially thought and may be overridden by inter-species variability.

Environmental conditions may influence C and N remobilization in trees (Millard and Grelet 2010), making it difficult to draw general ecological and functional patterns across studies. Unfortunately, comparative studies on C and N remobilization carried out under similar growth conditions and at the whole-plant scale are scarce (Stephens *et al.* 2001; Silla and Escudero 2003).

In Mediterranean forest plantations, drought is the main cause of seedling mortality (Villar-Salvador *et al.* 2012). Drought survival is linked to the capacity of the seedlings to divert large amounts of resources during the wet season to new root and shoot growth and to develop large deep roots shortly after planting and before the onset of the dry season (Padilla and Pugnaire 2007) (Villar-Salvador *et al.* 2012). This ability depends on the functional

characteristics of the plants, which can be influenced by the nursery cultivation regime (Villar-Salvador *et al*. 2004). Understanding how seedlings use currently acquired and stored resources to support growth is of practical importance for growers and could ensure transplanting success in the forest. For instance, if new growth mainly depends upon C and N remobilization, nursery practices should promote the accumulation of C and N reserves during autumn and winter to maximize growth capacity when the seedlings are transplanted in the spring.

In this study, our main aim was to compare the importance of C and N remobilization for new root and shoot growth in the seedlings of four evergreen woody species that coexist in Mediterranean forests: *Quercus ilex* subsp. *ballota* (Desf.) Samp. (holm oak), *Quercus coccifera* L. (kermes oak), *Olea europaea* L. (olive) and *Pinus hapelensis* Mill. (Aleppo pine). Specifically, we investigated whether the four species differed in: (1) the allocation pattern of winter-assimilated C, (2) the relative contribution to early growth of new roots and shoots of stored C and N *vs.* current photosynthesis and N uptake, and (3) the relative importance of different plant parts as sources of C and N. The selected species have distinct growth and ecological strategies and represent different phylogenetic groups. Three species are shade-tolerant broadleaves with resprouting capacity: *Quercus ilex* is a late-successional tree that dominates many forest communities in the central and western Mediterranean basin; *Quercus coccifera* is a slow-growing shrub common in holm oak and Aleppo pine forests and is a main component of mature plant communities on semiarid sites; *Olea europaea* is a mid-successional species and is quite common in holm and cork oak forests on mild winter sites. Finally, *P. halepensis* is a fast-growing, shade-intolerant pioneer conifer with no resprouting capacity, and is very common in disturbed and shallow soils on dry sites in the Mediterranean basin (Blanco *et al*. 1998). The two oaks have strong tap roots that store large amounts of resources and their shoot growth pattern is episodic, while *O. europaea* and *P. halepensis* lack strong tap roots and show a polycyclic shoot elongation pattern (Sánchez-Gómez *et al*. 2006; Willaume and Pagès 2006; Girard *et al*. 2010). We hypothesized that the oaks would be highly dependent on remobilized resources to support new growth in spring, and would preferentially use C and N stored in the roots. We assumed that *P. halepensis* and *O. europaea* would rely more on currently acquired resources. To test these hypotheses, we performed a dual ^{13}C and ^{15}N labeling experiment where seedling C reserves and soil N were enriched in ^{13}C and ^{15}N, respectively. This method allowed us to identify the contribution to the growth of new organs of (1) remobilized C and N, and (2) current photosynthesis and N uptake.

Material and methods

Plant material

For all four species, seeds from inland Iberian Peninsula provenances were sown in February 2003 in ®Forest Pot300 trays (50 cavities of 300 ml per tray; Nuevos Sistemas de Cultivo S.L., Girona, Spain) containing an 80:20 (v/v) mixture of peat and vermiculite. The seedlings were grown in the Centro Nacional de Recursos Genéticos Forestales "El Serranillo", MAGRAMA nursery (Guadalajara, central Spain). To avoid late spring frosts, the seedlings were cultivated in a greenhouse until late May and then moved outdoors under ambient conditions. The seedlings were cultivated according to standard cultivation protocols for Mediterranean nurseries (Villar-Salvador *et al.* 2004).

In mid January 2004, when the seedlings were 11 months old, 60 plants per species were moved to the INRA Nancy (Champenoux, France) and placed for 1 month in a controlled environment chamber (Dagard, Chambres froides modulables, 23600 Boussac, France) for winter acclimation with an 8-hour photoperiod and 50–95% relative humidity. Nine high-pressure mercury vapor discharge lamps (HQI-E 250w/N/SI OSRAM) provided a photosynthetic photon flux density (PPFD) of 250 μmol m^{-2} s^{-1} at the plant level. Day and night temperatures were 8 and 3 °C, respectively, similar to temperatures at many inland Iberian Peninsula locations during winter. The seedlings were kept well-watered and were not fertilized during this acclimation stage.

Labeling procedure

Carbon reserves were ^{13}C-labeled first by subjecting the seedlings to an enriched $^{13}CO_2$ atmosphere under winter temperature conditions as defined above. The seedlings were then moved to a greenhouse and grown under spring conditions, and the soil was enriched with ^{15}N. Fifty-six seedlings of each species, homogeneous in size, were chosen for the experiment. Thirty-six seedlings per species were submitted to four ^{13}C labeling cycles from 9th February to 11th March (Figure 1). Each labeling cycle took 4 days, and each species was labeled for 24 h. Plants were labeled in a controlled environment chamber (VTPH 5/1 000, Vötsch Industrie-technik GmbH, Reiskirchen-Lindenstruth, Germany) operating as a semi-closed system (for a full description see Vivin *et al.* 1995), and exposed to $^{13}CO_2$-enriched air (4.4 atom% ^{13}C) at a constant CO_2 concentration of 500 ppm. This was achieved by continuously mixing a small flow of $^{13}CO_2$ diluted in N_2 (Cylinder 1, 11 atom% ^{13}C, Eurisotop, CEA, France) with a flow of industrial CO_2 (Cylinder 2, 1.08 atom% ^{13}C). The chamber was

divided into two compartments: the upper compartment contained the canopy of the plants while the lower one encased the root plugs. Both parts were separated by an opaque PVC sheet, which was sealed with solvent-free putty. The roots were kept isolated from the shoots to prevent any dilution of the enriched $^{13}CO_2$ atmosphere through root respiration. Chamber temperature was 8±1 °C and relative humidity was 77%. Three high-pressure SONT sodium vapor discharge lamps (Philips Electronics N.V., Amsterdam, Netherlands) provided a PPF of 500 μmol m^{-2} s^{-1} at the plant level. Between labeling cycles, the saplings were returned to the growth chamber, under the environmental conditions described in the second paragraph of plant matherial. The remaining unselected seedlings were not labeled and were kept under the same environmental conditions in a different growth chamber.

On March 15 2004, the labeled seedlings were transplanted into 3-liter pots filled with perlite and transferred to a ventilated transparent greenhouse. The perlite had previously been washed with de-ionized water. Four blocks of 11 seedlings (eight labeled and three control seedlings) per species were randomly arranged in the greenhouse. The pots with seedlings were surrounded with empty pots to minimize edge effects. Periodically, the pots were rotated within the blocks. From March 24 to the end of the experiment on May 2004, each seedling (labeled and control) was fertilized daily with 40-80 ml of a complete nutrient solution (Le Blevennec 1986). The nutrient solution contained (mg l^{-1}): KNO_3 (101); $Ca(NO)_2$ (345); K_2HPO_4 (75); H_2KPO_4 (96); $MgSO_4$ $7H_2o$ (270); NaCl (10), Mn (0.2), Zn (0.10), Cu (0.012), B (0.10), Mo (0.025) and Fe (2.5). Furthermore, the nutrient solution for the labeled seedlings was ^{15}N (2 atom%) enriched with $K^{15}NO_3$ (^{15}N > 98 atom%, Spectra stable isotopes, Division of spectra gases Inc., Columbia, USA).

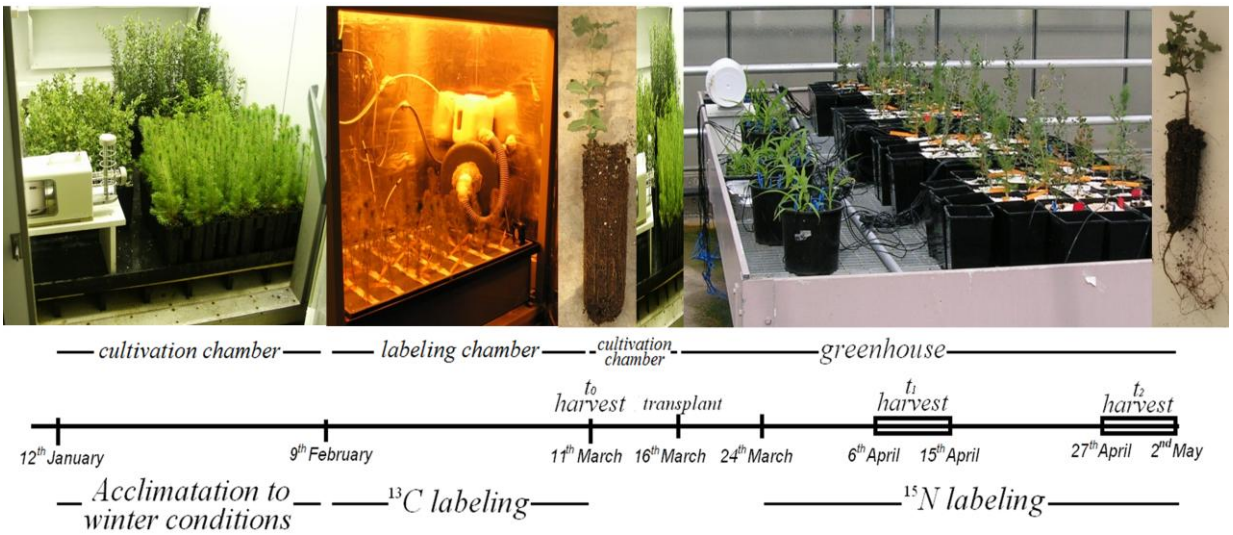

Figure 1. Flow diagram of the key events through the experiment.

Sampling

The plants were harvested at specific developmental stages, rather than at specific dates because most important functional changes in plants occur at specific ontogenetic or phenological stages (Sloan and Jacobs 2008). In addition, this approach facilitated species comparison. The following three developmental stages were defined:

t_0: Before transplanting and ^{15}N labeling, the seedlings showed no apparent growth due to the low temperatures in the culture chamber. Minimum and maximum average temperatures were 3 and 8 °C, respectively.

t_1: Seedlings had not yet started shoot elongation but had produced significant amounts of new roots. Most *P. halepensis*, *O. europaea*, *Q. ilex*, and *Q. coccifera* seedlings reached this stage 21, 23, 26 and 31 days, respectively, after transplanting. Minimum and maximum average temperatures in the greenhouse up to this developmental stage were 11 and 23 °C, respectively.

t_2: First shoot flush had ceased and most leaves were completely unfolded and mature. Most seedlings in *P. halepensis*, *O. europaea* and *Q. ilex* reached this stage 44 days after transplanting (t_0), while most *Q. coccifera* plants reached this stage 59 days after transplanting. Minimum and maximum average temperatures in the greenhouse between April 15 and May 2 were 14 and 28 °C, respectively.

At each developmental stage, ten labeled and four control seedlings per species were harvested and their roots were carefully washed in tap water to eliminate the peat, and then rinsed in de-ionized water to eliminate the nutrient solution. Roots that had protruded outside the plug into the pot growing media were sampled as new roots. The rest of the (old) roots were separated into coarse (> 2 mm diameter) and fine roots. Shoots were cut at the cotyledon insertion point and divided into old and new stems and leaves. All plant components were immediately frozen in liquid N and stored in a freezer at -80 °C, then freeze-dried, weighed to determine their mass and ground in a ball mill (Sodemi, St Ouen L´Aumône, France) for C and N analyses. The number of plant components varied at each developmental stage: four at t_0 (old leaves, old stems, old fine roots and old coarse roots); five at t_1 (new fine roots added); and six at t_2 (new shoots added).

Isotopic analyses and calculating C and N allocation

Total C and N concentrations, and $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic ratios in plant parts were measured with an elemental analyzer (NA 1500 NCS, Carlo Erba, Milan, Italy) coupled to a Delta-S isotopic ratio mass spectrometer (Finnigan–Mat, Thermoquest Corp., San Jose, CA). Analyses were carried out at the Plateforme Technique d'Ecologie Fonctionnelle (OC 081, INRA, Champenoux, France).

Isotopic abundance (A%) for C or N is defined as:

$$A\% = \frac{\text{Heavy isotope}}{\text{Light isotope} + \text{Heavy istotope}} \times 100 \quad (\%) \qquad (1)$$

where the heavy isotope is ^{13}C or ^{15}N and the light isotope is ^{12}C or ^{14}N, respectively. The tracer (^{13}C or ^{15}N) is defined as the difference between the A% of a given seedling component following administration of the tracer ($A_{labeled}\%$), and the A% measured for the same component in an unlabeled seedling ($A_{unlabeled}\%$):

$$\text{Heavy isotope}_{\ excess} = (A_{labeled}\% - A_{unlabeled}\%) \times 100 \quad (\%) \qquad (2)$$

The contribution of photo-assimilated C and absorbed soil N to seedling growth was calculated with isotopic dilution equations (Deléens *et al.* 1994). We assumed that, at spring growth, a fraction X of the C (or N) present in each seedling component came from newly assimilated C (or N) and the other fraction Y came from remobilized old C (or N) accumulated before. In our case, old C was labeled and old N was unlabeled. For either C or N, X+Y=1, and X can be calculated from:

$$A\%_{sample} = X \times (A\%_{new}) + Y \times (A\%_{old}) \qquad (3)$$

$$A\%_{sample} = X \times (A\%_{new}) + (1 - X) \times (A\%_{old}) \qquad (4)$$

where X, the proportion of new C (or N) in each seedling component, was estimated as:

$$X = \frac{A\%_{sample} - A\%_{old}}{A\%_{new} - A\%_{old}} \qquad (5)$$

$A\%_{sample}$ is either ^{13}C (or ^{15}N) abundance in the plant component at a specific developmental stage and $A\%_{old}$ is the ^{13}C abundance at t_o of the ^{13}C-

labeled plant component (or the ^{15}N abundance of the control plant component). We did not calculate these values but assumed that $A\%_{old}$ of plant parts corresponded to elements from reserves (C or N) and that the values were equal to A% of the bulk plant material (Pellicer *et al.* 2000; Cerasoli *et al.* 2004). For C, $A\%_{new}$ is ^{13}C abundance of the considered plant part in unlabeled plants at a specific developmental stage. For N, $A\%_{new}$ is the ^{15}N abundance of the labeled fertilizer.

The new C and N content of each seedling component at a specific development stage were calculated as the product of X (X_C for C and X_N for N) and the C or N content of the component considered. The old C and N content of each component was calculated as the difference between its total C or N content and its new C or N content, respectively.

Plant N uptake rate (N_u) between consecutive developmental stages was calculated as:

$$Nu = \frac{\text{Plant New N content}_{t_{n+1}} - \text{Plant N content}_{t_n}}{t_{n+1} - t_n} \times \frac{1}{\text{Fine root mass}_{t_n}} \qquad (\text{mg mg}^{-1}\,\text{d}^{-1}) \qquad (6)$$

Relative growth rate (RGR) between t_0 and t_1 or t_2 was calculated as:

$$RGR = \frac{\ln(\text{Plant mass}_{t_i}) - \ln(\text{Plant mass}_{t_0})}{t_i - t_0} \qquad (\text{mg g}^{-1}\,\text{d}^{-1}) \qquad (7)$$

where t_i is either t_1 or t_2. Partitioning of new C ($P_{C\ new,organ}$) and new N ($P_{N\ new,organ}$) into the old plant parts at each sampling date was determined as:

$$P_{new,organ} = \frac{\text{Content of new C or N into old plant parts}}{\text{Content of new C or N in the plant}} \times 100 \qquad (\%) \qquad (8)$$

Statistical analyses

We used a one-way ANOVA to assess species effect on the content of labeled C recovered after labeling at t_0. The effect of species and sampling moment on morphology, X_C, X_N, new and old C and N content were assessed by two-way ANOVA for each plant component separately. To assess if a given species favored specific plant organs for winter C storage, we compared the observed winter C content of a compartment after ^{13}C labeling with the winter C content expected in that compartment if the allocation was proportional to the size of the compartment. Expected winter C content of a component *i* after labeling was calculated as:

$$\frac{\text{Component mass}_i}{\text{Plant mass}} \times \text{Plant winter C content} \quad \text{(mg)} \quad (9)$$

The same approach was used to assess if specific plant components were stronger donators of old C and N during plant growth, *i.e.* if they proportionally reduced their old C and N content more than other compartments. We compared the observed reduction in old C for a given compartment with the reduction expected had it been proportional to the size of the compartment. We followed the same process for remobilized N. The expected reduction in old C (or N remobilization) for a given plant component was calculated as:

$$\frac{\text{Component}_i \text{ mass}}{\text{Plant mass}} \times \text{Plant C reduction (or N remobilized)} \quad \text{(mg)} \quad (10)$$

For winter C content, the reduction in old C and the amount of remobilized N, we performed an ANOVA test for each species with plant compartment and the interaction between plant compartment and observed/expected state as factors in the model. Fisher's Least Significant Difference test was used to identify differences between observed and expected means in each plant compartment. For all our analyses, data homocedasticity was checked with the Levene test. When ANOVA assumptions were not met, the data were transformed. Relationships between variables were analyzed with the Pearson correlation or Kendall Tau coefficient when the relationship was non linear. Statistical analyses were conducted with the STATISTICA 7.0 software (StatSoft, Inc, Tulsa, USA).

Results

C allocation under winter conditions

After one month of ^{13}C labeling under low temperature conditions, the amount of C incorporated (winter C) by the seedlings varied among species in the following order *P. halepensis* > *O. europaea* > *Q. ilex* > *Q. coccifera* (Figure 2). Leaves contained more winter C than stems for all species, and coarse roots contained more winter C than fine roots, except for *P. halepensis* where fine roots contained more winter C than coarse roots. Allocation of winter C inside the seedling varied significantly among species. Overall, the amount of winter C was allocated proportionally to the size of each plant compartment. In both *Quercus* species, for example, most winter C was allocated to old coarse roots. However, there were some exceptions. In *Q. ilex*, the leaves contained more

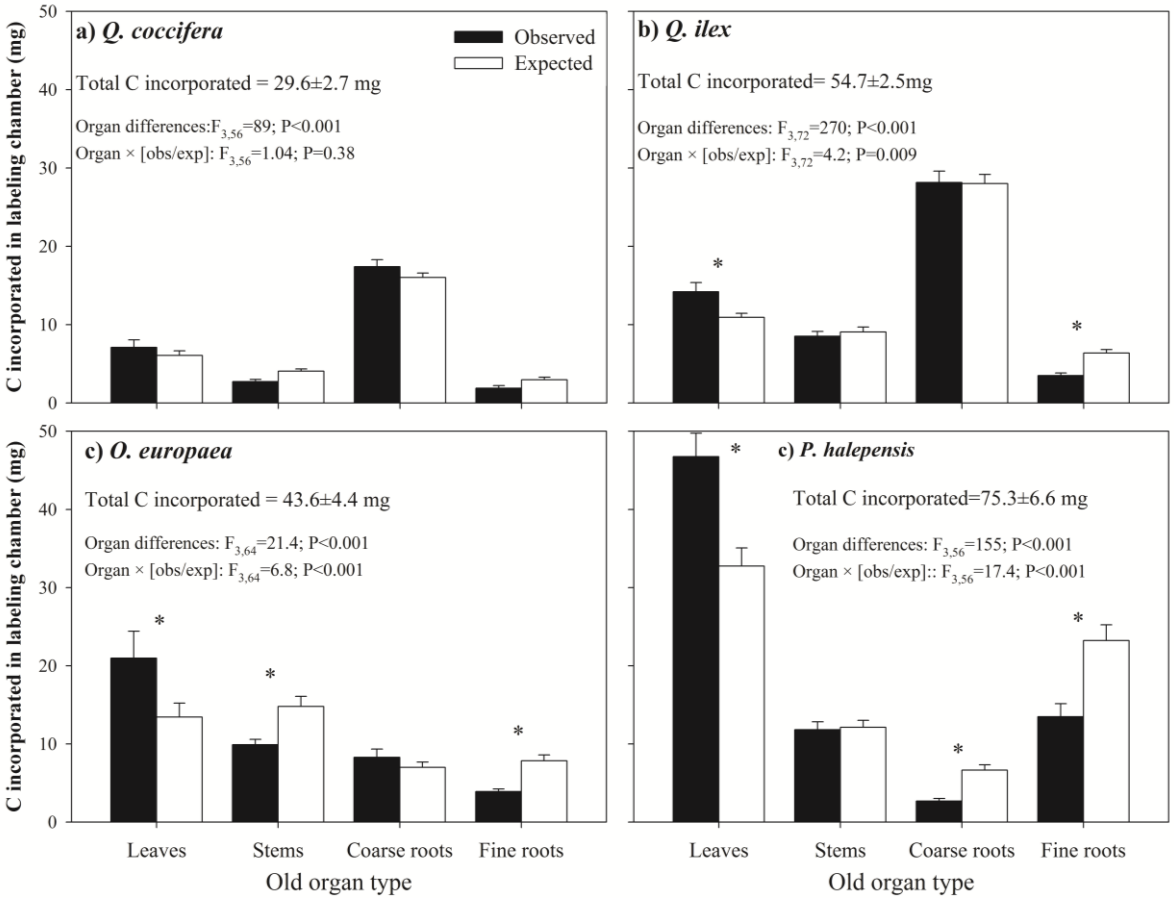

Figure 2. Observed *vs.* expected (according to organ size) labeled ^{13}C content in different organs in *Quercus coccifera* (a), *Q. ilex* (b), *Olea europaea* (c) and *Pinus halepensis* (d) seedlings after labeling in the chamber under low temperature conditions. In each subfigure, the total content of labeled ^{13}C per plant is shown. Data are means ± 1 standard error. The effects of compartment and the compartment × observed/expected factors on labeled ^{13}C content are shown in each subfigure. For each plant component, an asterisk indicates significant differences between observed and expected results.

winter C than expected from their size, while fine roots contained less winter C than expected. Unlike the oaks, most winter C in *O. europea* and *P. halepensis* was allocated in old leaves, which contained more winter C than expected from their size. On the other hand, old coarse and fine roots in *P. halepensis* and old stems and fine roots in *O. europaea* contained less winter C than expected.

Growth

At t_0, *P. halepensis* had the smallest and *Q. ilex* the largest seedlings, while *O. europaea* and *Q. coccifera* seedlings were intermediate in size (Figure 3). The four species also had differing sizes of old plant compartments (in all cases P<0.01).

Old root mass in both oaks was larger than in *O. europaea* and *Pinus halepensis*, which proportionally had more mass in old leaves and stems than the oaks. Absolute growth differed among species ($F_{3,854}$=678; P<0.001). At t_2, *O. europaea* had the highest and *Q. coccifera* the lowest absolute growth (59±9.9 and 24±3.8 mg for *O. europaea* and *Q. coccifera*, respectively), while the other two species had intermediate absolute growth (43±7.3 and 39±3.2 mg, for *Q. ilex* and *P. halepensis*, respectively). RGR between t_0 and t_1 was lower than RGR between t_1 and t_2 (Table 1). Until t_1, the oaks had lower RGR than *O. europaea* and *P. halepensis*. At t_2, *P. halepensis* had the highest RGR and oaks, especially *Q. coccifera*, the lowest, while *O. europaea* had an intermediate RGR.

In all species, old plant component mass increased throughout development except for old leaves, which showed only a slight increase in *O. europaea* and *Q. coccifera* and no change in *Q. ilex* and *P. halepensis* (Development stage × Species interaction, $F_{6,99}$=2.78; P<0.01). Overall, old component mass increased very little between t_0 and t_1 and in most cases, this mainly occurred in the stems and roots (Figure 3). Between t_0 and t_2, old stems and roots showed the highest increase in mass among old organs (stems: $F_{2,101}$=23.54; P<0.001; coarse roots: $F_{2,102}$=13.70; P<0.001; fine roots: $F_{2,108}$=5.16; P<0.01). The results were similar across species except for *Q. ilex* where old stem mass increment was smaller than in the other species (Development stage × Species interaction, $F_{6,101}$=3.34; P<0.01).

New roots showed significant differences among species ($F_{3,64}$=7.6; P<0.001) (Figure 3). *Q. coccifera* had the highest new fine root mass and *Q. ilex* the lowest at both development stages (t_1=0.057±0.007 and t_2=0.26±0.02 mg for *Q. coccifera*; t_1=0.033±0.009 and t_2=0.15±0.01 mg for *Q. ilex*), whilst *O. europaea* and *P. halepensis* had intermediate mass values with very little difference between them (t_1=0.059±0.008 and t_2=0.18±0.02 mg for *O. europaea*; t_1=0.048±0.005 and t_2=0.21±0.01 mg for *P. halepensis*). New shoots appeared between t_1 and t_2 and were bigger in *Q. ilex* and *O. europaea* than in *Q. coccifera* and *P. halepensis*, whose new shoot growth was similar. For all species, new shoots were two to five times greater than new fine roots.

New and old C composition of plants

The fraction of new C (X_C; C derived from current photosynthesis) increased in most old components throughout development, except in *Q. ilex* where no change or even a slight decrease in X_C was noted (Table 1). X_C in *Quercus* species showed the following order among the old components: leaves > stems > coarse roots > fine roots. In *O. europaea* the order was: leaves > fine roots ≥

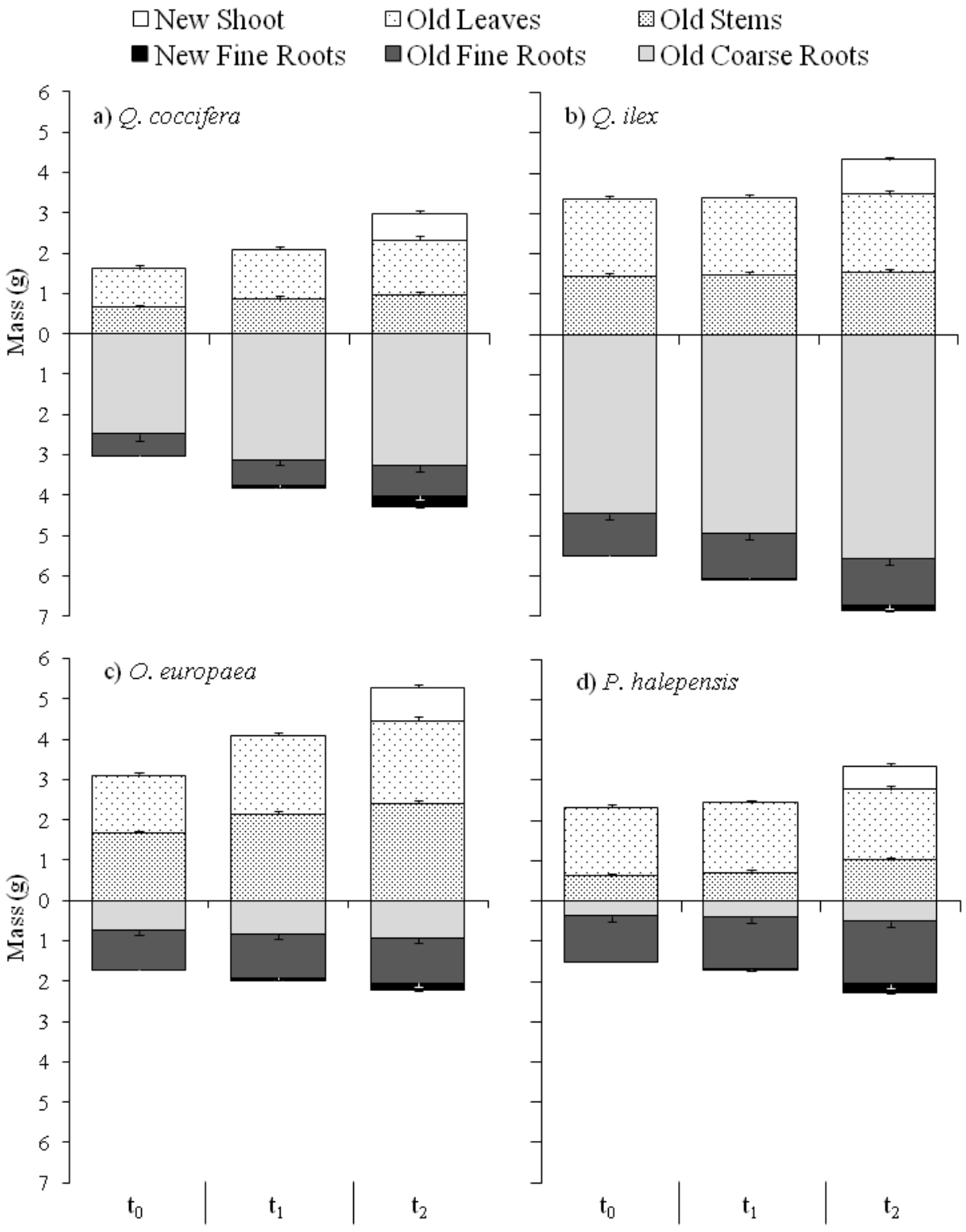

Figure 3. Mass of different plant components in *Quercus coccifera*, *Q. ilex*, *Olea europaea* and *Pinus halepensis* seedlings at different growth stages. (t_0): after ^{13}C labeling and before transplanting to spring growth conditions; (t_1): shoot elongation had not yet started but seedlings had grown significant amounts of new roots; (t_2): first shoot flush of growth had finished and most leaves had completely unfolded and matured. Data are means ±1 standard error.

Table 1. Fraction of new C (X_C; C derived from current photosynthesis) and N (X_N; N taken up from the soil) in different plant components for *Quercus ilex*, *Q. coccifera*, *Olea europaea* and *Pinus halepensis* seedlings sampled before shoot elongation (t_1) and at the end of the first shoot flush (t_2). Values are means ± 1 standard error. F-values and significance results of two-way ANOVA are shown in the last three columns. Where *: 0.01<P≤0.05; **: 0.001<P≤0.01; ***: P≤0.001, ns: P>0.05.

	Q. coccifera		*Q. ilex*		*O. europaea*		*P. halepensis*		Species (1)	Develop. stage (2)	1 × 2
	t_1	t_2	t_1	t_2	t_1	t_2	t_1	t_2			
RGR (mg g^{-1} d^{-1})	0.5±0.9	2.8±0.7	2.8±0.8	4.4±0.4	8.6±0.8	7.4±0.5	7.5±1.2	10.6±0.6	32.0***	5.6**	3.2*
X_C (%)											
New shoots		82±4.4		57±7.2		47±3.2		39±4.1	14.9 ***		
Old leaves	52±2.6	66±3.7	51±4.5	49±3.3	27±3.7	40±5.5	24±2.7	37±4.1	25.0 ***	12.4 *	2.27 ns
Old stem	9±2.7	34±6.9	45±3.2	34±4.7	6±1.4	7±1.6	23±4.0	33±3.9	31.1 ***	7.7 **	8.4 ***
Old coarse roots	10±0.9	20±4.9	21±1.7	23±2.4	12±3.2	24±5.4	29±3.9	41±3.8	12.8 ***	13.6 ***	1.09 ns
Old fine roots	6±1.1	14±4.8	8±2.0	7±0.8	14±3.6	22±6.7	26±2.9	23±2.6	8.1***	3.91*	0.67 ns
New fine roots	38±4.3	46±6.5	64±3.3	62±5.4	16±3.5	61±2.1	45±4.2	88±0.6	23.6 ***	59 ***	17.8 ***
Partitioning of new C into old organs (%)	99±0.2	76±4.3	99±0.3	85±1.7	99±0.1	64±3.7	98±0.4	85±1.3	2.6*	794***	15.6***
X_N (%)											
New shoots		70±4.0		66±4.0		52±2.9		22±2.5	27.6 ***		
Old leaves	2±0.3	7±1.1	2±0.2	3±0.5	5±0.5	5±0.7	2±0.2	16±2.0	12.6 ***	103***	22.7 ***
Old stem	11±1.1	31±2.4	10±1.2	16±1.2	6±0.8	20±1.2	4±0.4	25±1.6	17.7 ***	272 ***	14.8 ***
Old coarse roots	9±1.0	24±2.6	9±1.0	9±1.1	6±0.7	17±1.4	8±0.5	27±0.9	14.4 ***	135 ***	14.6 ***
Old fine roots	10±0.8	28±2.8	9±0.8	14±1.6	11±1.2	31±2.9	5±0.3	20±0.8	20.9 ***	226 ***	10.6 ***
New fine roots	43±7.3	88±2.8	25±2.6	69±4.4	24±2.4	72±4.9	19±1.7	89±2.2	10.5 ***	292 ***	5.5 ***
Partitioning of new N into old organs (%)	90±1.9	31±2.6	94±1.2	32±3.8	93±1.2	41±3.2	87±1.1	47±1.5	4.3 **	1098***	10.0***

coarse roots > stems, and in *P. halepensis*: coarse roots ≥ leaves > fine roots ≥ stems. Overall, old leaves, new shoots and new fine roots had the highest X_C values while old fine roots had the lowest values.

At the end of first flush, the contribution of new C to the construction of new organs was generally greater than the contribution of old C, and differed among species (Table 1). At t_1, new C represented most (> 50%) of the C content in *Q. ilex* new roots while in *O. europaea* new C represented less than 20%. New C represented less than half of the C content in new fine roots in *P. halepensis* and *Q. coccifera* (see also Figure S2 in Supplementary Material). At t_2, X_C in new roots was greater than 60% in all species except for *Q. coccifera*, where it represented less than 50%. In all species, in new shoots new C represented around 50% or more of total C except for *P. halepensis*, where it was < 40%. Globally, *Quercus coccifera* had the highest X_C and *P. halepensis* the lowest, while *Q. ilex* and *O. europaea* had similar X_C values, intermediate between *Q. coccifera* and *P. halepensis*.

Old C decreased throughout development and differed among species (Figure S1 a and c and Table S1 in Supplementary Material). In all species, old leaves experienced the greatest reduction in old C, which was proportionally greater than the expected reduction according to their initial C content (Figure 4). Old coarse roots in the *Quercus* species and *O. europaea* and old stems in *Q. ilex* also showed a strong decrease in old C. However, the reduction in old C in coarse roots for both *Quercus* species was lower than expected, while in old coarse roots for *O. europaea* and stems for *Q. ilex*, it was higher than expected. For all species, the reduction in old C in old fine roots was lower than expected, except for *O. europaea*.

Part of the old C was respired and part was remobilized to supply new growth. The species differed in the amount of remobilized C at t_2 ($F_{3,26}$=70.8; P<0.001), which was lower in the two *Quercus* species than in *O. europaea* and *P. halepensis*, which were quite similar (Figure 4). The amount of C respired also differed among species at t_2 ($F_{3,26}$=204; P<0.001). Respired C was highest in *Q. ilex*, whose respired C values were 2.3, 3.7, and 4.1 times higher than *Q. coccifera*, *O. europaea* and *P. halepensis*, respectively.

For all species, most new C was partitioned into old components (Table 1), more so at t_1 (> 90%) than at t_2 (> 60%). The highest partitioned new C values were observed for *P. halepensis* and *Q. ilex*, the lowest for *O. europaea*, and intermediate values were found for *Q. coccifera*.

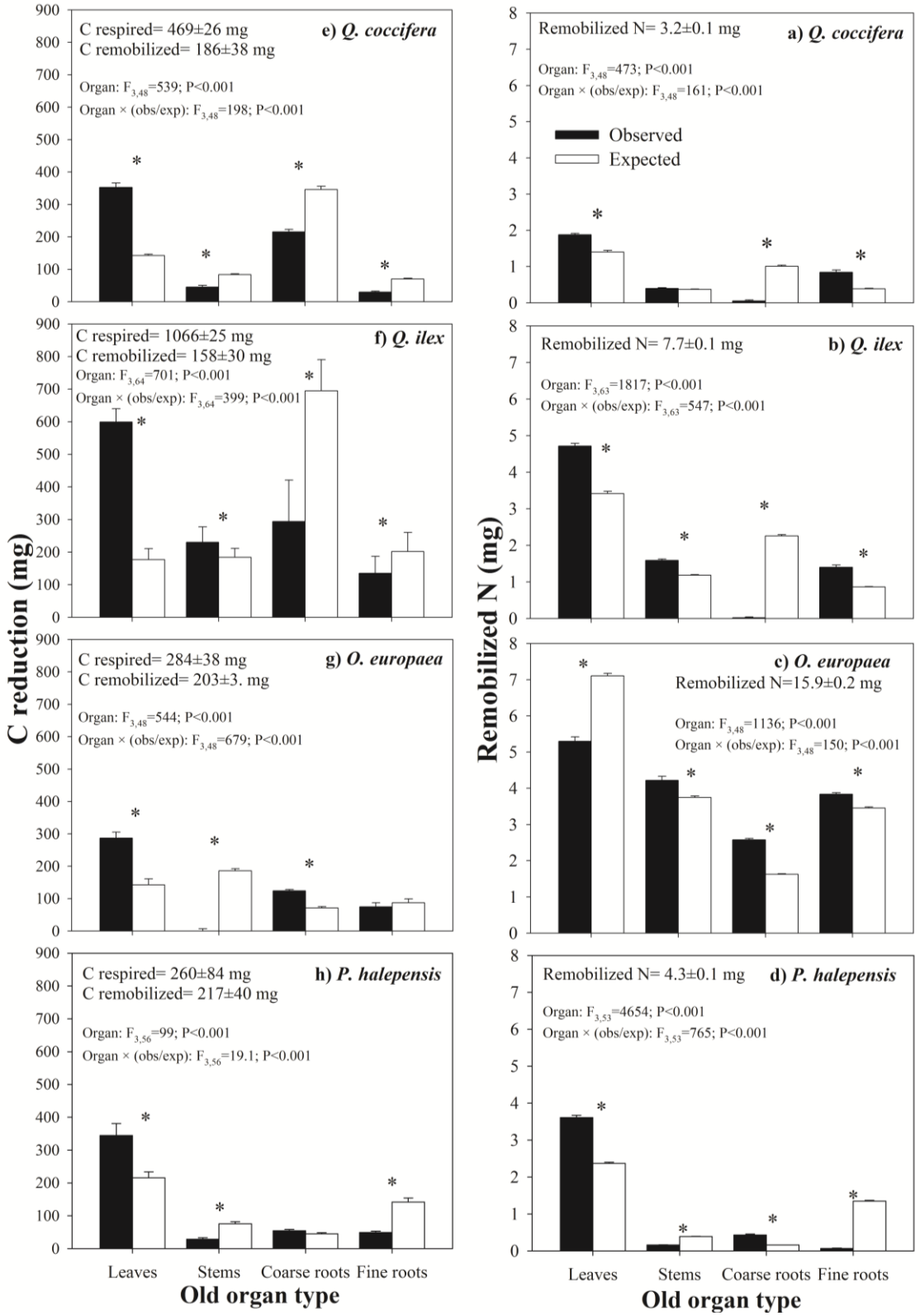

Figure 4. Observed *vs.* expected (according to organ size) amount of reduction in old C and remobilized N for different old components in *Quercus coccifera* (a), *Q. ilex* (b), *Olea europaea* (c) and *Pinus halepensis* (d) seedlings at the end of the study. In each subfigure, the total amount of the amount of respired and remobilized C or remobilized N per plant is given. The effects of compartment and the compartment × observed/expected factors on labeled ^{13}C content are shown in each subfigure. For each plant compartment, an asterisk indicates significant differences between observed and expected results.

New and old N composition of plants

The fraction of new N (X_N; N taken up from the soil) increased in most components throughout development with a magnitude that varied among species (Species × Development stage interaction, Table 1). In all species and at all developmental stages, X_N was lower in old components than in new ones.

In new roots, X_N was lower at t_1 (< 43%) than at t_2 (> 69%). At t_1, *Q. coccifera* and *P. halepensis* had the highest and lowest values, respectively, whilst *O. europaea* and *Q. ilex* had intermediate X_N values. At t_2, *Q. coccifera* and *P. halepensis* had the highest X_N values (>85%), while *O. europaea*, and especially *Q. ilex*, had the lowest X_N values (Table 1).

In *Q. coccifera* new shoots, most N was new. In contrast, new N represented only a small fraction of the N in new shoots in *P. halepensis*, while the percentage was 52-66% in *O. europaea* and *Q. ilex*. At t_1, new N was mainly partitioned into old organs (> 87%). At t_2, partitioning of new N into old organs decreased and showed significant differences among species with *P. halepensis* and *Quercus* species having the highest and lowest values, respectively and *O. europaea* showing intermediate values of N partitioning into old organs (Table 1).

Total old N content at t_0 differed among species as follows: *O. europaea* > *Q. ilex* > *P. halepensis* ≥ *Q. coccifera* (Figure S2 a and c in Supplementary Material). The amount of old N remobilized to new roots and shoots also differed among species (Table S1). *Olea europaea* had the highest N remobilization, while *Q. ilex* and *P. halepensis* had intermediate values, and *Q. coccifera* the lowest (Figure 4). Overall, the amount of N remobilized from old components was not closely related to their size. Old leaves supplied the most remobilized N in all species and amounts were greater than expected from component size in all species except *O. europaea*, where they were lower than expected. Old fine roots in *Q. coccifera*, *Q. ilex* and *O. europaea* and old stems in *Q. ilex* and *O. europaea* were also significant contributors of remobilized N. N remobilized from old fine roots was greater than expected in all species except *P. halepensis*, where it was lower than expected. Similarly, N remobilization from old stems in *Q. ilex* and *O. europaea* occurred at a higher rate than expected. In the two *Quercus* species, surprisingly little N was remobilized from coarse roots despite their high N content. However, in *O. europaea* and *P. halepensis*, coarse roots remobilized more N than expected.

N_u was greater from t_1 to t_2 than from t_0 to t_1, when N_u values were 0.05, 0.12, 0.14 and 0.25 mg N g^{-1} d^{-1} for *Q. ilex*, *O. europaea*, *P. halepensis* and *Q. coccifera*, respectively. From t_1 to t_2, N_u values were 0.79 mg N g^{-1} d^{-1} in both *Q. coccifera* and *O. europaea* and 0.64 and 0.34 mg N g^{-1} d^{-1} in *Q. ilex* and *P. halepensis*, respectively.

Relation between variables

Across species, both X_N and X_C in new shoots (Figure 5a and b) and dissimilarity in X_N and X_C between new shoots and fine roots ($X_{N,new\ shoots}$ / $X_{N,new\ fine\ roots}$ and $X_{C,new\ shoots}$ / $X_{C,new\ fine\ roots}$ ratios) were negatively related to RGR (Figure 5c, and Kendall Tau=-1; P=0.042 for X_C dissimilarity). Partitioning of N taken up by roots into old organs was positively related to RGR (Figure 5d) and negatively related to the dissimilarity in X_N between new shoots and fine roots (r=-0.99, P=0.002). Partitioning of new C into old organs was not related either to RGR and X_C or the $X_{C,new\ shoots}/X_{C,new\ fine\ roots}$ ratio. Remobilized C and N, measured as the amount of labeled C and unlabeled N in new organs at t_2, was not related either to RGR or X_C and X_N, respectively. Absolute growth was positively correlated with the old N content of the seedlings at both the intra- and inter-specific levels (Kendall Tau=1, P=0.042; Figure 5e). Across species, absolute growth rate was positively related to remobilized N (Figure 5f).

Discussion

All four species studied assimilated labeled C under low winter temperature conditions. Evergreen trees from other temperate biomes are also known to assimilate C during winter as long as low temperatures do not limit photosynthesis (Hansen *et al.* 1996; Miyazawa and Kikuzawa 2005). Although some of the winter C is rapidly consumed by respiration, the remainder is stored and increases carbohydrate reserves, which can fuel spring growth (Hansen *et al.* 1996; Körner 2003). In our study, the seedlings showed no growth under winter conditions, so the labeled C assimilated before the beginning of spring (in the labeling chamber) must have been stored rather than transformed into structural carbohydrates. Carbon assimilated in winter accumulated throughout the plant and the amount of winter C stored in the different organs was directly related to their size. Shoots were the main winter C sink in *P. halepensis* and *O. europaea*, while in the *Quercus* seedlings, the coarse roots –the largest compartment in these seedlings– played this role. Dickson *et al.* (1990) found that deciduous oaks also allocate almost all fixed C to roots when shoot

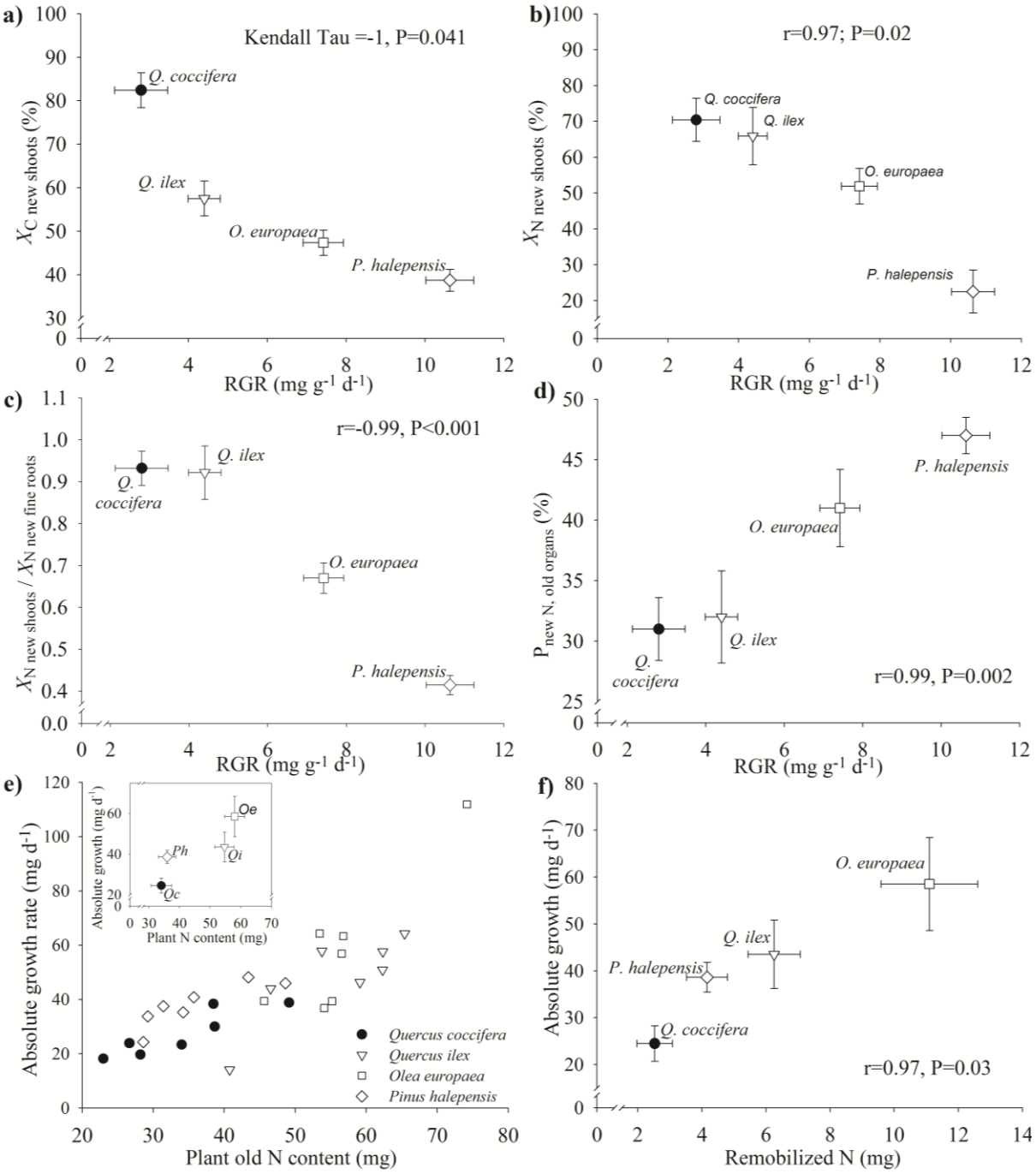

Figure 5. Relations between the relative growth rate (RGR) across species and the fraction of new C (X_C; current photosynthesis) in new shoots (a), fraction of new N (X_N; soil N) in new shoots (b), dissimilarity in X_N between new shoots and fine roots (X_N new shoots/X_N new fine roots) (c) and partitioning of N taken up from the soil into old organs ($P_{new\ N,old\ organs}$) (d). Subfigure (e) shows the relation between plant absolute growth and plant old N content at the within-species scale (inter-specific scale in inserted figure). Finally, subfigure (f) represents the relation between absolute growth and remobilized N across species. Each point in subfigures (a), (b), (c), (e) and (f) is the species mean value and bars are 1 SE. In Figure (e), each point represents one plant and correlations for each species are: *Q. coccifera* (r=0.89; P=0.02), *Q. ilex* (r=0.81; P=0.03), *O. europaea* (r=0.80; P=0.06) and *P. halepensis* (r=0.95; P=0.001).

elongation is arrested during the growing season. Similarly, shade-tolerant broadleaf species and trees with episodic shoot growth, such as oaks, also show greater root C storage than do continuously growing, shade-intolerant species (Canham *et al.* 1999). The two *Quercus* species in our study differed, despite their relatedness, in their allocation of winter C. *Q. ilex* allocated more winter C to foliage than expected from their size, at the expense of allocation to fine roots, whereas in *Q. coccifera*, the allocation of winter C to the different organs was closely dependent on their size. Our results for *Q. ilex* are consistent with Cerasoli *et al.* (2004) findings for the Mediterranean oak, *Q. suber*, which concentrated 30% of winter C in leaves although foliage only represented 21% of plant mass, while the roots, representing 62% of the plant mass, contained less than 50% of the winter C. Results in *P. halepensis* and *O. europaea* seedlings indicate that the leaves are priority sites for winter C storage in these species: not only is foliage the largest compartment, the leaves also contained more winter C than expected from their size (Figure 2). (Hansen *et al.* 1996) found contrary results for 3-year-old *Pinus sylvestris* L. saplings; the roots were the main sink for winter C while foliage played a secondary role. These contrasting results suggest that winter C allocation may differ among *Pinus* species.

Growth of new fine roots and shoots is crucial for seedling establishment and survival in dry ecosystems (Padilla and Pugnaire 2007; Villar-Salvador *et al.* 2012). The contribution of remobilized C and N to this new organ growth varies widely both among and within species (Millard and Grelet 2010; Brüggemann *et al.* 2011). Tree age, soil fertility, climatic conditions, competition and depredation all affect the contribution of remobilized C and N to plant growth (Salifu and Timmer 2003; Silla and Escudero 2003; Millett *et al.* 2005; Vizoso *et al.* 2008; Millard and Grelet 2010). In our study, there were also notable differences among species in how remobilized C and N contributed to new organ growth depending on species growth capacity, type of organ and developmental stage. Soon after transplanting in early spring, remobilized N was the main N source for new fine root growth in all species; then by mid spring, most N in new fine roots was being taken up from the soil, as indicated by low and high X_N values at t_1 and t_2, respectively (Table 2). Interestingly, we found that contribution of remobilized C and N to new shoot growth increased with RGR. In accordance with (Cornelissen *et al.* 1996; Antúnez *et al.* 2001), RGR for both *Quercus* species in our study was lower than for *O. europaea* and *P. halepensis*; furthermore, *Q. coccifera* had lower RGR than *Q. ilex* Remobilization was responsible for most C and N in new shoots for the fastest-growing species, *P. halepensis*, while new fine roots contained mostly recently assimilated C and N. This resulted in very different proportions of new C and

N in new shoots and in new fine roots for *P. halepensis*. By contrast, both new shoots and new fine roots were highly enriched in new C and N in oaks, the slowest-growing species, especially in the slowest-growing species, *Q. coccifera*; indicating that current photosynthesis and soil N were the main sources for new growth. Consequently, the proportion of remobilized C and N in shoots and in roots was similar in oaks. Finally, *O. europaea* had intermediate growth and intermediate values for the proportion of new C and N in new shoots and new fine roots. Similar to our findings for the two *Quercus* species, the contribution of remobilized C to new leaf and stem growth was shown to be low in spring for *Q. suber*, *Pinus nigra* Arn. ssp. *laricio*, and *Pinus uncinata* Ramond (Cerasoli *et al.* 2004; Maillard *et al.* 2004; Felten *et al.* 2007). In the evergreen *Vaccinium vitis-ideae* L., N remobilization supplied 47-69% of N in new shoots shortly after first flush (Grelet *et al.* 2001), which is similar to our values for *Q. ilex*, *O. europaea* and *P. halepensis*. In *P. nigra*, (Maillard *et al.* 2004) found that remobilized N made up approximately 70 and 20% of new shoot and root N, respectively.

The fact that shoot enrichment in remobilized C increased with RGR probably indicates that current photosynthesis could not meet C demand for new organs in the faster-growing species, which led to greater support by stored C. As for N, why did fast-growing species use less N taken up from the soil for new shoot growth? Silla and Escudero (2003) concluded that Mediterranean *Quercus* species remobilize a higher proportion of stored N to support growth when N taken up by roots does not meet plant N demand. In our study, N taken up by roots represented 84-94% of new organ N demand in early spring, and 98-137% in mid spring across species (data not shown). This indicates that N uptake *per se* was not a limiting factor for new organ growth. We suggest that competition for new N between old and new organs could be the underlying mechanism that explains the relationship between RGR and the proportion of new N in new shoots: competition between old and new organs for recently acquired N increased with RGR, which likely reduced the amount of soil N remaining for new organ growth and, consequently, increased the demand for remobilized N. Three results support this hypothesis. Firstly, the old organs in all species coped with most of the N taken up by the roots in early spring as indicated by very high partitioning of new N (> 87%) in old organs (see Table 2). Consequently, the amount of new N available for allocation to new organs was very low, satisfying only 21-36% of new fine root N demand. Consistent with this we found that new root growth in all species was mainly sustained by remobilized N in early spring. Secondly, partitioning of soil N to old organs increased with RGR (Figure 5d), evidencing that sink strength of old organs for new N was higher in fast growing species. Thus, less new N was

available for new growth due to high allocation of new N in old organs. High N demand in old organs in spring could be explained by radial growth and/or replenishment of N reserves (Kagawa *et al.* 2006). Thirdly, dissimilarity between new shoots and roots in the proportion of new N was negatively related to proportion of new N in old organs. New shoots, which had a greater N demand than did new roots, are likely to be more affected by the competition between old and new organs for new N than new roots (see Figure S3 in Supplementary Material). This explains the greater dissimilarity in the proportion of new N between new shoots and new roots with increasing RGR (Figure 5c). Differences in N_u among species might also have affected competition between old and new plant parts by increasing N source-sink differences (Nambiar and Fife 1991; Hansen *et al.* 1996; Dyckmans and Flessa 2001). N_u was lowest in *P. halepensis*, the fastest growing species, while oaks with the lowest RGR, had higher N_u; this higher N uptake rate might have alleviated the competition between old and new organs for new N in the oaks.

Our findings have interesting ecological and functional implications. To our knowledge, this is the first time that a connection has been made between RGR and new organ composition in remobilized C and N. Leaf specific area, leaf area ratio and net assimilation rate have been identified as major determinants of high RGR in plants (Cornelissen *et al.* 1996; Antúnez *et al.* 2001). Fast-growing species, such as *P. halepensis*, that rapidly colonize disturbed areas (Barbéro *et al.* 1998), may rely on remobilized resources to support fast new shoot growth in spring, which can increase performance in competitive environments (Bausenwein *et al.* 2001) and help seedling establishment in spring. By contrast, slow-growing species, such as evergreen oaks, are likely to rely more on current photosynthesis and soil N to support new growth, with remobilization playing a secondary role. Our results do not support our initial hypothesis that oaks would rely more on stored resources while *P. halepensis* and *O. europaea* would mainly use currently assimilated resources to support new organ growth in spring. Instead, our findings indicate that, rather than supporting seasonal growth, oaks probably use stored resources for respiration, disturbance recovery, persistence under prolonged stress conditions and cold and drought acclimation (Canham *et al.* 1999; del Tredici 2001). Consistent with this hypothesis, for both *Quercus* species the old coarse roots, which are the largest store sites of C and N experienced much lower reduction in C and N than expected according to their size during spring growth (Figure 4).

The amount of old C recovered from new organs at the end of the study was lower than total old C reduction in old plant parts. This indicates that part of the C released from old organs (Figure S1) was respired or given off as

exudates (Loescher *et al.* 1990). Though remobilized C did account for most of the reduction in old C on *P. halepensis* and *O. europaea*, C remobilization was lower in the *Quercus* species where most old C was respired during spring growth. Unfortunately, we were not able to assess the contribution of each organ to total remobilized C as we did not distinguish between respired and remobilized C. In all species, old C stores were to a great extent replenished by currently fixed C, as observed for other species by Chapin III *et al.* (1990), Loescher *et al.* (1990), Cerasoli *et al.* (2004). Finally, contrary to our results in the *Quercus* species, Cerasoli *et al.* (2004) found that stored C did not fuel respiration during spring growth in the evergreen oak *Q. suber*.

Leaves are usually the main sites for C and N remobilization in evergreen woody plants, with woody stems and roots playing the major role in deciduous species (Nambiar and Fife 1991; Millard and Proe 1993; Millard *et al.* 2001; Grelet *et al.* 2001; Palacio *et al.* 2007b). In our study, old foliage was indeed a major source of C and N: leaves showed the greatest reduction in C and N and, in most cases, the reduction was proportionally greater than expected from their size. *Pinus halepensis* and *Q. ilex* are good examples of the importance of leaves as N sources: old foliage supplied *ca.* 84 and 61% of remobilized N, respectively. A similar pattern was reported for *Pinus sylvestris* and *Q. suber* (Millard *et al.* 2001; Cerasoli *et al.* 2004). On the other hand, Silla and Escudero (2003) observed that woody compartments supplied equal or greater amounts of N than did old leaves in *Q. ilex*. This does not concur with our results for *Q. ilex*; however, for *O. europaea* and *Q. ilex*, we did observe that old roots and stems were also main contributors of remobilized N.

Absolute plant growth is a N-source driven process controlled by the amount of stored N available (Millard and Grelet 2010). Our results confirm this process at a within-species scale. Indeed, within each of the four species, absolute growth was positively correlated with total plant old N content even though the new N supplied during the experiment was the same for all individuals. Interestingly, we also found evidence that the amount of remobilized N to new organs, as determined by the size of the N pool in the plant at the beginning of the growing season, may also control absolute growth differences across species (Figure 5).

Conclusions

We conclude that winter C accumulates throughout the plant and is allocated depending on the size of the organ, with leaves being priority winter C storage sites in all species except *Q. coccifera*. A substantial part of the stored winter C was respired and the remainder was remobilized to support new

growth in spring. Remobilization was the main N source for new fine root growth in all species soon after transplanting in early spring, but soil N supplied most N in new fine roots in mid spring. We also showed that the importance of remobilized C and N for the construction of new organs in spring depends on species relative growth rate. Specifically, the contribution of remobilized C and N to new shoot growth and the dissimilarity between new shoots and roots in the proportion of new C and N were greater in fast- than in slow-growing species. Absolute growth was positively related to the amount of stored N both within and across species. Species with higher absolute growth remobilized greater amounts of old N. Old leaves were important sources of remobilized C and N, but old stems and old fine roots also played an important role in *O. europaea* and *Q. ilex*. As seedling establishment depends on the formation of large deep roots shortly after transplanting and before the onset of the dry season (Padilla and Pugnaire 2007), our results can help provide seedling nurseries with guidelines to promote new root and shoot growth after field planting. For example, nursery practices should promote C and N storage in *P. halepensis* prior to planting, because this species relies heavily on stored reserves for new growth. Similarly, nurseries should promote for traits conferring high C assimilation and soil N acquisition in oaks, especially for *Q. coccifera*, which primarily use external C and N sources to support new growth. Finally, both C and N storage and high external acquisition capacity must be promoted in *O. europaea*, which use both sources equally to supply new organ growth.

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Supplementary material

Table S1. Two way ANOVA results for species and developmental stage effects on the amount of new (current photosynthesis and soil N) and old (reserves) C and N in different components of *Quercus ilex*, *Q. coccifera*, *Olea europaea* and *Pinus halepensis*. Data are F values. Where *: 0.01 < P ≤ 0.05; **: 0.001 < P ≤ 0.01; ***: P ≤ 0.001, ns: P > 0.05; † P = 0.07.

	New shoots	Old leaves	Old stems	Old coarse roots	Old fine roots	New fine roots
C old (mg)						
Species (1)	5.1 **	38 ***	112 ***	193 ***	453 ***	2.1 n.s.
Develop. stage (2)		59 ***	6.7 **	12 ***	3828***	105 ***
1 × 2		2.5 *	4.24 ***	3,7 **	429 ***	2.3 ns
C new (mg)						
Species (1)	6.9 **	5.4 **	11 ***	41 ***	8.5 ***	4.1 **
Develop. stage (2)		0.06 ns	6.8 *	6.7 *	0.6 ns	93 ***
1 × 2		3.3 *	3.1 *	1.9 ns	0.23 ns	3.0 *
N old (mg)						
Species (1)	14 ***	26 ***	167 ***	102 ***	110 ***	3.2 *
Develop. stage (2)		2.6 †	3.1 *	0.24 ns	6.8 **	0.09 ns
1 × 2		0.41 ns	2.5 *	0.73 ns	2.4 *	1.5 ns
N new (mg)						
Species (1)	13 ***	16 ***	23 ***	54 ***	41 ***	5.8 **
Develop. stage (2)		58 ***	139 ***	35 ***	106 ***	305 ***
1 × 2		17 ***	6.2 **	6.7 ***	11 ***	5.4 **

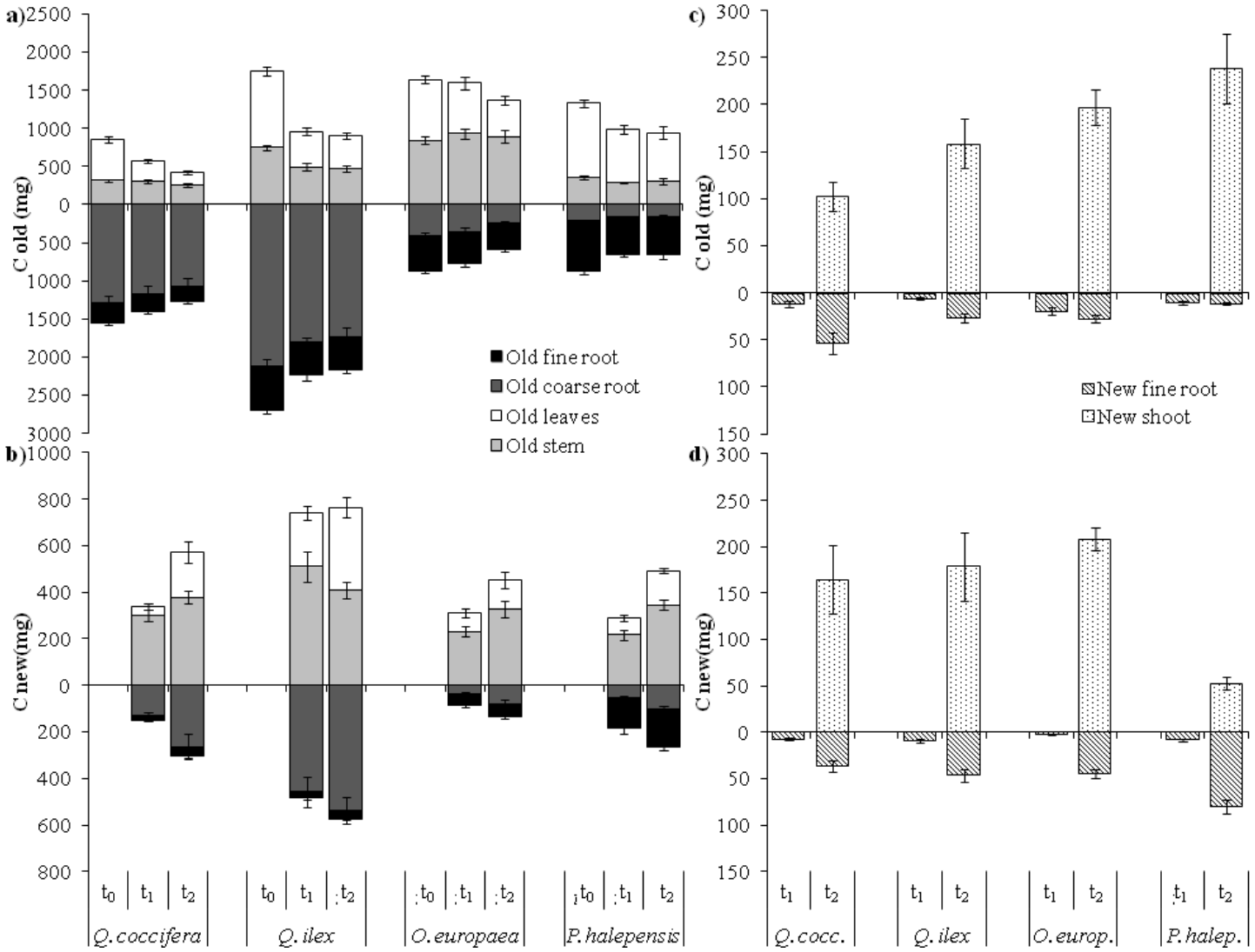


Figure S1. Old (reserves) and new C (current photosynthesis) content in new and old compartments of *Quercus ilex*, *Q. coccifera*, *Olea europaea* and *Pinus halepensis* seedlings measured at different growth stages. (t_o): after ^{13}C labeling and before transplanting to spring growth conditions; (t_1): shoot elongation had not yet started but seedlings had grown significant amounts of new roots; (t_2) first shoot flush of growth had finished and most leaves had completely unfolded and matured. Data are means ±1 standard error.

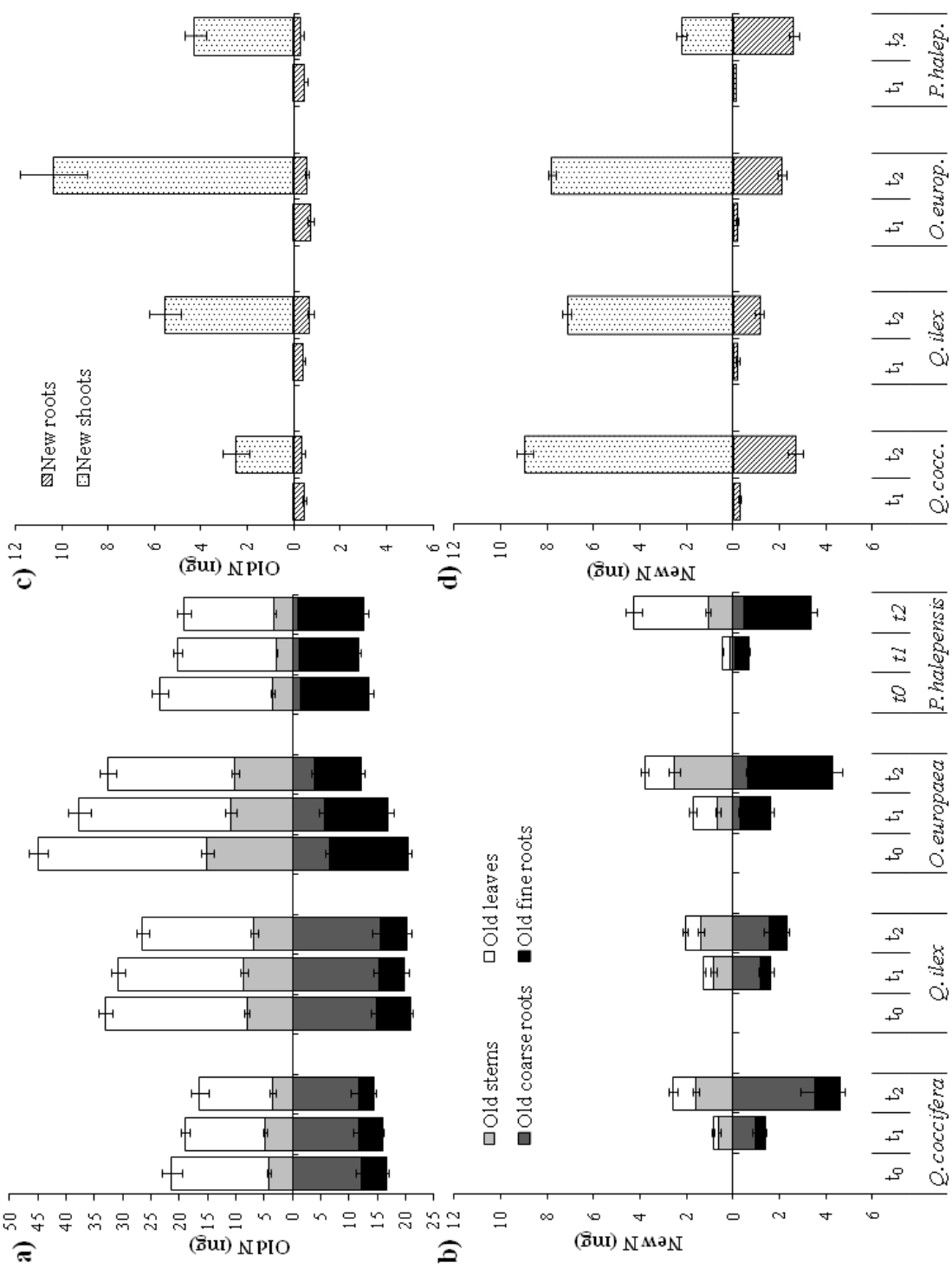
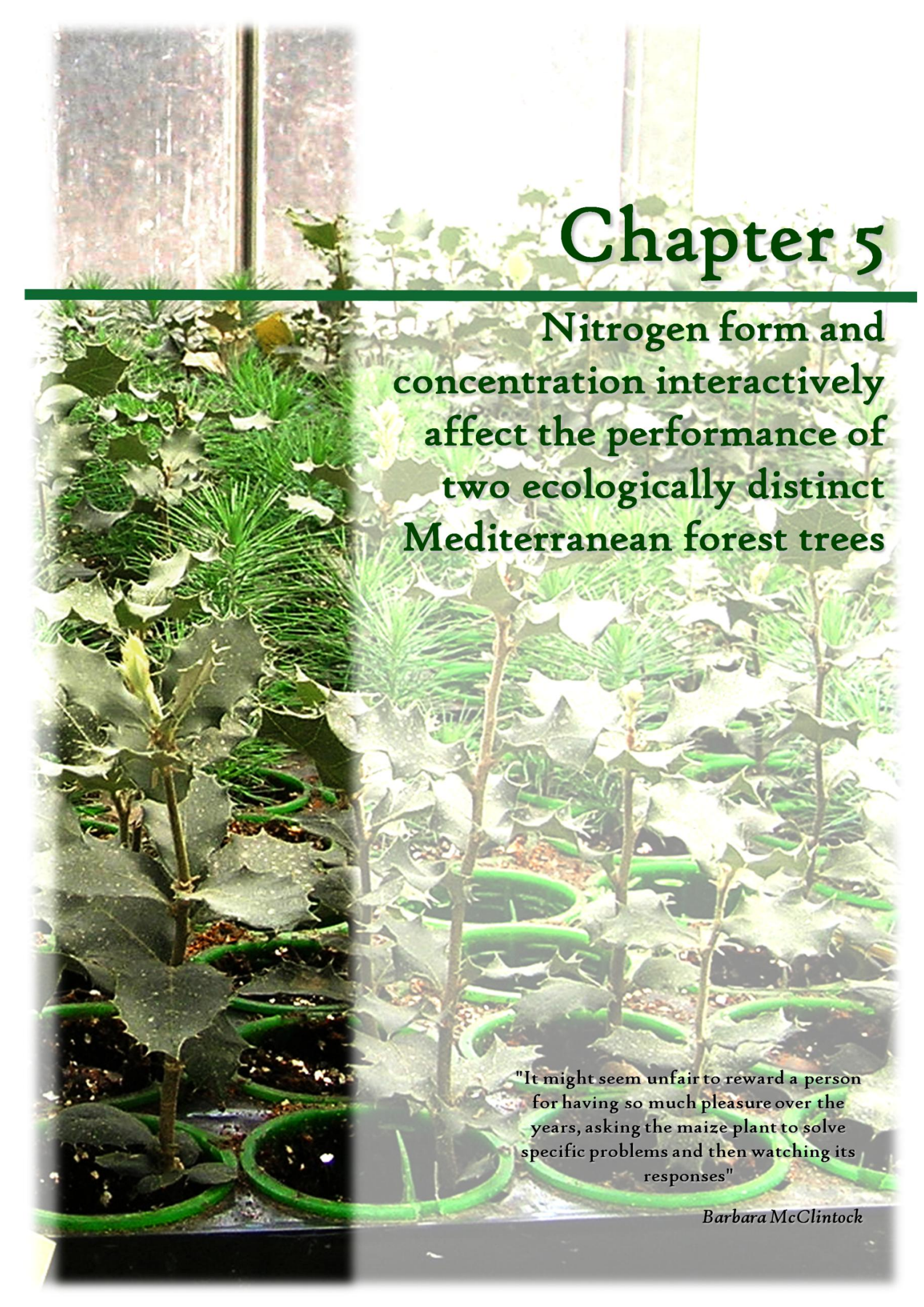


Figure S2. Old (reserves) and new (soil N) N content in new and old compartments of *Quercus ilex*, *Q. coccifera*, *Olea europaea* and *Pinus halepensis* seedlings measured at different growth stages. (t_0): after ^{13}C labeling and before transplanting to spring growth conditions; (t_1): shoot elongation had not yet started but seedlings had grown significant amounts of new roots; (t_2): first shoot flush of growth had finished and most leaves had completely unfolded and matured. Data are means ±1 standard error.



Chapter 5

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

"It might seem unfair to reward a person for having so much pleasure over the years, asking the maize plant to solve specific problems and then watching its responses"

Barbara McClintock

Este capítulo reproduce el texto del siguiente manuscrito:

This chapter reproduces the text of the following manuscript:

Uscola M., Oliet Palá J.A. , Villar-Salvador P., Díaz-Pinés E. & Jacobs D. 2013. Nitrogen form and concentration interactively affect the performance of two ecologically distinct Mediterranean forest trees (2nd revision in **European Journal of Forest Research**).

Fotography: Glasshouse culture of *Pinus halepensis* and *Quercus ilex* seedlings in Real Jardín Botánico de Alcala facilities.

By: M. Uscola

La fuente de nitrógeno y su concentración interactúan afectando el desarrollo en dos especies forestales mediterráneas de ecología contrastada

Resumen

La mayoría de los estudios que analizan los efectos de las fuentes de N inorgánicas sobre el crecimiento y el estado nutricional de especies forestales se han llevado a cabo en especies boreales o templadas utilizando una única especie. Sin embargo, son escasos los estudios comparativos de especies de otros biomas. En este trabajo se evaluó la respuesta de dos especies arbóreas mediterráneas de ecología contrastada, *Quercus ilex* L. y *Pinus halepensis* Mill., al cultivo con diferentes fuentes de N inorgánicas y a la fertilización foliar con aminoácidos. Los plantones fueron fertilizados con diferentes proporciones de NH_4^+ y NO_3^- a dos concentraciones 1 y 10 mM N. Además, se analizó el efecto de la fertilización foliar con aminoácidos (aplicada semanalmente a una concentración de 10 mM N) en plantones que habían recibido una fertilización edáfica de 1 mM N con una mezcla equimolar de NO_3^- y NH_4^+. A baja concentración de N las distintas formas químicas de N apenas mostraron efectos sobre las plantas. Sin embargo, a alta concentración, los plantones presentaron distinto desarrollo según la fuente de N. El NH_4^+ a alta concentración causó toxicidad, ya que redujo el crecimiento, especialmente en la encina. El NO_3^- a 10 mM N incrementó fuertemente el crecimiento en el pino pero tuvo efectos mínimos en la encina. El NH_4^+ favoreció la absorción de P e inhibió la de K en ambas especies y a ambas concentraciones, mientras que el NO_3^- produjo el efecto inverso. Mezclas equimolares de ambas fuentes de N produjeron desarrollos intermedios a los producidos en NO_3^- y NH_4^+ aplicados individualmente. La fertilización foliar con aminoácidos incrementó la tasa fotosintética en ambas especies, además favoreció ligeramente el crecimiento del pino. Concluimos que las distintas respuestas de las especies ante las fuentes inorgánicas de N están relacionadas con su ecología. El pino, un árbol pionero que crece principalmente en suelos ricos en NO_3^-, se desarrolla mejor con ésta fuente de N y tiene una mayor plasticidad ante la disponibilidad de N en el suelo. La encina, un árbol tardío en la sucesión que principalmente se desarrolla en suelos ricos en NH_4^+, presenta una baja respuesta a las fuentes de N o a su concentración.

Palabras clave: Amonio; fertilización foliar; crecimiento; nitrato; Pinus halepensis; Quercus ilex.

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

Abstract

Most studies examining inorganic N form effects on growth and nutrition of forest trees have been conducted on single species from boreal or temperate environments, while comparative studies with species from other biomes are scarce. We evaluated the response of two Mediterranean trees of contrasting ecology, *Quercus ilex* L. and *Pinus halepensis* Mill., to cultivation with distinct inorganic N forms and amino acid foliar fertilizer. Seedlings were fertilized with different NH_4^+ / NO_3^- proportion at either 1 or 10 mM N. Amino acid foliar fertilization (10 mM N applied weekly) was tested in plants that had also received an equimolar 1 mM N mixture of NO_3^- + NH_4^+ in soil. N-forms had negligible effects at low N in both species. Ten mM NH_4^+ caused toxicity as it reduced growth, but the effect was greater in oak than in pine. Ten mM NO_3^- strongly increased growth in pine with minor effects in oak. NH_4^+ enhanced P uptake and inhibited K uptake in both species and at both concentrations, while the opposite occurred with NO_3^-. Equimolar NO_3^- + NH_4^+ plants had intermediate performance between NO_3^- and NH_4^+ plants. Amino acid foliar fertilization increased photosynthesis in both species and slightly improved growth in pine. We concluded that responses to inorganic N forms were related to species ecology. The pine, a pioneer tree that grows on NO_3^--rich soils, improved performance with NO_3^- and had strong plasticity to changes in N supply. The oak, a late successional tree that mainly thrives on NH_4^+-rich soils had low responsiveness to N form or concentration.

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

Introduction

Nitrogen (N) is a macronutrient limiting primary productivity in natural and managed terrestrial ecosystems (LeBauer and Treseder 2008). N is present in soils as inorganic forms, such as ammonium (NH_4^+) and nitrate (NO_3^-), and organic forms, such as amino acids (Christou *et al.* 2006). Plants have the potential to acquire all types of N forms (Paungfoo-Lonhienne *et al.* 2008) but they frequently show preferential uptake for the most abundant N form in their habitat or successional stage (Kronzucker *et al.* 2003; Weigelt *et al.* 2005). Thus, early successional species tend to exhibit high NO_3^- but low NH_4^+ and amino acid uptake rates, whereas late successional species preferentially use NH_4^+ and amino acids and also show low responsiveness to changes in N availability (Reich *et al.* 1995; Kronzucker *et al.* 2003; Weigelt *et al.* 2005).

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

As a cation, NH_4^+ is adsorbed in the substrate, thereby reducing N leachate losses after fertilization compared with NO_3^-. This has important environmental benefits (Raven *et al.* 1992) and increases crop N use efficiency. However, NH_4^+ fertilization tends to acidify the substrate because it induces proton efflux in the rhizosphere, reduces concentrations of other cations in

plants and causes toxicity when applied at high rates, hindering plant growth, especially of roots (Öhlund and Näsholm 2001; Britto and Kronzucker 2002). In contrast to NH_4^+, NO_3^- can be commonly supplied at higher concentration without harmful effects on plants but easily leaches from substrate causing potential environmental impacts (Landis *et al.* 1989; Cruz *et al.* 1993).

Forest plantations in Mediterranean regions frequently exhibit poor performance (Oliet *et al.* 2009b). Seedling outplanting performance strongly depends on seedling morphology and physiology (Grossnickle 2012; Villar-Salvador *et al.* 2012). Nitrogen fertilization greatly influences seedling N reserves, morphology, photosynthesis rate and stress tolerance and, therefore, it frequently enhances outplanting performance (Islam *et al.* 2009; Oliet *et al.* 2009b). However, inadequate N fertilization can result in nutritional and morphological imbalances and delay stress acclimation in plants (Islam *et al.* 2009; Andivia *et al.* 2011). Conventional fertilization programs in forest container nurseries provide between 100 and 150 ppm (7-10 mM) N to cultivated plants (Landis *et al.* 1989). However, when the objective is to N load seedlings, such as fall fertilization or in exponential fertilization regimes, fertilizer N concentration can be greater than 150-200 ppm (10-14 mM) N (Hawkins *et al.* 2005; Metcalfe *et al.* 2011). Fertilization in forest nurseries is usually accomplished by applying fertilizers with equal proportions of NH_4^+ and NO_3^- (Landis *et al.* 1989). Because N form affects the functional attributes of seedlings, the optimal proportion of NH_4^+ and NO_3^- must be determined for each species to maximize fertilization efficiency, promote high quality plants and minimize environmental contamination. Mineral nutrition of Mediterranean species is comparatively less well understood (Oliet *et al.* 2004) and as far as we know, knowledge of N-forms response in Mediterranean tree species has been limited to only two species, *Ceratonia siliqua* L. and *Pinus pinaster* Ait. (Cruz *et al.* 1993, 1997; Warren and Adams 2002).

Along with root uptake, plants can also absorb nutrients through leaves, and so foliar fertilization is sometimes used to supply nutrients in crop species, especially when nutrients cannot be easily taken up from soil and/or during high nutrient-demand periods (Fageria *et al.* 2009). Amino acids can potentially improve effectiveness of foliar fertilization relative to inorganic N compounds because they allow the plant to rapidly translocate N to other organs with a low energetic cost in N assimilation and protein synthesis than inorganic N (Hsu 1986). Also amino acids use would prevents leaf injury (Boynton 1954). Amino acids are used as foliar fertilizers in horticultural crops, but few reports have confirmed the beneficial effect of this N form (Plummer and Kethley 1964; Maini 2006). Additionally, foliar fertilization with amino acids is rarely used to

cultivate forest tree species, so little is known about the efficiency of this application method in forest tree species (Landis *et al.* 1989).

We investigated whether two ecologically distinct Mediterranean trees have different functional responses to inorganic N forms (NH_4^+ and NO_3^-) and amino acid foliar fertilization. We addressed this question using seedlings of *Quercus ilex* L. *ballota* (Desf) Samp. (holm oak) and *Pinus halepensis* Mill. (Aleppo pine), which are widely distributed in the Mediterranean basin and commonly used in afforestation. *P. halepensis* is a fast growing shade-intolerant pioneer tree (Zavala *et al.* 2000) that mostly thrives on limestone soils where NO_3^- is usually the dominant N form (Gimeno-García *et al.* 2001). *Q. ilex* is a slow growing, shade-tolerant late successional tree (Zavala *et al.* 2000). NH_4^+ is the main N form in holm oak forest soils (Bonilla and Rodá 1992; Gallardo *et al.* 2005). According to previous literature and given the predominant N-form in Aleppo pine and holm oak forests soils, we hypothesized that these species would show variable responses to N forms and concentration. Thus, we predicted that the pine will perform better when fertilized with NO_3^-, while the oak will have improved performance when fertilized with NH_4^+ or equimolar mixtures of NH_4^+ and NO_3^-. To fulfill our objective, we evaluated the morpho-physiological performance of seedlings grown for six months with three proportions of NO_3^- and NH_4^+ at two N levels. In addition, we evaluated an amino acid foliar fertilization treatment.

Material and methods

Plant material, growing conditions and experimental design

The experiment was conducted in the greenhouse facilities of the Botanical Garden Juan Carlos I at the Universidad de Alcalá (Madrid, Spain). Seeds of *Q. ilex* ssp. *ballota* (Desf) Samp. (holm oak) and *P. halepensis* Mill. (Aleppo pine) from inland Spain provenances were planted germinants into Super-Leach™ containers (Bardi S.A.L., Navarra, Spain). This container has 35 cavities of 305 ml. Growing media was unfertilized peat moss, pH 5.5±0.1 (Kekkilä Bo, Kekkilä Oyi, Finland). A 2×2 full factorial experimental design was used for each species: factor N concentration (low, 1 mM N; and high, 10 mM N) and factor NH_4^+/NO_3^- proportion (only NH_4^+ or NO_3^- and equimolar amounts of both N forms, hereafter [N+A]). In addition, one isolated amino acid foliar fertilization treatment per species was included. Each fertilization treatment had 25 seedlings per species. Containers were re-arranged twice per week throughout the experiment duration.

Nutrient solutions were prepared according to Ingestad (1979) and Landis *et al.* (1989). The low N solution (1 mM) composition varied according to N form (Table 1). P and K concentration in the low N solution were 0.33 and 1 mM, respectively, and they were ten times more concentrated in the high N solution. A 10 mM N concentration was chosen because it represents a typical concentration used in nursery culture, especially for N loading (Hawkins *et al.* 2005; Oliet *et al.* 2009b). These concentrations were previously found to support conifer growth at deficient and sufficient levels, respectively (Hawkins *et al.* 1999; Metcalfe *et al.* 2011). The low N treatment had similar inorganic N concentration to those *Q. ilex* forest soils (Bonilla and Rodá 1992). Both nutrient solutions were supplemented with 0.1 g l^{-1} of a commercial micronutrient mixture (Hortrilon, Compo, Barcelona, Spain). Electrical conductivity (EC) and pH of nutrient solutions were measured periodically. pH ranged between 6.8 and 7.1, with no significant differences among treatments. EC of 1 mM N solutions ranged from 317±25 to 416±34 μS cm^{-1} for the NO_3^- and NH_4^+ solutions, respectively, while EC of 10 mM N solutions ranged between 2048±235 and 2940±236 μS cm^{-1}, respectively. [N+A] solutions showed intermediate EC values between NO_3^- and NH_4^+ solutions (391±29 and 2850±122 μS cm^{-1} for 1 and 10 mM N solutions, respectively).

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

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

The experiment was conducted from 18 February to 23 July 2009. Light transmission of the greenhouse was 60 %, and daily mean temperature varied from 16 to 29 ºC. Fertilization started on 11 March and to ensure accurate fertilizer delivery and avoid leachate, between 20 and 40 mL of fertilizer solution was applied individually to each seedling twice a week with a syringe

(Figure 1). In addition, seedlings were watered monthly with at least 60 mL of nutrient solution to flush out accumulation of salts.

Foliar fertilization began two weeks after soil fertirrigation. We sprayed 2.6 mL to a bulk of 25 seedlings of a commercial amino acid fertilizer (free amino acids: 10% w/w; N: 8.8% w/w Welgro Amino, Massó S.A, Barcelona, Spain) that included 18 amino acids. Glycine (13.0 %), proline (7.2 %) and alanine (5.0 %) were the most abundant amino acids. Nitrogen concentration of the solution was 10 mM. The solution was homogenously sprayed to a set of 25 plants per species once a week. These plants were also fertilized with 1 mM of [N+A] applied to the growing media as per the non-foliar treated 1 mM [N+A] seedlings.

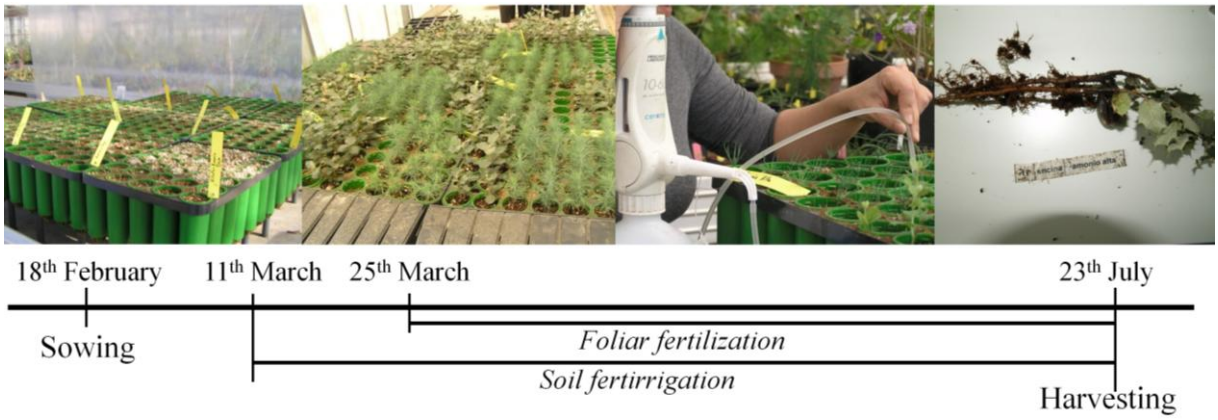


Figure 1. Flow diagram of the key events through the experiment.

Morphology and nutrient concentration

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

The same 15 seedlings per species used for morphological determinations were used for N, P and K concentration analysis. Five composite samples were analyzed, with each composite sample obtained by randomly pooling three seedlings. Samples were ground in a ball mill (PM100, Retsch Haan, Germany).

Determination of N concentration was done by the standard Kjeldahl method and that of P concentration followed the methodology described in Allison *et al.* (1962) using an auto-analyzer (CFA SAN++, Skalar, Breda, The Netherlands). K concentration was determined from perchloric acid extracts in an auto-analyzer (SAN ++) by gas segmented continuous flow coupled to a flame photometer (Sherwood Model 410, Cambridge, UK).

Gas exchange and photosynthetic pigments

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

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

Chemical composition of growing media extracts

The growing media of each seedling used for morphology and nutrients analysis was collected before washing the roots. Five composite samples were formed by pooling the substrate of three randomly chosen plants per specie and treatment. Additionally, nine containers were filled with growing media; three of these were left without any seedling and the other six contained one seedling per container (three oak and pine seedlings, respectively). The nine containers were not fertilized and were irrigated only with deionized water. These

containers were used as reference samples for substrate analysis. Reference samples were analyzed individually. Growing media from foliar fertilized seedlings were not analyzed. Growing media extracts were obtained by shaking aliquots of growing media composite samples for 2 h with deionized water under saturated conditions, and filtered with a 40 μm pore size filter (DP400 130, Albet, Germany) in a Buchner´s funnel (BR-1611, JP Selecta, Spain) and stored at -30 °C until analysis. Electrical conductivity and pH of the saturated extract were measured with an EC-Meter Basic 30+ (CRISON, Spain) and micropH 2000 meters (CRISON, Spain), respectively. An extraction-distillation approach was used to determine NO_3^- and NH_4^+. From the saturated extracts, 25 mL were analyzed for total inorganic N and NH_4^+ concentration by the steam-distillation method of Mulvaney (1996) in a steam distiller (Kjeltec^{TM} 2100, FOSS, Denmark) and for pH in an automatic titrator with potentiometric (702 titrino, Metrohm, Switzerland). NO_3^- concentration was calculated as the difference between total inorganic N and NH_4^+ concentration, as nitrite concentration was considered negligible, due to absence of anoxic conditions in the growing media. All analyses were made in duplicate and averaged.

Statistical analysis

The effect of N form and concentration on plant performance was assessed by two-way ANOVA for a fully randomized design with foliar fertilization as an isolated treatment. For the chemical composition of growing media extracts the ANOVA included a reference sample without any seedling, (but foliar fertilization treatment was not included). Species were analyzed separately because they showed strong heterocedasticity when analyzing both species together, according to Levene´s test. When significant effects of factors were detected, Fisher's Least Significant Difference test (LSD) was used to identify differences between treatment means. Significance level was established at α = 0.05. Several variables were heterocedastic and were analyzed by the Kruskal-Wallis non parametric test and the average planned comparisons non parametric test was used for treatment multiple comparisons. Statistical analyses were conducted with STATISTICA 6 software (StatSoft, Inc, Tulsa, USA).

Results

Morphology

N form and concentration affected all morphological variables in both species (Table 2). However, the effect of the interaction of N concentration and N form was significant for all parameters in both species (Table 2 and Figure 2).

Plant fractions and seedling total mass did not differ among N forms at 1 mM N in Q. *ilex*, both total seedling and plant fractions had larger mass at 1 mM N than at 10 mM N, except for 10 mM NO_3^-, which overall did not differ from the 1 mM N treatments (Figure 1a). At 10 mM N, the mass of plant fractions and total plant mass in NH_4^+ or [N+A] fertilized seedlings was lower than the mass of NO_3^- fertilized seedlings. Ten mM NH_4^+ Q. *ilex* seedlings showed generalized leaf necrosis and 32% of the plants were dead by the end of cultivation. Mortality or leaf necrosis was not found in the remaining treatments. Irrespective of N form, 10 mM N Q. *ilex* seedlings had lower total root and coarse root mass than those grown at 1 mM N. Fine root mass in 10 mM NH_4^+ and [N+A] seedlings was lower than in the remaining treatments. Root fibrosity (fine root mass / total root mass ratio) was higher for NO_3^- than for NH_4^+ or [N+A] fertilized seedlings (21, 16, and 18%, respectively). Foliar fertilization did not affect morphology relative to 1 mM N in Q. *ilex* seedlings

N forms did not affect either total or plant fractions mass at 1 mM N in *P. halepensis*. In contrast, at 10 mM N an increase occurred in total and fraction mass in NO_3^- seedlings in relation to the other N forms (significant N source × fertilizer N interaction, Table 2, Figure 1b). Ten mM [N+A] and, especially, 10 mM NO_3^- seedlings had larger mass than those of their equivalents grown with 1 mM N, while total and plant fraction mass in seedlings grown at 10 mM NH_4^+ was similar to that in 1 mM NH_4^+ plants. Only pines fertilized with 10 mM NH_4^+ had foliar necrosis and 8% of these seedlings were dead by the end of the study. Notably, 10 mM NH_4^+ seedlings had lower coarse and fine root mass relative to the remaining treatments. Fine root mass contribution to total root mass was higher for NO_3^- than for NH_4^+ or [N+A] fertilized Aleppo pine seedlings (67, 60 and 58%, respectively). Foliar-fertilized *P. halepensis* seedlings had higher total mass relative to the 1 mM [N+A] and NO_3^- treatments and higher fine root mass than all N forms at 1 mM. Similarly, foliar-fertilized plants had greater total mass than NH_4^+, similar than [N+A] and smaller total mass than NO_3^- high-fertilized seedlings (Figure 2b).

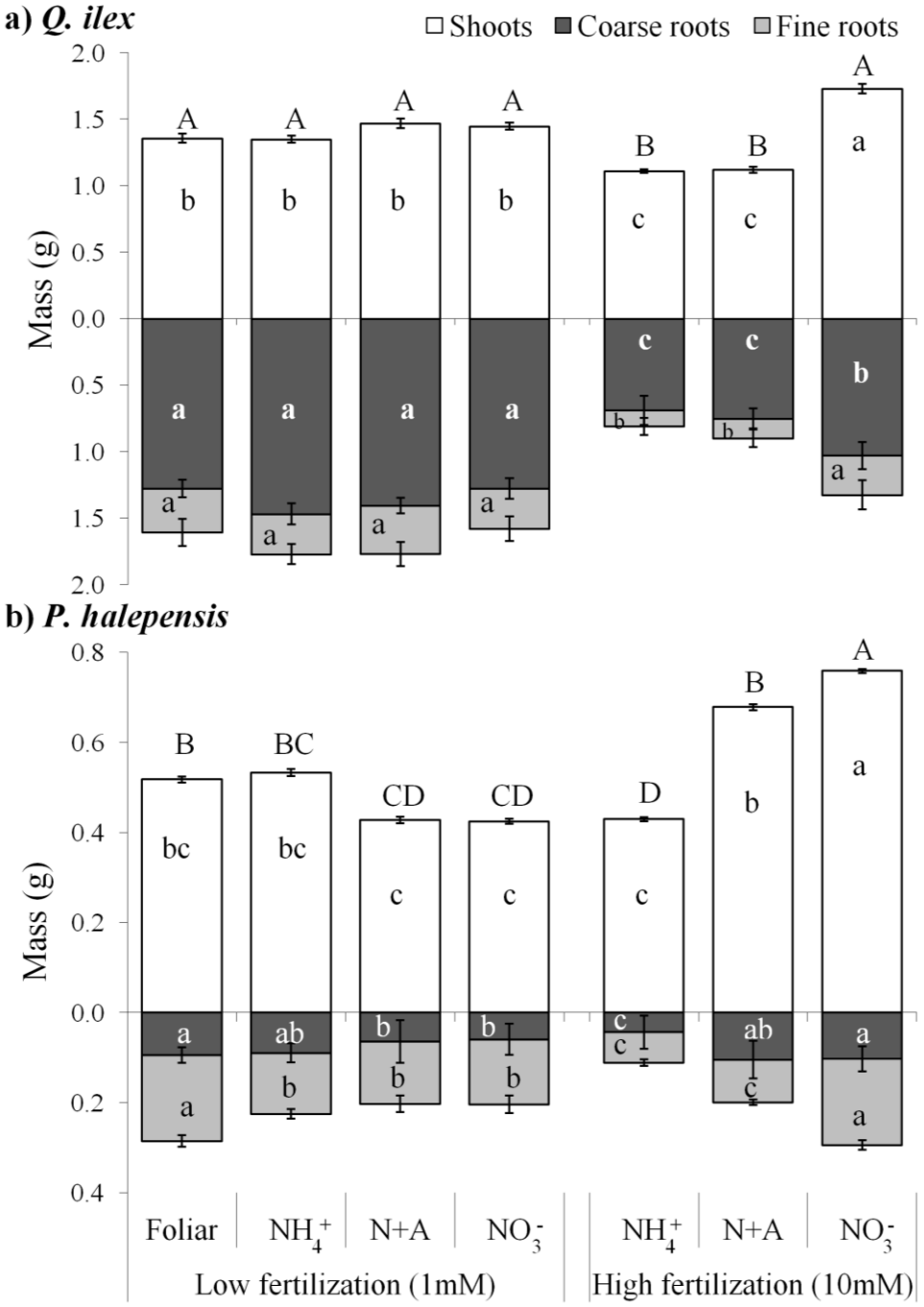


Figure 2. Mass (mean ± SE, n = 15) by fractions of a) *Quercus ilex* and b) *Pinus halepensis* seedlings as affected by fertilizer N form (only NH_4^+ or NO_3^-, and equimolar amounts of NO_3^- and NH_4^+ [N+A]), and fertilizer N concentration (high- 10 mM and low- 1 mM). Foliar fertilization with amino acids is included as an isolated treatment. Within a fraction, different lower-case letters indicate statistical differences among treatments. Different capital letters denote significant differences in total plant mass at α=0.05 using Fisher's LSD. To facilitate treatment comparison, mass scale is different for each species.

Table 2. Summary of two-ways ANOVA (p-values) for N forms (NH_4^+, NO_3^-+NH_4^+ equimolar concentration, and NO_3^-), and N concentration ([N]) (high- 10mM and low- 1mM) on dry weight of plant fractions and plant nutritional status of *Quercus ilex* and *Pinus halepensis* seedlings. [1] Non-homocedastic variables were analyzed by Kruskal Wallis test.

	N form	[N]	N form × [N]
Quercus ilex			
Plant mass	0.001	<0.001	<0.001
Shoot mass	<0.001	0.03	<0.001
Fine roots mass	0.012	<0.001	<0.001
Coarse roots mass	0.62	<0.001	<0.001
Plant N	0.049	<0.001	0.032
Plant P	<0.001	<0.001	<0.001
Plant K	<0.001	<0.001	0.016
Pinus halepensis			
Plant mass	<0.001	<0.001	<0.001
Shoot mass	[1]<0.001		
Fine roots mass	[1]<0.001		
Coarse roots mass	0.012	0.019	<0.001
Plant N	[1]<0.001		
Plant P	<0.001	<0.001	0.24
Plant K	<0.001	<0.001	0.68

Nutrient concentration

Fertilization increased plant N, P and K concentration in oak, but this increment varied with N form (interaction N concentration × N form, Table 2, Figure 3a). At 1 mM N, there were no differences in N concentration among N forms. Consequently, no differences in N content were observed among 1 mM N seedlings (data not shown). However, at 10 mM N, NO_3^--fertilized seedlings had lower N concentration than those grown with NH_4^+ or [N+A]. Although all seedlings received the same proportion of macronutrients, NO_3^- seedlings had lower P concentration than those grown with NH_4^+ and [N+A] at both N concentration levels. An opposite response has shown for K, its concentration was higher in NO_3^- than in [N+A] or NH_4^+ plants. Variation in nutrient root and shoot concentration in *Q. ilex* followed the same trend that was described at the plant level except for N concentration. Variation in plant N concentration was attributed to changes in root N concentration rather than shoot N

concentration, which did not differ between N forms (data not shown). Mean plant N content across treatments in holm oak ranged between 128 to 133 mg N seedling^{-1} (P>0.05).

Foliar fertilization did not affect plant nutrient concentration relative to 1 mM N seedlings in Q. *ilex* (Figure 3a). Plant N, P and K concentration in foliar fertilized seedlings was lower than in 10 mM N seedlings, with the exception of plant P concentration for 10 mM NO_3^- fertilized seedlings. N content of foliar fertilized oak seedlings did not differ from the N content in the remaining 1 mM N treatments (data not shown).

Concentrations of N, P and K were higher with increasing fertilization in pine seedlings. Seedlings grown with NO_3^- had the lowest N and P concentration and NH_4^+ seedling had the highest, while seedlings grown with [N+A] showed intermediate values (Table 2 and Figure 3b). Plant K concentration did not differ between NO_3^- and [N+A] seedlings, but was higher than in NH_4^+ cultivated pines within each N concentration. Variation in root and shoot nutrient concentration in pine followed the same trend as described for the plant level, except for K concentration. Shoot K concentration was lower in NH_4^+ than in [N+A] and NO_3^- (P<0.001), while root K concentration rising with increasing proportion of NH_4^+ in the fertilizer (P<0.001) (data not shown). Across treatments in pine mean plant N content was 42-62 mg N seedling^{-1}. It was significantly higher at 10 mM than at 1 mM N (P<0.001) while N form had not influence on plant N content (data not shown).

Foliar fertilized pine seedlings had lower N concentration than 1 mM NH_4^+ seedlings and exhibited no difference from either 1 mM [N+A] or NO_3^- plants (Figure 3b). Foliar fertilization increased P concentration relative to the 1 mM NO_3^- seedlings in pine, but showed no difference relative to the other N-forms at 1 mM N. Foliar fertilized pines also had higher K concentration than 1 mM NH_4^+ seedlings, no differences with 1 mM NO_3^- and [N+A] seedlings, and lower K concentration than all N forms at 10 mM N. Plant N content for foliar fertilized seedlings was only lower than for 1 mM NH_4^+ fertilized plants, and P content was only higher than 1 mM NO_3^- plants (data not shown).

Gas exchange and photosynthetic pigments

N form at low N concentration did not affect A in either species. At high N concentration, however, an increase in the proportion of NO_3^- in the fertilizer enhanced A (N form × N concentration interaction, Table 3). Foliar-fertilized pines and oaks had higher A than the remaining treatments.

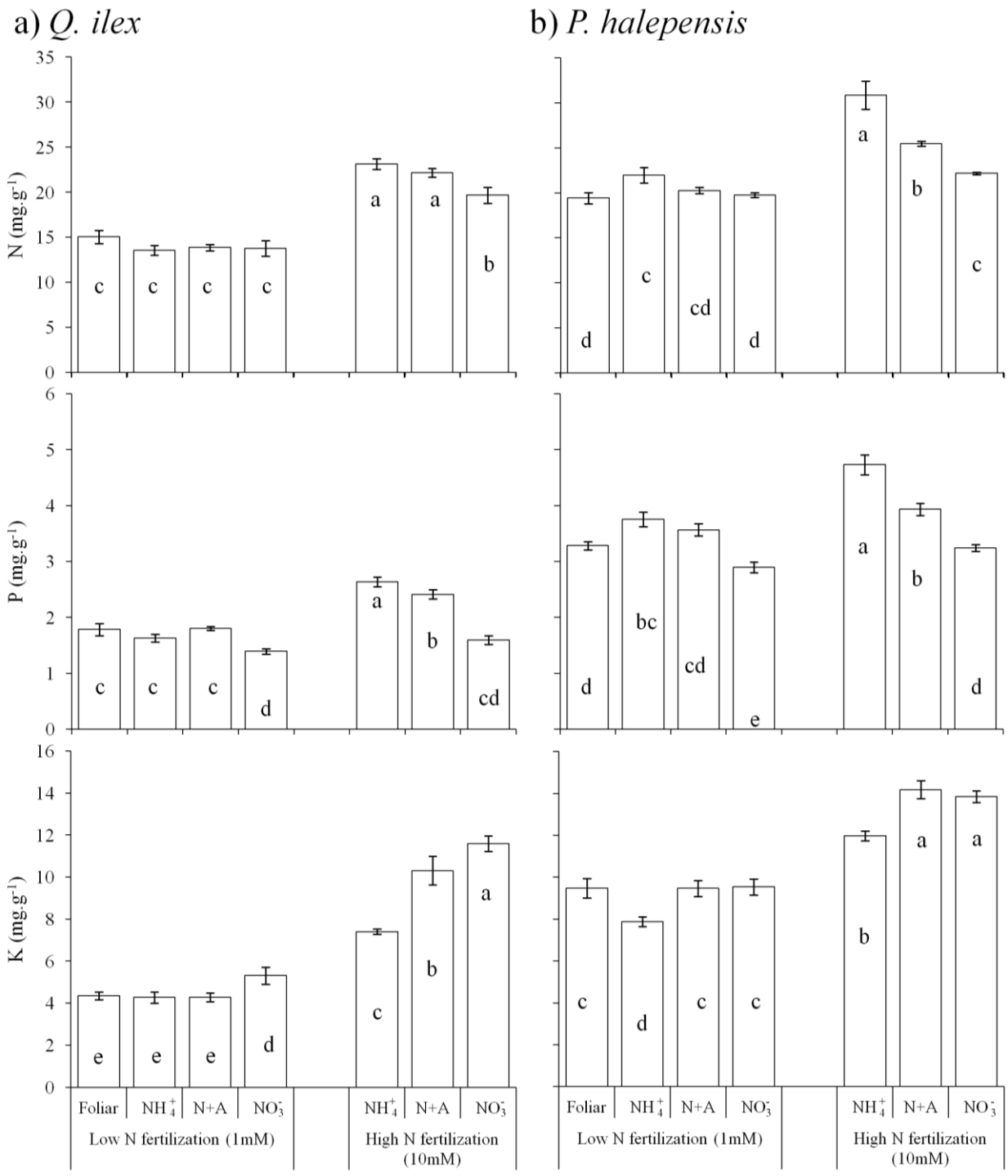


Figure 3. N (upper), P (middle) and K (lower) whole plant concentration (mean ± SE, n=5) of a) *Quercus ilex* and b) *Pinus halepensis* seedlings as affected by fertilizer N form (only NH_4^+ or NO_3^-, and equimolar amounts of NO_3^- and NH_4^+ [N+A]), and fertilizer N concentration (high- 10 mM and low- 1 mM). Foliar fertilization with amino acids is included as an isolated treatment. Means followed by different letters denote significant differences at α=0.05 using Fisher's LSD.

fertilizer enhanced *A* (N form × N concentration interaction, Table 3). Foliar-fertilized pines and oaks had higher *A* than the remaining treatments.

All photosynthetic pigments increased with N fertilization in both species, while N form had no influence (Table 3). Foliar fertilized *Q. ilex* seedlings had lower chlorophyll concentration than the remainder treatments while carotenoid concentrations were lower only than the N forms applied at 10 mM N. *Pinus halepensis* seedlings had lower chlorophyll and carotenoid concentrations than the N forms applied at 10 mM N and similar to those at 1 mM N.

Growing media chemical characteristics

Single growing media (without plants) had higher pH than growing media with seedlings (4.8±0.1 for single samples and 4.1±0.2 and 4.2±0.2 for samples with oak and pine seedlings in average, respectively; P=0.019). Growing media pH of all holm oak fertilization treatments was lower than the reference sample without seedling pH (Table 4). NO_3^- fertilized plants had higher growing media pH than [N+A] or NH_4^+ in seedlings fertilized at 1 mM N, but not at 10 mM N (N form × Fertilizer N concentration interaction). Effect of N form and concentration on growing media pH in *P. halepensis* showed similar trends as in *Q. ilex*. Only the 1 mM NO_3^- plants had higher growing media pH than that of the 10 mM NH_4^+ plants.

Growing media EC and NH_4^+ and NO_3^- concentrations had very similar patterns across treatments in both species. EC in 10 mM N seedlings was 6 to 10 times higher than the EC in 1 mM N seedlings (Table 4). NO_3^- seedlings had lower EC than NH_4^+ plants, while [N+A] fertilization resulted in intermediate EC values in both species. The EC of the reference sample without seedling was lower than all fertilization treatments in both species. Growing media NH_4^+ and NO_3^- concentrations were greater at 10 mM than at 1 mM N. Growing media NH_4^+ and NO_3^- concentrations raised with the increase in the proportion of NH_4^+ and NO_3^- in the fertilizer, respectively. However this effect was greater at 10 mM than at 1 mM N (significant interaction N form × N concentration, Table 4). NH_4^+ and NO_3^- concentrations in the reference sample without seedlings in both species were similar to that in the growing media of seedlings grown at 1 mM and lower than in the growing media of 10 mM N seedlings.

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

	Foliar	Low N (1 mM)			High N (10 mM)			*P* > F		
		NH_4^+	[N+A]	NO_3^-	NH_4^+	[N+A]	NO_3^-	N form	[N]	N form × [N]
Quercus ilex										
A (mmol m^{-2} s^{-1})	20.2±1.4a	13.2±1.3bc	8.8±1.1d	12.1±0.9c	11.3±1.6cd	13.3±1.4bc	15.1±0.9bc	0.09	0.07	0.03
Chlorophylls (mg m^{-2})	952±26c	1106±43b	1086±34b	1070±49b	1114±94b	1357±93a	1385±59a	0.38	0.015	0.22
Carotenoids (mg m^{-2})	145±7c	164±8c	154±9c	159±8c	180±13b	203±8a	200±5a	0.83	0.001	0.41
Pinus halepensis										
A (mmol m^{-2} s^{-1})	36.7±3.9a	26.0±1.3cd	23.2±1.1d	24.6±0.9d	23.5±1.6d	27.2±0.4c	31.6±2.2b	0.17	0.09	0.03
Chlorophylls (mg m^{-2})	984±37b	982±28b	1078±24b	1002±37b	1227±20a	1189±37a	1208±41a	0.67	<0.001	0.19
Carotenoids (mg m^{-2})	150±4d	144±3d	160±6c	148±3d	174±4b	172±5b	183±6a	0.52	<0.001	0.22

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

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

Discussion

Effects of inorganic N forms

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

Nitrogen forms induced distinct changes in seedling functional performance at high N concentration, which were species-dependent. First, high NH_4^+ fertilization caused toxicity in both species as it reduced growth yet increased plant N concentration (Salifu and Timmer 2003). Toxicity of NH_4^+ at high concentration has also been reported in several boreal conifers (Öhlund and Näsholm 2001; Rothstein and Cregg 2005). Although *Quercus* species are classified as NH_4^+-tolerant (Britto and Kronzucker 2002), the oak was more sensitive than the pine to high NH_4^+ fertilization, as both growth reduction relative to 1 mM N plants and mortality were greater in the former than in the latter species (Figure 2). This result, therefore, does not support our hypothesis that oak will perform better with NH_4^+. Secondly, consistent with our hypothesis, high NO_3^- fertilization greatly promoted pine growth compared to plants fertilized with 1 mM N, while it had a negligible effect on oak. Growth

stimulation in pine under high NO_3^- can be explained, in part, by an increase in photosynthetic capacity (Table 3) as was similarly reported for *Pinus radiata* D. Don (Bown *et al.* 2010). Although high NO_3^- fertilization increased *A*, this effect cannot be ascribed to either higher shoot N (Field and Mooney 1983) or higher photosynthetic pigment concentration in high NO_3^- seedlings. This might be explained by the same N content being differentially partitioned to and within the photosynthetic apparatus, a process that varies depending on N form in fertilizer (Warren *et al.* 2000). Performance of seedlings grown at high [N+A] was closer to that of high NH_4^+ seedlings than that of NO_3^- plants suggesting that the effects of NH_4^+ overshadowed those of NO_3^- in equimolar mixtures, which may be explained by NO_3^- uptake inhibition by NH_4^+ (Kamminga-Van Wijk and Prins 1993; Öhlund and Näsholm 2001). This was more evident in *Q. ilex* than in *P. halepensis*. N form not only influenced N uptake but also uptake of P and K, probably reflecting the need of plants to equilibrate the electrochemical balance in cells (Britto and Kronzucker 2002). Specifically, NH_4^+ uptake competes with (and reduces) uptake of other cations, such as K, and enhances uptake of anions, such as P (Sotiropoulos *et al.* 2005; Rothstein and Cregg 2005); yet, the opposite effect occurs for NO_3^- uptake whereby K uptake is enhanced (Lang and Kaiser 1994; Britto and Kronzucker 2002). Our results suggest that this effect is magnified under higher N concentration.

Growing media pH affects growth and nutrition of plants (van den Driessche 1971; Garnett and Smethurst 1999). Although the fertilizer pH was neutral, growing media pH was lower than the pH recommended for nursery cultivation of forest species (5.5 and 6.5 for conifers and hardwoods, respectively, Landis *et al.* 1989). Optimum growing media pH varies among species. Growth and needle N concentration in *Pseudotsuga menziesii* (Mirb.) Franco were highest at pH 4.5, while performance in *Picea sitchensis* (Bong.) Carr. was lowest (van den Driessche 1971). In this study it was also observed that the effect of different N forms on plant performance was independent of the growing media pH. Rygiewicz *et al.* (1984a; 1984b) showed that uptake rate of both NH_4^+ and NO_3^- by *P. menziesii* was constant over a pH range from 3.0 to 5.5. Finally, in the Mediterranean pine *P. pinaster*, the greatest root biomass was found at pH 3.5 (Arduini *et al.* 1998). As far as we know, no published research has examined pH effects on the functional performance of the studied species here. However, as growing media pH differences among treatments were small we believe that performance differences in our study are attributed to N forms rather than to pH. Specifically, growth reduction in holm oak under high NH_4^+ fertilization cannot be attributed to changes in substrate pH, given that high

NH_4^+ fertilization did not influence this parameter, as similarly reported by Guo *et al*. (2002).

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

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

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

Results of this study have important implications for better understanding the ecology of these widespread Mediterranean trees as we demonstrate that they have different N response patterns. The pine, a pioneer species, is more plastic to changes in N supply and shows improved performance with NO_3^- as an N source, while the oak, a late successional species, has relatively low responsiveness to N form or N concentration and is more sensitive to high NH_4^+ concentration than pine. Similar to our results with N, *Q. ilex* has also been shown to have lower plasticity to light and water than *P. halepensis* (Baquedano and Castillo 2006; Puértolas *et al.* 2010). Absence of morphological plasticity to nutrients in oak relative to pine may be associated with two mechanisms. On one hand, holm oak has larger seeds than Aleppo pine, which allows for a prolonged reliance on seed N reserves during seedling development (Villar-Salvador *et al.* 2010), making oak seedlings relatively independent of soil N. On the other hand, holm oak may have low sufficiency levels to N supply as reported for other oak species (Salifu and Jacobs 2006). Moreover, our results with Aleppo pine did not conform to those observed in many boreal or temperate conifers. Metcalfe *et al.* (2011) showed higher growth in two conifers when fertilizer was enriched in NH_4^+ at 10 mM. van den Driessche (1971) reported higher growth in three conifers with NH_4^+ or equimolar mixtures of both inorganic N using 3.1 mM N solutions. Kamminga-Van Wijk and Prins (1993) found higher NH_4^+ uptake rates than NO_3^- in *P. menziesii* in a broad range of N concentrations from 0 to 1 mM. Results in Aleppo pine are consistent with its ecology, as it grows on degraded limestone soils, where NO_3^- concentration is frequently much higher than that of NH_4^+ (Gimeno-García *et al.* 2001). Similar to Aleppo pine, pioneer trees of wet temperate areas and calcicole herbs grow better under NO_3^- than NH_4^+, which has been linked to inability to avoid excess tissue NH_4^+ accumulation even when grown at low NH_4^+ concentration (0.1-3 mM N) (Gigon and Rorison 1971; Kronzucker *et al.* 2003).

Seedling size and tissue nutrient concentration affect outplanting performance (Oliet *et al.* 2009b; Oliet *et al.* 2009a; Villar-Salvador *et al.* 2012; Grossnickle 2012). In our study, except for plants grown with 10 mM NH_4^+, which exceeded the optimal N range, all treatments had N concentrations within optimum ranges of N and P for Aleppo pine (Oliet *et al.* 2006, 2009a) and holm oak (Villar-Salvador *et al.* 2004a). However, the plants grown at low N concentration were K deficient, while those grown at high N concentration have values within the optimal K ranges (Landis *et al.* 1989). In contrast to tissue nutrient concentration, plant size was smaller than the sizes recommended for 1-year old seedlings for both species (see Villar-Salvador *et al.*

2004a; Oliet *et al*. 2009a; 2009b). This is because our experiment was 3-4 months shorter than the typical cultivation length in Mediterranean nurseries. However, our experiment provides new insights for nursery cultivation of these species as we demonstrate that N form at high concentration strongly affects growth and nutritional status. Thus, both species should preferentially be grown with NO_3^- as an N source when fertilized at high N concentration, instead of using mixtures of both N forms. To validate the results of this study, however, further experiments testing intermediate inorganic N concentration values (especially in oak) and field outplanting performance are needed in both species.

Effect of amino acid foliar fertilization

Amino acid foliar fertilization had minor effects on seedling performance in relation to the other low N treatments. The low effectiveness of amino acid foliar fertilization suggests that both species have very low amino acid uptake, as indicated by the lack of N content differences between foliar-fertilized seedlings and the remaining 1 mM N treatments. This may be due to low leaf surface ion exchange capacity and/or outer cell wall water permeability in both species (Swietlik and Faust 1984). Consistent with the latter contention both species have low residual transpiration (Villar-Salvador *et al*. 2004b; Pardos *et al*. 2009). Relative ineffectiveness of amino acid foliar fertilization might also be due to low fertilizer amino acid concentration. Most commercial amino acid foliar fertilizers are designed for horticultural plants (Saborío 2002), and their concentration is probably too small to be effective for sclerophyllous tree species, which have a higher degree of water impermeable cuticles than do malacophyllous species (Larcher 1995). Furthermore, effectiveness of foliar fertilization is strongly reduced if the plant lacks nutritional deficiencies (Wójcik 2004). N concentration in 1 mM seedlings was close to the optimum recommended leaf N concentration (*P. halepensis* - Oliet *et al*. 2009a and *Q. ilex* - (Oliet *et al*. 2009b), thereby limiting amino acid leaf uptake.

Foliar fertilization did, however, stimulated *A* in both species, which cannot be explained by higher photosynthetic pigment or tissue N concentration of foliar-fertilized seedlings (Field and Mooney 1983; Bondada and Syvertsen 2003). Higher *A* in foliar fertilized pines could be explained by increased hydraulic conductivity mediated by higher fine root production (see Figure 2b) (Grossnickle 2005).

Foliar fertilization was more effective in pine than in oak, slightly increasing pine growth. This is consistent with oak lower plasticity to nutrient

availability than pine, as observed in the first part of this study. Differences in foliar fertilization effectiveness between species might be explained by differences in stomata distribution and cuticle composition (Burkhardt 2010) (i.e., pine has amphistomatic leaves, while oak has hypostomatic leaves). Moreover, the adaxial leaf surface of holm oak is covered by a dense layer of dead trichomes (Safou and Saint-Martin 1989), which might also hinder absorptive capacity in oak relative to pine.

Conclusions

This study demonstrates that the effect of N availability on holm oak and Aleppo pine performance is not straightforward as it depends on N form and species. N form affected the performance of both species, but only at high N concentration. Ten mM NH_4^+ generated toxicity in both species, while 10 mM NO_3^- promoted pine growth and did not increase oak growth relative to N forms at low N concentration. High N concentration increased photosynthetic pigment concentration and reduced root development in both species. With increasing proportion of NH_4^+ in the fertilizer at 10 mM, both species showed decreased fine root formation. NH_4^+ promoted uptake of P and inhibited that of K, while NO_3^- caused the reverse response. An equimolar mixture of NO_3^- and NH_4^+ produced intermediate responses in most attributes between those observed with only NH_4^+ and only NO_3^-, although responses in oak tended to be more similar to NH_4^+ than to NO_3^-. Foliar fertilization with amino acids promoted photosynthesis of both species, with a slight stimulation of growth in pine. These results provide further insight into the functional and ecological differences of these species and indicate that N-forms should be considered in the cultivation of both species.

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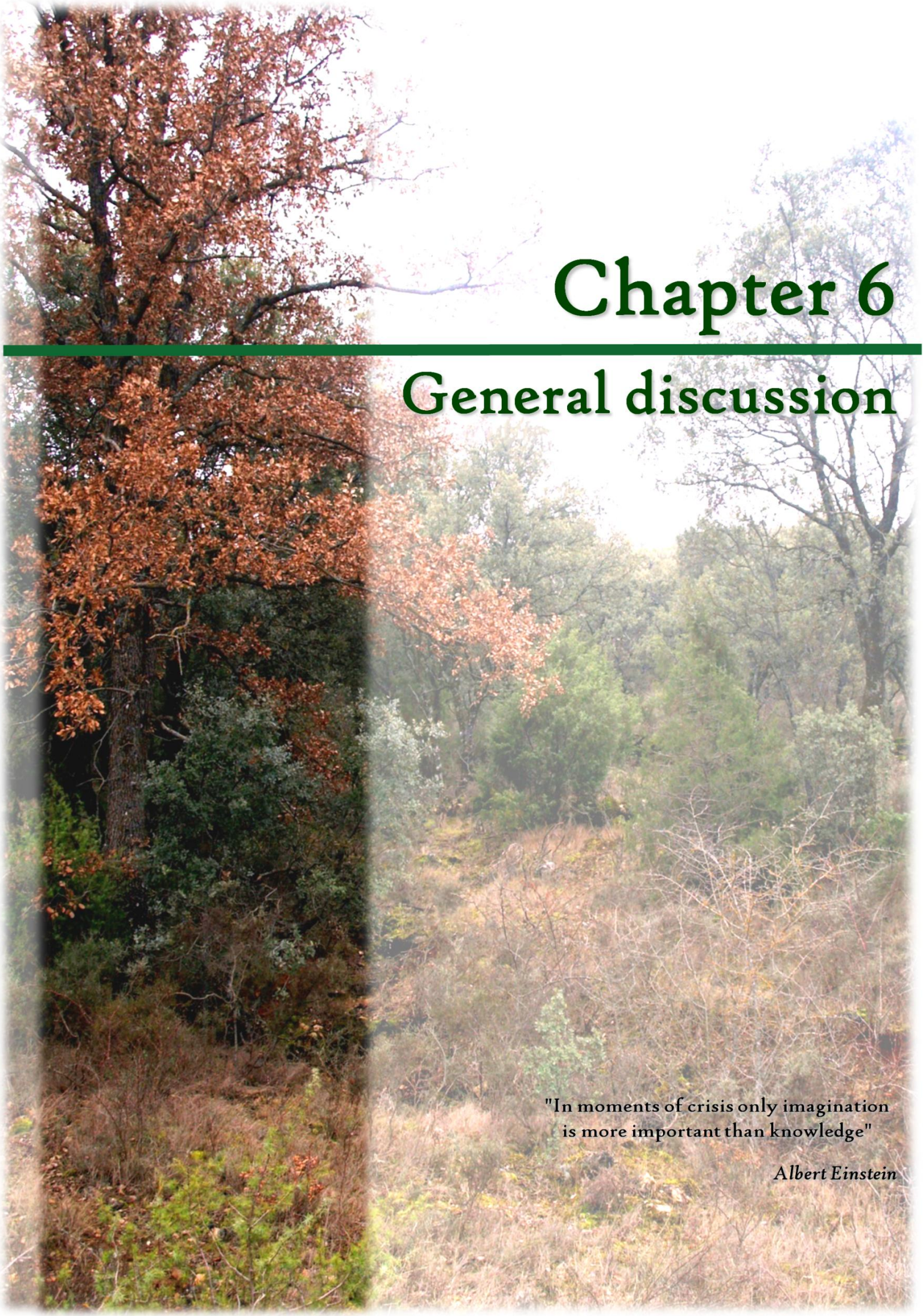

Chapter 6

General discussion

"In moments of crisis only imagination
is more important than knowledge"

Albert Einstein

Fotography: Mediterranean oak forest in
Puebla de Valles (Guadalajara)

By: M. Uscola

Ecophysiology of nitrogen in Mediterranean plants: strategies of nitrogen forms absorption, functional responses, and use of reserves for growth

The experiments carried out in this Thesis illustrate that Mediterranean forests species exhibit important differences in the acquisition, allocation and remobilization of N and in the functional responses to this resource. In addition, we support the idea that N should not be considered as a single resource, as plants are able to use different chemical form of N (Jones *et al.* 2005; Näsholm *et al.* 2009; Paungfoo-Lonhienne *et al.* 2010). All this points out that N plays a central role in the functioning and structure of Mediterranean forest communities.

All species were able to take up not only inorganic N forms, but also intact amino acids using both, the foliar (*Chapter 2*) and root pathway (*Chapter 3*). The ability to absorb intact amino acids is not surprising as species from several ecosystems do also have this ability (Jones *et al.* 2005; Näsholm *et al.* 2009; Masclaux-Daubresse *et al.* 2010). However, this depends greatly on the species and the pathway considered, with the root uptake of *intact* amino acids being less efficient than the foliar absorption. Several results support this idea. First, slopes of the ^{15}N *vs.* ^{13}C regressions in foliar fertilized seedlings were steeper than the slopes of soil fertilized plants. Second, dual labeled glycine was detected in all the seedlings that were foliar sprayed with glycine, but intact glycine was only detected in part of the seedlings used for root uptake study. Species had different capacity of take up intact amino acids. In the case of root uptake, mycorrhizal symbiosis type and colonization intensity enhance species differences. Nevertheless, it was not possible to quantify these species differences as experimental conditions between root and shoot absorption were not the same and, also, due to the limitations of the different methodologies used (i.e. GC-MS and IRMS) (see *Chapter 2* and *3* for further information) (Jones *et al.* 2005; Warren 2012).

The importance of organic N compounds for plant nutrition has been shown in arctic, alpine and boreal ecosystems (Kielland 1994; Näsholm *et al.* 1998; McKane *et al.* 2002). These Thesis results suggest that organic N can also be an important N source in Mediterranean ecosystems. First, all the species were able to take up, in some degree, intact glycine. Moreover, intact glycine uptake efficiency, i.e. uptake from a N source standardized by its soil N

availability, was, at least, similar to NO_3^- uptake efficacy (*Chapter 2*). Second, the amino acid pool in the experimental soil was of similar magnitude as the inorganic N pool (*Chapter 2*). Additionally, soil organic N concentration is also frequently higher than inorganic N in many Mediterranean communities (Delgado-Baquerizo *et al*. 2013). Finally, some species, such as *Rosa canina* and *Juniperus thurifera* had notable preference for glycine over inorganic N forms. A limitation of my study is that only glycine absorption was measured. This amino acid is not the most abundant amino acid either in the soil used in our experiment or in soils of many other ecosystems (Yu *et al*. 2002; Andresen *et al*. 2008; Werdin-Pfisterer *et al*. 2009). Absorption rate of different amino acids can differ widely due to molecular weight differences and ionic state differences (Persson and Nasholm 2001; Weigelt *et al*. 2005; Sauheitl *et al*. 2009). Therefore, using only glycine, together with the limitation of having precise estimations of intact amino acid uptake by plants (*Chapter 2 and 3*; Jones *et al*. 2005; Warren 2012) can provide an imprecise picture of the importance of amino acid absorption for plant nutrition. However, I have some indications that point out that the contribution of intact amino acids could be comparable to that of NO_3^- and/or NH_4^+. Finally, other small organic molecules such as proteins or peptides can contribute to enhance organic N importance for Mediterranean plants (Paungfoo-Lonhienne *et al*. 2008).

N absorption rate depended on N forms, species and input pathway. Interspecific differences in N uptake were mainly associated to plant growth form and morpho-anatomical differences of roots and leaves. In particular, plant architecture and leaf structure attributes, but not cuticle permeability to water, were major factors driving foliar N absorption across species. Plant architecture determines interception and retention of deposited nutrients. Specific leaf structural attributes, such as high stomatal density and presence of trichomes, seem to speed leaf N uptake (*Chapter 3*; Adriaenssens *et al*. 2010). Root architecture and the ability to metabolize the N compounds mainly explained total N root uptake differences across species (*Chapter 2*). The species that developed a more fibrous root systems (*i.e*. higher specific root length) and exhibited higher N reduction capacity had greater N root uptake than the plants showing the opposite traits. Whereas the uptake differences of N sources by roots was species-dependent (*Chapter 2*), differences in the absorption rates of N sources by leaves were independent of species identity and were controlled by the physico-chemical characteristics of the compounds (*Chapter 3*).

Although leaves can absorb N, the roots are the main pathway for N acquisition. For instance, foliar N uptake was 36 times lower than root N

uptake in *Pinus halepensis* and *Quercus ilex* despite the fertilizer solutions used for foliar fertilization were 10 times more N-concentrated than the solution used for root N fertilization (Figure 1). Foliar absorption of N is more limited due to the cuticle (Wójcik 2004; Fageria *et al.* 2009) as it strongly depends on the nutrient concentration gradient between the exterior and the tissue, it is much more dependent on the plant nutritional status than is root N uptake (Wójcik 2004; Fageria *et al.* 2009). Therefore, foliar absorption is much lower than root uptake in plants without nutrient deficiency. In spite of the lower N uptake capacity of foliage relative to that of root, foliar fertilization can improve plant N nutritional status by direct uptake or indirectly by stimulating root N uptake (*Chapter 3*; Dong *et al.* 2002) through fine root production and photosynthesis (*Chapter 5*; Maini 2006). Therefore, foliar N absorption may not only reduce competition for soil N, but also might enhance plant competitive capacity. Foliar nutrition seems to have additional positive effects on species. For example, Maini (2006) found that amino acid leaf fertilization increased nitrate reductase activity, stress resistance and fruit setting.

N utilization seems to drive the relative growth rate (RGR) of plants. Plants show wide variation in RGR when cultivated under the same environmental conditions (Poorter 1990; Cornelissen *et al.* 1996). Inherent growth capacity determines the competitive capacity and resource demand of

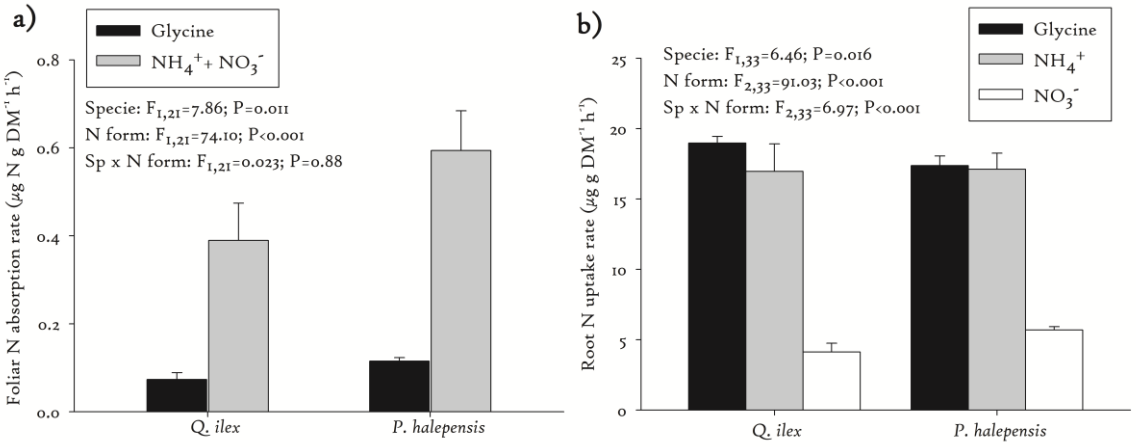

Figure 1. Uptake rate of glycine, NH_4^+ and NO_3^- by shoots (a) and roots (b) in *Quercus ilex* and *Pinus halepensis* seedlings. Data are mean ± 1 SE. For root N uptake measurement, plants were fertilized with three 1 mM N solutions that included the three N sources in equimolar amounts but where only one N form was ^{15}N-labeled. Plants were harvested 6 h after labeling. In order to quantify foliar N uptake, shoots were sprayed with a 10 mM N solution containing either $^{15}NH_4^{15}NO_3^-$ or ^{15}N-labeled glycine, two times per day for three days (Source: Uscola unpublished data). Cuticular conductance was not considered as a covariate in the foliar uptake sub-experiment. Note differences in scale between both fractions.

plants and, consequently, RGR plays an import role in the dynamic and structure of plant communities (Grime and Hunt 1975; Poorter 1990). High RGR is linked to a suite of traits involving high allocation of resources to leaves at the expense of other organs, and high tissue N and P concentration and specific leaf area (Poorter 1990). Surprisingly, RGR, which impels N demand, does not seem to drive N uptake capacity in Mediterranean forest species. This result was confirmed not only in *Chapter 2* but also in *Chapter 3*, where *Pinus halepensis,* the fastest growing species, did not have the highest N uptake rate, while *Quercus coccifera*, the slowest growing species, had the fastest N uptake. My Thesis provides evidence of other mechanisms related with N economy that seem to also control the RGR of plants. The first mechanism is the high use of N reserves to support fast growth (*Chapter 4*; Millard and Grelet 2010). Similarly, evidence supporting the importance of N reserves for RGR, is the high and positively relation between the RGR and N remobilization potential (Figure 2). The species with higher N availability for resorption in leaves had higher RGR. Although leaves are the main N source for remobilization in evergreen woody plants (*Chapter 4*; Millard and Grelet 2010), species can use other organs to supply N reserves for growth (*Chapter 4*). For example, coarse roots were important sources of remobilized N in *Quercus coccifera* but not in *Q. ilex*, while fine roots or stems greatly contributed to supply N in *Olea europaea*. The contribution of other organs than leaves for N potential remobilization was not considered in N resorption potential (Figure 2), which underestimates the potential importance of other reserves in species differences.

The second mechanism related to the N economy of plants that might drive RGR differences across species is the preference for NH_4^+ as a N source. The fastest growing species had also highest NH_4^+ preference, while the slowest growing species showed less preference for NH_4^+ and their N form absorption pattern was very similar to the pattern of N forms availability in the soil. The potential benefit of using NH_4^+ instead of NO_3^- is the reduction of the energetic costs of N metabolization, which might increase the proportion of energy and resources invested to growth. Several studies have shown a higher NH_4^+ preference in wet temperate plant communities (Harrison *et al.* 2008; Maire *et al.* 2009; Schulz *et al.* 2011; Boudsocq *et al.* 2012), and also in a range of plant communities in Australia (Turnbull *et al.* 1996). We suggest that higher NH_4^+ preference can also provide other benefits to plant that might contribute to increase RGR. Mediterranean forests on limestone soils are usually P-limited due to high soil pH (Sardans *et al.* 2004, 2005; Sardans and Peñuelas 2005). NH_4^+ enhances anion absorption as phosphorous (P) to maintain internal

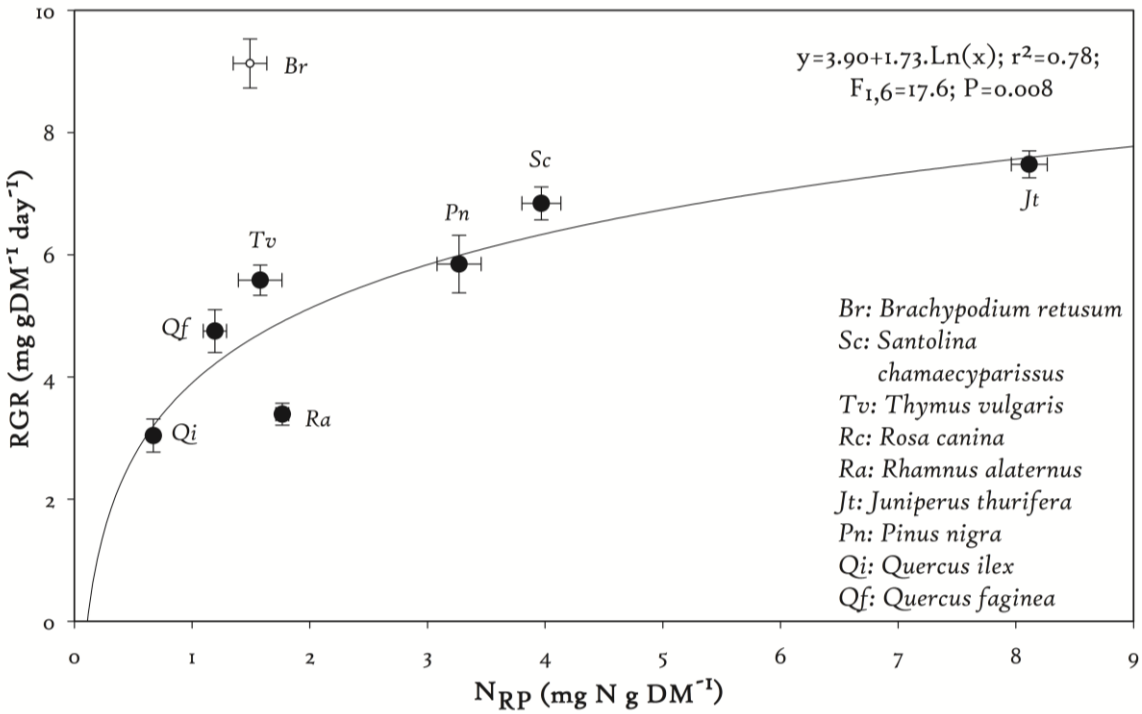

Figure 2. Relationship between relative growth rate (RGR) and N resorption potential (N_{RP}) in Mediterranean forest species (*Chapter* 2). N_{RP} was calculated multiplying the N content in green leaves at the beginning of growing season and N resorption efficiency and standardized by plant mass. In this analysis, I assumed that leaves were the main N source for remobilization/resorption (*Chapter* 4; Millard and Grelet 2010). *R. canina* it is a deciduous species and lacked leaves at the beginning of the growing season. *Brachypodium retusum* was also excluded from the regression analysis.

electrochemical equilibrium (*Chapter* 5, Britto and Kronzucker 2002; Sotiropoulos *et al.* 2005; Rothstein and Cregg 2005). NH_4^+ uptake reduces soil pH more than NO_3^- absorption (Helali *et al.* 2010; Metcalfe *et al.* 2011), which might help P or Fe absorption in high pH carbonate rich soils, such as limestone soils (Tyler 1992; Zohlen and Tyler 2000). Thus, higher NH_4^+ preference could have additional advantages in Mediterranean ecosystems, such as reducing several nutrient limitations.

Water and light have been considered the main resources constraining plant life in Mediterranean forest ecosystems (Zavala *et al.* 2000; Sánchez-Gómez *et al.* 2006; Gómez-Aparicio *et al.* 2006). My Thesis suggests that N can also be an important driver for community structure in Mediterranean ecosystems. I provide evidences for different N-based fundamental niche differences among Mediterranean plants as we demonstrate differences in uptake capacity, N form preference (*Chapter* 2) and internal storage and

remobilization (*Chapter 4*), which might have distinct dimension in N fundamental niche. Thus, coexistence of species that have a similar use pattern of water and light could also be possible through niche complementary due to different N use (Davies *et al.* 1998; Kahmen *et al.* 2006). A first dimension of N fundamental niche differentiation is N uptake and N form preference. Regarding ecological succession, pioneer species grow faster and have higher NH_4^+ preference than mid and late successional species (*Chapter 2*). Fundamental niche segregation was also related to differences in N acquisition between growth forms, with the smaller forms having faster N uptake than the taller growth forms. Co-occurring species within a succesional stage also segregated their fundamental niche by different N uptake capacity and N form preference. Pioneer species had both different N uptake rates and NH_4^+ preferences intensity among them. But mid and late succesional species had similar N uptake rate, and segregation by N forms preferences were related to the community structure, as has been shown for other ecosystems (McKane *et al.* 2002; Weigelt *et al.* 2005; Aanderud and Bledsoe 2009): while dominant species preferred NH_4^+, the subordinate species preferred the remaining N forms.

We suggest that another way by which plants segregate their N fundamental niche is how plants use N reserves for growth (*Chapter 3*), which ultimately might affect the timing of soil N acquisition. The species that mainly relied on soil N in mid spring probably can reduce competition for soil N when coexisting with species that strongly rely on N reserves to meet their N demands (*Chapter 4*). Thus, the pattern of N reserves utilization in spring could enable the species to segregate through time the use of soil N.

Finally, contrary to other studies (Guo *et al.* 2002), we could not detect a clear effect of N forms at a concentrations similar to those in natural soils on growth and physiological performance at a intraespecific level (*Chapter 5*). However, we cannot discard that exposure to N forms at low concentration for longer periods (our experiment only lasted 5 months) will affect the functional attributes of seedlings, as occurred at high concentration.

Practical implications

The results of my Thesis have practical implications to forest restoration, especially for seedling cultivation and outplanting performance. As plant nutrition is one of the most important issues in the cultivation of forest species (Villar-Salvador *et al.* 2012; Grossnickle 2012), my Thesis point out that

not only knowledge of the water and light ecology is important but also the knowledge of N ecology can be useful for forest restoration culture practices. In this respect, two processes related to N economy are relevant. On the one hand, species N absorption capacity and preferences for N forms. On the other hand, the effect of N sources on the functional performance of seedlings. High seedling quality can be achieved during nursery production considering these aspects. For example, NH_4^+ fertilization at high concentration reduces growth but also the fine root fraction, which can hinder the ability to take up nutrients and water after transplanting (*Chapter 5*). In contrast, NO_3^- fertilization at high N concentration does not reduce fine root growth.

Nitrogen form not only affects the seedling N concentration, but also the concentration of other mineral nutrients (*Chapter 5*). Seedling field performance could be improved by producing seedlings with higher reserves of other elements than N, such as P or K. Meeting species N uptake rate (*Chapter 2*) with N fertilization rate, can improve fertilization efficiency and reduce N lixiviation from nurseries and associated environmental drawbacks.

New root and shoot growth is essential to ensure survival in forest plantations (Villar-Salvador *et al.* 2012). Therefore, survival and growth of seedlings in the field can be improved if we know how species use their reserves for new growth. I have shown that the growth of the root system in the early spring, when seedlings become established due to the growth of new roots out of the plug, strongly depends on N reserves (*Chapter 4*). In Mediterranean climates, where summer drought is the main cause of plantation failure, seedlings that produce deep roots before summer drought onset can survive to the dry season by accessing to water and nutrients accumulated deep in the soil. In fast-growing species which depends strongly on N reserves for new growth during the mid spring, nursery cultivation practices must be designed to enhance N and C storage. In slow-growing species, which rely less on the reserves, nursery growers must promote attributes that maximize resource acquisition in the field, *i.e.* large plants with high photosynthetic capacity and broad, fibrous roots.

Foliar fertilization can be an interesting option for N-loading seedlings in nursery cultivation and for quickly supplying nutrients to seedlings planted in very poor soils (*Chapter 3*). However, it is necessary to determine the optimum fertilizer N concentration for each species, since the foliar absorption capacity varies widely between species. Furthermore, foliar fertilization can be a effective tool to the odd supply nutrients to seedlings (*Chapter 3*) in forest

plantations carried out on very nutrient-poor soils or on soils that impose severe nutrient constraints, such as P or Fe uptake on gypsum or limestone soils in very dry areas. Moreover, foliar fertilization in the nursery can also have other advantages, by not only providing nutrients, but also enhancing root fibrosity and photosynthesis rate.

Future research on ecological process related to N

Many aspects of the N ecology of Mediterranean plants have to be further investigated. Several subjects related to my Thesis are:

1) Which is the contribution of intact organic N uptake to N nutrition in Mediterranean ecosystems? As I previously mentioned, uptake rate of different amino acids can greatly vary. Experiments testing different amino acids or small proteins carried out on a suitable time scale are needed to know the real contribution of intact organic N uptake to plant nutrition in natural ecosystems.

2) How important are soil microorganisms for soil N competition? Nitrogen uptake is highly dependent on the interaction with soil microbes. An added difficulty for N form uptake studies under field conditions is the extent of plant-soil microbe competition for N, which greatly influences the amount of soil N available to plants (Bardgett *et al.* 2003; Harrison *et al.* 2008). Plant species probably differ in how effectively they compete with micro-organisms for N sources (Näsholm *et al.* 2000; Weigelt *et al.* 2005). When we designed my Thesis, one of the objectives of the *Chapter 2* was to know the role of microbes as N competitors. Unfortunately, we had some problems in our isotopic analysis of microbial mass and these results could not be included in the Thesis.

3) Is phylogeny affecting the relationships between attributes across species? Cross-species differences and relationships between traits observed in *Chapter 2* may be partially conditioned by species phylogeny. Therefore, we need to perform phylogenetic analyses to know the specific weight of phylogeny in our results.

4) How important is the foliar pathway for the nutrient input in plants of Mediterranean ecosystems? The contribution of foliar uptake of water and nutrients has been demonstrated to be important for carnivores and epiphytic plants (Benzing *et al.* 1976; Adamec 2002; Lambers 2008), but also for other plants such as *Sequoia sempervirens* (coastal redwood) (Burgess and Dawson 2004). Also, anthropogenic N deposition can affect distinctly to the species of

natural ecosystems as a consequence of the differences in the ability to foliar N absorption (Sparks 2009). One of the problems to determinate the contribution of N atmospheric inputs in natural ecosystems is the low availability of data on the amount of different N forms deposited on foliage and, especially, the concentration of N forms in solutions under specific circumstances, such as very small rain events or after morning dew.

5) Is nutrient remobilization and resorption a general driver of the relative growth rate in plants? To provide further evidence of the linkage between relative growth rate and N and C remobilization (*Chapter 4*), it is necessary to carry out analyses of published data and carry out new experiments with a higher number of species. Moreover, C reserves were considered in broad sense but we did not distinguish either between structural and non-structural carbohydrates or between different non-structural carbohydrates. Moreover, we do not know what N compounds are stored and remobilized. Future studies should analyze the isotopic enrichment of specific C and N compounds.

6) Do N forms affect the morpho-physiology of plants at low concentration? We could not observe important functional response of plants to different proportions of N forms at low concentration. However, we believe that N forms at low concentration do have deeper effects on plant functional performance but probably this effect can only be detected with longer time frameworks, especially in slow-growing species. Accordingly, we suggest that experiments on N form should be carried out for longer periods.

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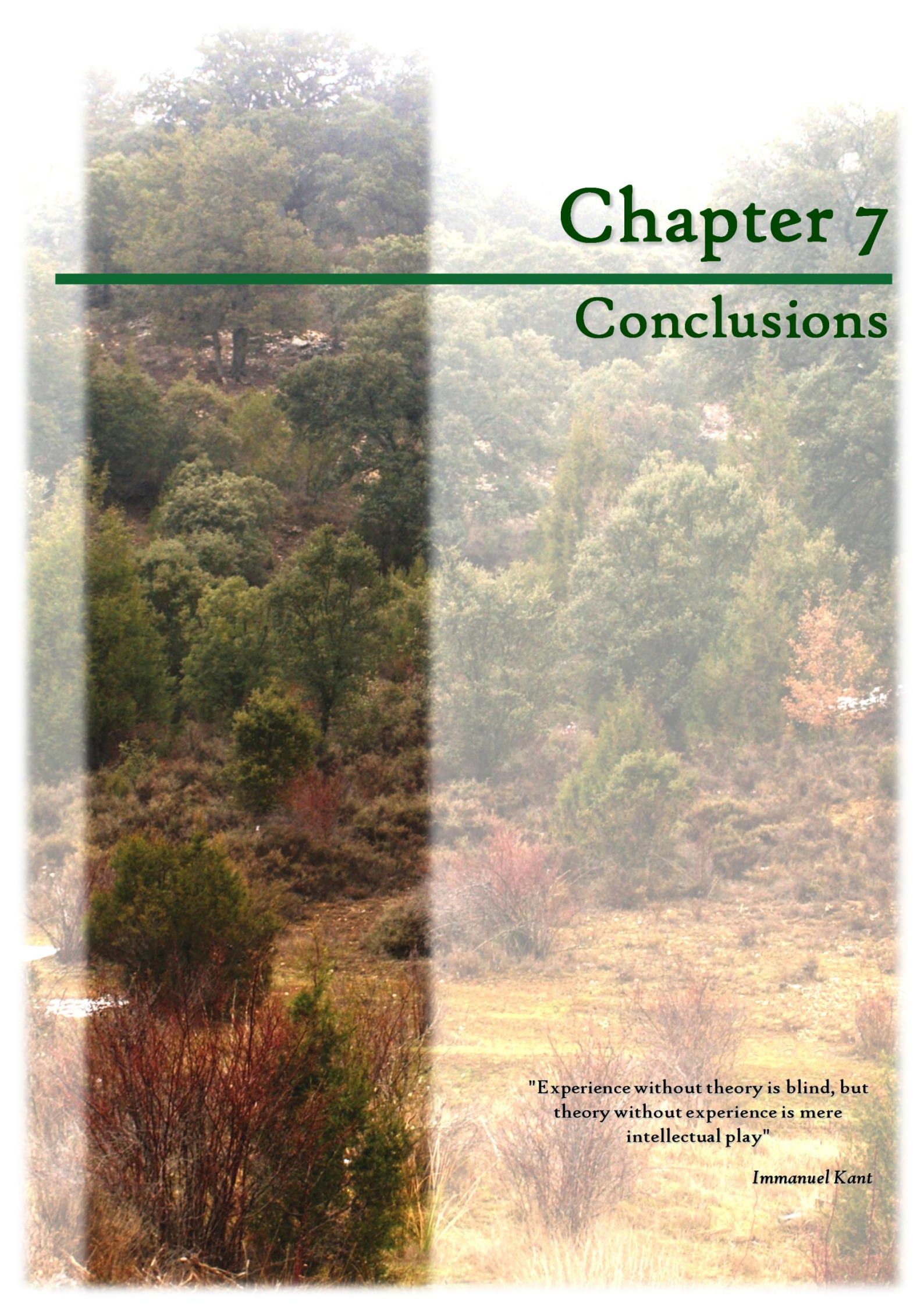

Chapter 7

Conclusions

"Experience without theory is blind, but theory without experience is mere intellectual play"

Immanuel Kant

Fotography: Mediterranean oak forest in
Tamajón (Guadalajara)

By: M. Uscola

Conclusiones generales

La tasa de absorción total de N por las raíces fue mayor en la especie herbácea y en los caméfitos que en los arbustos y árboles. Las diferencias entre especies se explican por diferencias en la estructura de la raíz y la capacidad de asimilación del N, de forma que las especies con una mayor longitud específica de raíces y mayor actividad nitrato reductasa absorbieron más rápidamente el N. Ni la velocidad de crecimiento ni la concentración de N en hojas se relacionaron con la tasa de absorción total de N.

Todas las especies absorbieron por las raíces glicina intacta y N inorgánico (NH_4^+ y NO_3^-). La capacidad de absorción radical de glicina intacta se relacionó positivamente con la colonización de micorrizas vesículo arbusculares, aunque no se encontró relación con la absorción de fuentes de N inorgánicas. La colonización con ectomicorrizas fue muy baja y no se detectó que afectara a la absorción de ninguna fuente de N.

La mayor parte de las especies prefirieron el NH_4^+ como fuente de N en detrimento del NO_3^-, es decir, que proporcionalmente absorbieron el NH_4^+ por encima de su abundancia relativa en el suelo. La preferencia por la glicina no mostró un patrón claro entre especies.

Las especies pioneras presentaron una mayor preferencia por el NH_4^+ que las más tardías de la sucesión. Entre las especies pioneras existieron notables diferencias tanto en la tasa de absorción total de N como en la preferencia por el NH_4^+. Igualmente, entre las especies de las etapas intermedias–tardías de la sucesión, los árboles dominantes utilizaron preferentemente el NH_4^+ como fuente de N, mientras que los arbustos subordinados prefirieron la glicina o el NO_3^-.

Quercus ilex y *Pinus halepensis* absorbieron por las hojas tanto fuentes de N inorgánicas como orgánicas, incluida la glicina intacta, siendo la tasa de absorción mayor en *Q. ilex* que en *P. halepensis*. Estas diferencias se explicaron por la mayor capacidad de *Q. ilex* de intercepción y retención de la solución y, posiblemente, por su mayor densidad de estomas y la presencia de tricomas.

La tasa de absorción por las hojas difirió entre fuentes de N siendo mayor la absorción de urea, seguida de NH_4^+, glicina y NO_3^-. Dichas diferencias se explican por las propiedades físico-químicas de los compuestos. Un incremento en la permeabilidad de la cutícula al agua aumentó la absorción de las fuentes de N, exceptuando la de NO_3^-.

Las plantas perennifolias mediterráneas fueron capaces de asimilar y almacenar C bajo condiciones de bajas temperaturas. El C asimiladose distribuyó se asignó según el patrón de distribución de biomasa de la planta. En las quercíneas, la mayor parte del C de invierno se distribuyó a raíces gruesas, mientras que en *Olea europaea* y *Pinus halepensis* se incorporó principalmente a la parte aérea. Sin embargo, las hojas fueron, en general, sitios preferentes de almacenamiento de C de invierno ya que contuvieron mucho más C de invierno del esperado por su tamaño.

Todas las especies dependieron en gran medida de las reservas de N para la construcción de las raíces nuevas al principio de la primavera. En cambio, en la mitad de la primavera, la mayor parte del N en las raíces nuevas provino del N del suelo y del C asimilado en el momento.

Las especies difirieron notablemente en su dependencia de las reservas de C y N para construir los brotes. Las quercíneas, usaron mayoritariamente C y N recientemente adquirido, mientras que *O. europaea* y, especialmente *P. halepensis,* usaron principalmente C y N removilizado de las reservas.

Las hojas viejas fueron la principal fuente de C y N utilizado para apoyar el crecimiento y metabolismo en primavera, ya que presentaron una gran reducción en su contenido de C y N que, además, fue mayor de lo esperado por su contenido inicial.

Las fuentes de N inorgánicas a alta concentración condicionaron el desarrollo morfológico y fisiológico de *Q. ilex* y *P. halepensis*. Sin embargo, el efecto de las fuentes de N a baja concentración fue muy pequeño. *Pinus halepensis*, un árbol pionero, se desarrolló mejor con NO_3^-, tuvo una mayor plasticidad ante cambios de disponibilidad del N y experimentó menos toxicidad al NH_4^+. *Quercus ilex*, un árbol tardío en la sucesión, presentó una baja respuesta a las fuentes de N o a su concentración y sufrió mayor toxicidad por el NH_4^+.

La fertilización foliar con aminoácidos tuvo un efecto muy leve sobre el desarrollo de las plantas. Estimuló ligeramente la fotosíntesis y la proporción de raíces finas pero no incrementó el crecimiento ni la concentración de nutrientes.

La estrategia de utilización del N parece condicionar la velocidad de crecimiento de las plantas. Las especies de rápido crecimiento usaron en mayor proporción las reservas de N para apoyar el crecimiento en primavera y mostraron una elevada preferencia por el NH_4^+ como fuente de N. Sin embargo, la velocidad de crecimiento no se relacionó con la tasa de absorción de N.

Esta Tesis evidencia que el N no debe ser considerado como un único recurso, sino que deben considerarse sus distintas formas químicas y que el N orgánico puede ser una importante fuente de N en ecosistemas mediterráneos. La constatación de que las especies difirieron en el uso del N y sus formas químicas, y que dicho uso tuviera implicaciones para el crecimiento, indica que las especies forestales mediterráneas presentan nichos fundamentales diferenciados en base al N. Por tanto, concluimos que el N debe desempañar un papel importante en el funcionamiento y la estructura de comunidades forestales mediterráneas.

General conclusions

Total N uptake rate by roots was higher in the herb and the chamaephytes than in the shrubs and trees. Root structure and assimilation capacity explained interspecific differences. Species having higher specific root length and nitrate reductase activity had higher total N uptake rates. Neither the relative growth rate nor the leaf N concentration were related to N uptake rates.

All the species absorbed intact glycine and inorganic N (NH_4^+ and NO_3^-) by roots. Intact glycine uptake capacity was related to the colonization with vesicular arbuscular mycorrhizae, though no relationship was found with inorganic N uptake. Colonization of ectomycorrhizae was very low and I could not detect any effect on the absorption of any N form.

Most species preferred NH_4^+ as a N source at the expense of NO_3^-, *i.e.* NH_4^+ was proportionally absorbed over its relative abundance in the soil. Glycine preference showed no clear pattern among species.

Pioneer species had higher preference for NH_4^+ than mid–late successional species. Among pioneer species, total N uptake rates and NH_4^+ preference showed notable differences. Similarly, mid to late successional species only differed in their N form preference; while dominant trees preferred NH_4^+, subordinate shrubs preferred glycine or NO_3^-.

Quercus ilex and *Pinus halepensis* absorbed through leaves both inorganic and organic N, including intact glycine. *Quercus ilex* had higher foliar N absorption than *P. halepensis*, which were explained by higher interception and retention of solutions in *Q. ilex* and probably due to higher stomatal density and presence of trichomes.

Foliar absorption was highest for urea, followed by NH_4^+, NO_3^- and glycine. Differences among N forms were explained by differences in physico-chemical properties. An increase in cuticule permeability to water increased foliar absorption of N sources, except in NO_3^-.

Mediterranean evergreen species were able to assimilate and store C under winter conditions. Carbon assimilated under winter conditions was partitioned throughout the plant depending upon the size of the plant organs. Thus,

Quercus species allocated winter C to coarse roots, while *Olea europaea* and *P. halepensis* allocated it into shoots. However, leaves were, in general, priority storage sites because they contained more winter C than expected by their size.

In early spring, remobilization was the main N source for new fine root growth. By mid spring, soil and photosynthesis supplied most N and C, respectively, for growth of new fine root.

Species used differently N and C reserves for growth of new shoots. *Quercus* species mainly used recently assimilated N and C, while *O. europaea* and especially *P. halepensis* used N and C reserves for new shoots growth.

Old leaves were the main source of C and N source for metabolism and new growth in spring as they showed the greatest reduction in C and N and, in most cases, the reduction was proportionally greater than expected by their size.

Inorganic N forms at high N concentration affected plant the performance of *Q. ilex* and *P. halepensis*. However, N forms had low effects at low concentration. The pine, a pioneer tree improved performance with NO_3^-, had strong plasticity to changes in N supply and experienced less toxicity to NH_4^+. The oak, a late successional tree had low responsiveness to N form or concentration and showed higher toxicity to NH_4^+.

Foliar fertilization with amino acids had low effect on plant performance. It slightly increased photosynthesis and the proportion of new fine roots but did not increase either growth or nutrient concentration.

The N use strategy seems to determine the relative growth rate in plants. Fast-growing species used N reserves to a greater extent for new growth and preferred NH_4^+ as an N source. However, the relative growth rates was not related to N absorption rate.

This Thesis evidences that N should not be considered as a single resource but as multiple sources constituted by its different chemical forms, and that organic N can be an important N source in Mediterranean ecosystems. The statement that species used differently N (forms) and that this use had implication for growth, indicates that Mediterranean forest species have different N-based fundamental niche. Thus, I conclude that N is an important driver for the functioning and community structure in Mediterranean ecosystems.

Appendix

CURRICULUM VITAE

Mª MERCEDES USCOLA FERNÁNDEZ

Julio 2013

Apellidos: USCOLA FERNANDEZ Nombre: Mª MERCEDES
D.N.I.: 53438094-D Fecha de nacimiento: 15/08/1979 Sexo: MUJER

Situación profesional actual

Organismo: Universidad de Alcalá
Facultad, Escuela o Instituto: Facultad de Ciencias
Depto./Secc./Unidad: Departamento de Ciencias, Unidad Docente de Ecología
Dirección postal: Carretera N-II, km. 33.500, Alcalá de Henares, 28805
Teléfono: +34 91 885 64 08 Fax: +34 91 885 49 29
Correo electrónico: mercedes.uscola@uah.es
Categoría profesional: personal de investigación en formación (FPU)
Fecha de inicio: 03/09/2008

Formación académica

Titulación Superior	Centro	Fecha
Ldo. Ciencias Ambientales	Universidad de Alcalá	30/02/2005
M.O. Restauración de ecosistemas	Universidad de Alcalá	31/02/2009

Doctorado	Centro	Fecha
FASE DOCENCIA	Universidad de Alcalá	31/06/2007
Trabajo Investigación Tutelado	Universidad de Alcalá	14/06/2008
Diploma de Estudios Avanzados	Universidad de Alcalá	28/10/2009

Actividades anteriores de carácter científico profesional

Puesto	Institución	Fechas
Personal investigador	Universidad de Alcalá	01/06/08-02/09/08
Profesora FP (producción forestal)	APHISA	15/09/06-15/09/07
Coordinadora de voluntariado en Parques Nacionales	ACA-TRAGSA	05/09/07-20/09/07 05/11/07-20/09/07
Técnico al Desarrollo del proyecto	TECNIBERIA, asociación de empresas de ingeniería y consultaría	01/04/06-31/07/06
Profesora	CEECO	2003-2004

Idiomas (R = regular, B = bien, C = correctamente)

Idioma	Habla	Lee	Escribe
Inglés	C	C	C
Francés	B	B	B

Participación en Proyectos de I+D financiados en Convocatorias públicas.

Título del proyecto: *Tolerancia y estrategias ecofisiológicas de los pinos ibéricos durante la fase juvenil en respuesta al estrés hídrico, las bajas temperaturas y la disponibilidad de nutrientes* (AGL2011-24296/FOR)

Entidad financiadora: Ministerio de Ciencia e Innovación (MICIIN)

Entidades participantes: Universidad de Alcalá, Universidad Politécnica de Madrid, Universidad de Granada, IRTA, Purdue University

Duración, desde: Enero 2012 hasta: Diciembre 2014

Investigador principal: Pedro Villar-Salvador

Número de investigadores participantes: 6

Título del proyecto: *Programa de actividades de I+D de la Comunidad de Madrid sobre restauración del medio natural REMEDINAL 2* (S2009/AMB/1783)

Entidad financiadora: Comunidad de Madrid

Entidades participantes: Universidad Rey Juan Carlos, Universidad Complutense de Madrid, Universidad Autónoma de Madrid, Universidad de Alcalá, Universidad Politécnica de Madrid, INIA, Museo Natural de Ciencias Naturales (CSIC)

Duración, desde: Enero 2010 hasta: Enero 2013

Investigador principal: José M. Rey Benayas (Adrián Escudero es el Coordinador de la Red)

Número de investigadores participantes: 8 (en la Universidad de Alcalá)

Título del proyecto: *Efectos de la revegetación (pasiva y activa) en la dinámica y diversidad de plantas leñosas y aves en paisajes agrarios* (CGL2007-60533/BOS)

Entidad financiadora: Ministerio de Ciencia y Tecnología (CICYT)

Entidades participantes: Universidad de Alcalá

Duración, desde: Septiembre 2007 hasta: Septiembre 2010

Investigador principal: José Mª Rey Benayas

Número de investigadores participantes: 12

Título del proyecto: *Optimización de la nutrición de encina (Quercus ilex L.) en vivero: bases fisiológicas, técnicas de fertilización e implicaciones post-trasplante* (AGL2006-12609-C02-01/FOR)

Entidad financiadora: Ministerio de Ciencia y Tecnología (CICYT)

Entidades participantes: Universidad de Alcalá, Universidad de Alicante, Universidad de Córdoba, Universidad de Huelva, Centro Nacional de Mejora Forestal "el Serranillo" y CEAM

Duración, desde: Diciembre 2006 hasta: Diciembre 2009

Investigador responsable: Juan Oliet Palá

Número de investigadores participantes: 11

Título del proyecto: *Programa de actividades de I+D de la Comunidad de Madrid sobre restauración del medio natural REMEDINAL* (S-0505/AMB/0355)

Entidad financiadora: Comunidad de Madrid

Entidades participantes: Universidad Rey Juan Carlos, Universidad Complutense de Madrid, Universidad Autónoma de Madrid, Universidad de Alcalá, Universidad Politécnica de Madrid, INIA, Museo Natural de Ciencias Naturales (CSIC).

Duración, desde: Enero 2006 hasta: Enero 2009

Investigador responsable: José M. Rey Benayas (Adrián Escudero Coordinador de la Red)

Número de investigadores participantes: 10 (en la Universidad de Alcalá)

Publicaciones o Documentos Científico-Técnicos

(CLAVE: L = libro completo, CL = capítulo de libro, A = artículo, R = "review", E = editor, S = Documento Científico-Técnico restringido.)

Artículos en revistas internacionales

Autores (p.o. de firma): **M. Uscola**, P. Villar-Salvador, P. Gross, P. Maillard

Título: *Growth capacity determines the contribution of carbon and nitrogen remobilization to seedling growth in Mediterranean evergreen woody plants.*

Ref. revista: New Phytologist (submitted)

Clave: A Volumen Páginas: Fecha: 2013

Autores (p.o. de firma): **M. Uscola**, P. Villar-Salvador, J.A. Oliet Palá, C.R. Warren.

Título: *Foliar absorption and root dynamics translocation of nitrogen sources in seedlings of two Mediterranean trees*

Ref. revista: Tree Physiology (submitted)

Clave: A Volumen: Páginas: Fecha: 2013

Autores (p.o. de firma): **M. Uscola**, J.A. Oliet, P. Villar-Salvador, E. Díaz-Pinés, D.F. Jacobs

Título: *Nitrogen form and concentration interactively affect the performance of two ecologically distinct Mediterranean forest trees*

Ref. revista: European Journal of Forest Research (en 2ª revisión)

Clave: A Volumen: Páginas: Fecha: 2013

Autores (p.o. de firma): P. Villar-Salvador, J. Puertolas, B. Cuesta, J.L. Peñuelas, **M. Uscola,** N. Heredia, J.M.Rey-Benayas

Título: *Increase in size and nitrogen concentration enhances seedling survival in Mediterranean plantations. Insights from an ecophysiological conceptual model of plant survival*

Ref. revista: New Forests

Clave: A Volumen: 43 Páginas: 755- 770 Fecha: 2012

Capítulos de libro (Libros científico-técnicos)

Autores (p.o. de firma): P. Villar-Salvador, **M. Uscola,** N. Heredia

Título: *Quercus coccifera* L. (Coscoja)

Libro: Producción y manejo de semillas y plantas forestales.

Clave: CL Volumen: en prensa Páginas: 192-205 Fecha: 2013

Editorial (si libro): Ministerio de Agricultura, Alimentación y Medio Ambiente. J. Pemán (Ed. Ppal), R. Navarro, A. Prada, R. Serrada, J.L. Nicolás (eds).

Lugar de publicación: Madrid

Autores (p.o. de firma): P. Villar-Salvador, J.L. Nicolas Peragón, N. Heredia, **M. Uscola**

Título: *Quercus ilex* L. (encina)

Libro: Producción y manejo de semillas y plantas forestales

Clave: CL Volumen: en prensa Páginas: 226-249 Fecha: 2013

Editorial (si libro): Ministerio de Agricultura, Alimentación y Medio Ambiente. J. Pemán (Ed. Ppal), R. Navarro, A. Prada, R. Serrada, J.L. Nicolás (Eds).

Lugar de publicación: Madrid

Autores (p.o. de firma): P. Villar-Salvador, J. Oliet, N. Heredia, **M. Uscola,** P. Goikoetxea

Título: *Quercus faginea* Lam., *Quercus humilis* Mill.

Libro: Producción y manejo de semillas y plantas forestales

Clave: CL Volumen: en prensa Páginas: 206-225 Fecha: 2013

Editorial (si libro): Ministerio de Agricultura, Alimentación y Medio Ambiente. J. Pemán (Ed. Ppal), R. Navarro, A. Prada, R. Serrada, J.L. Nicolás (eds).

Lugar de publicación: Madrid

Capítulos de libro (que pertenecen a contribuciones a congresos)

Autores (p.o. de firma): **M. Uscola**, P. Villar-Salvador, J.A. Oliet, C. Warren

Título: *Eficacia de la fertilización foliar con diferentes fuentes de nitrógeno en la sobrecarga de plántulas de P. halepensis Mill. y Q. ilex L.*

Libro: Avances en la restauración de sistemas forestales: técnicas de implantación. C. Martínez Ruiz, F.J. Laricio Leza, B. Fernández Santos (Eds.)

Clave: CL Páginas: 121-127 Fecha: 2013

Editorial (si libro): SECF-AEET

Lugar de publicación: Madrid

Autores (p.o. de firma): **M. Uscola,** Villar-Salvador, P., Heredia, N.

Título: *Efecto de la fertilización edáfica con nitrato y amonio y la fertilización foliar con aminoácidos sobre el crecimiento de dos especies forestales mediterráneas, Quercus ilex y Pinus halepensis.*

Libro: Presente y Futuro de la Nutrición Mineral de las Plantas.

Clave: CL Páginas: 109-120 Fecha: 2008

Editorial (si libro): Universidad de Granada

Lugar de publicación: Granada

Autores (p.o. de firma): N. Heredia, Villar-Salvador, P., P. Millard, Cuadrado , **M. Uscola.**

Título: *La disponibilidad de nitrógeno en el suelo no influye en la cantidad de nitrógeno removilizado de la bellota en las primeras etapas de desarrollo de las plántulas de Quercus ilex.*

Libro: Presente y Futuro de la Nutrición Mineral de las Plantas.

Clave: CL Volumen: Páginas: 121-134 Fecha: 2008

Editorial (si libro): Universidad de Granada

Lugar de publicación: Granada

Estancias en Centros extranjeros

(estancias continuadas superiores a un mes)

CLAVE: D = doctorado, P = postdoctoral, I = invitado, C = contratado, O = otras (especificar).

Centro: Department of Forestry and Natural Resources, Purdue University

Localidad: Lafayette Indiana País: Estados Unidos de América

Fecha: SEPTIEMBRE-OCTUBRE 2011 Duración: 8 (semanas)

Tema: N concentration and N forms effects on growth and physiology of two Mediterranean species

Clave: D

Centro: School of Biological Sciences, University of Sydney

Localidad: Sydney País: Australia

Fecha: ENERO-MARZO 2011 Duración: 12 (semanas)

Tema: Preferencia por distintas fuentes de N en ecosistemas mediterráneos calizos de alta montaña (estudio en 9 especies)

Clave: D

Centro: INRA Institute Nationale de Recherche Agroforestiere

Localidad: Nancy-Champenoux País: Francia

Fecha: JUNIO-SEPTIEMBRE 2009 Duración: 18 (semanas)

Tema: Evaluación de la utilización de las reservas en el nuevo crecimiento (radical y aéreo) de cuatro especies mediterráneas (*Pinus halepensis, Quercus ilex, Quercus coccifera y Olea europaea var. sylvestris*)

Clave: D

Centro: centro de Mejoramiento Genético y Banco de Semillas Forestal. UNAM

Localidad: LEON País: NICARAGUA

Fecha: JUNIO-SEPTIEMBRE 2004 Duración: 12 (semanas)

Tema: Evaluación y difusión de germoplasmas mejorados de musa

Clave: O, beca de colaboración

Contribuciones a Congresos

Autores: **M. Uscola**, P. Villar Salvador, C. Warren, J. A. Oliet Palá

Título: *Eficacia de la fertilización foliar con diferentes fuentes de nitrógeno en la sobrecarga de plántulas de P. halepensis Mill. y Q. ilex L.*

Tipo de participación: comunicación oral

Congreso: II Reunión Conjunta AEET-SECF 2012.

Publicación: cuadernos SECF

Lugar de celebración: Palencia Fecha: Noviembre / 2012

Autores: **M. Uscola**, J.A. Oliet-Palá, P. Villar-Salvador, E. Díaz Pines, D.F. Jacobs

Título: *Respuesta funcional de las plántulas de Quercus ilex y Pinus halepensis al cultivo con diferentes fuentes de nitrógeno*

Tipo de participación: poster

Congreso: III Jornadas Técnicas de los Jardines Botánicos: hacia la sostenibilidad

Publicación: actas de las jornadas

Lugar de celebración: Real Jardín Botánico Juan Carlos I Fecha: Junio / 2012

Autores: **M. Uscola**, P. Villar-Salvador, J.A. Oliet-Palá

Título: *Absorción foliar y radical de compuestos de nitrógeno inorgánicos y orgánicos en dos especies arbóreas mediterráneas*

Tipo de participación: poster

Congreso: III Jornadas Técnicas de los Jardines Botánicos: hacia la sostenibilidad

Publicación: actas de las jornadas

Lugar de celebración: Real Jardín Botánico Juan Carlos I Fecha: Junio / 2012

Autores: **M. Uscola**, P. Villar-Salvador, P. Maillard

Título: *Las especies mediterráneas leñosas perennes usan de forma distinta las reservas de carbono y nitrógeno para el crecimiento de nuevos órganos (brotes y raíces)*

Tipo de participación: comunicación oral

Congreso: 2º Maratón Científico INIA-CIFOR

Publicación: actas de las jornadas

Lugar de celebración: Madrid Fecha: Julio / 2012

Autores: **M. Uscola**, P. Villar-Salvador, P. Maillard

Título: *Mediterranean evergreen woody species use differently carbon and nitrogen reserves for leaf and root growth*

Tipo de participación: poster

Congreso: 12th EEF Congress

Publicación: actas de las jornadas

Lugar de celebración: Ávila Fecha: Septiembre / 2011

Autores: N. Heredia, **M. Uscola**, P. Villar-Salvador, J. Oliet

Título: *Factores que determinan el uso de las reservas de N para el crecimiento de nuevos órganos en plantas leñosas mediterráneas*

Tipo de participación: comunicación oral

Congreso: I Reunión de la Red Española de Isótopos Estables

Publicación: actas de las jornadas

Lugar de celebración: Toledo Fecha: Junio / 2010

Autores: **M. Uscola**; P. Villar-Salvador; J.A. Oliet-Pala

Título: *Absorción radical y foliar de fuentes de nitrógeno inorgánico y orgánico en Pinus halepensis y Quercus ilex*

Tipo de participación: comunicación oral

Congreso: IX congreso de la AEET Reunión científica de la Asociación Española de Ecología Terrestre

Publicación: actas de las jornadas

Lugar de celebración: Jaén Fecha: Octubre / 2009

Autores**: M. Uscola**, P. Villar-Salvador, J. Oliet-Palá

Título: *Foliar and root uptake of inorganic and organic N sources in two Mediterranean tree species*

Tipo de participación: poster

Congreso: Second mini symposium on the use of stable isotopes in tree physiology and forest ecology

Publicación: actas de las jornadas

Lugar de celebración: Nancy Fecha: Julio / 2009

Autores: **M. Uscola,** P. Villar-Salvador, N. Heredia.

Título: *Efecto de la fertilización edáfica con nitrato y amonio y la fertilización foliar sobre el crecimiento de dos especies forestales mediterráneas Quercus ilex y Pinus halepensis*

Tipo de participación: comunicación oral

Congreso: XII Simposio Ibérico Sobre Nutrición Mineral de las Plantas. Nutriplant

Publicación: actas de las jornadas

Lugar de celebración: Granada Fecha: Octubre / 2008

Autores: N. Heredia, P. Villar-Salvador, P. Millard, J. Cuadrado, **M. Uscola**

Título: *La disponibilidad de nitrógeno en el suelo no influye en la cantidad de nitrógeno removilizado de la bellota en las primeras etapas de desarrollo de las plántulas de Quercus ilex.*

Tipo de participación: comunicación oral

Congreso: XII Simposio Ibérico Sobre Nutrición Mineral de las Plantas. Nutriplant

Publicación: actas de las jornadas

Lugar de celebración: Granada Fecha: Octubre / 2008

Cursos y seminarios recibidos

- Seminario "**Introducción al método comparado**". Alcalá de Henares del 28 de junio al 2 de julio de 2011. Organizado por el CSIC, Universidad de Valencia y Universidad de Alcalá e impartido por el Dr. Miguel Verdú del Campo (10 horas).
- Seminario "**Introducción al diseño de experimentos y los modelos lineales mixtos**". Alcalá de Henares del 15 al 24 de junio de 2010. Organizado por la Universidad de Granada y la Universidad de Alcalá e impartido por el Dr. Luis Cayuela Delgado (20horas).
- Seminario "**Análisis de datos ecológicos en R**". Alcalá de Henares del 5 al 13 de mayo 2009. Organizado por la Universidad de Alcalá e impartido por el Dr. Luís Cayuela Delgado (20horas).
- XII Seminario de Conservación de la Naturaleza. "**Conservación y Gestión de los bosques en la Comunidad de Madrid**". Soto del Real 25 y 26 de junio de 2008. Organizado por: Centro de Investigaciones Ambientales de la Comunidad de Madrid (CIAM), Fundación Biodiversidad (FIDA), Universidad Complutense de Madrid, Universidad Autónoma de Madrid, y Universidad de Alcalá (20 horas).
- Congreso Nacional sobre "**Retos y nuevas perspectivas en la revegetación de sistemas forestales**" I Reunión conjunta del Grupo de Trabajo de Repoblaciones Forestales (SECF) y el Grupo de Trabajo de Restauración Ecológica (AEET) IV Reunión del Grupo de Trabajo de Repoblaciones Forestales (SECF). Alcalá de Henares, 21-23 de Noviembre de 2007. Organizado por Universidad de Alcalá, SECF y AEET (30 horas).
- Seminario sobre **Gestión Ambiental Aplicada** al Ámbito de Actividades de las Fuerzas Armadas, orientado a graduados civiles. Alcalá de Henares 26-30 de Junio de 2006. Organizado por Universidad de Alcalá, TRAGSATEC y Ministerio de Defensa (40 horas).
- Seminario Avanzado de **Planificación y Gestión de Residuos** en el marco de un Desarrollo sostenible. Alcalá de Henares 5-15 de Junio de 2006. Organizado por AECI, Ministerio de Medio Ambiente y Universidad de Alcalá (40 horas).
- Seminario avanzado de **gestión integral de cuencas hidrológicas** en el contexto del Mediterráneo. Contribución al cumplimiento de los Objetivos de Desarrollo del Milenio (ODM) Alcalá de Henares 18-22 de Mayo de 2006. Organizado por AECI, y Ministerio de Medio Ambiente (40 horas).

EXPERIENCIA DOCENTE

Horas de docencia

Se especifica asignatura, titulación a la que pertenece, curso y número de horas impartidas

- Revegetación, Máster Oficial de Restauración de Ecosistemas, 2012-2013, (12 horas)
- Ecología y bienestar humano, Grado Biología sanitaria, 2011-2012, (36 horas)
- Fundamentos de ecología aplicada, Licenciatura de Biología, 2011-2012, (10 horas)
- Revegetación, Máster Oficial de Restauración de Ecosistemas, 2011-2012, (12 horas)
- Revegetación, Máster Oficial de Restauración de Ecosistemas, 2010-2011, (10 horas)
- Fundamentos de Ecología, Licenciatura de Biología 2010-2011, (10 horas)
- Técnicas aplicadas al trabajo de campo, Grado de Ambientales 2010-2011, (24 horas)

Proyectos dirigidos

Se especifica tipo de proyecto, convocatoria de presentación, titulo, alumno y calificación.

- **Proyecto Fin de Grado** de Ambientales septiembre 2012 "*Absorción foliar y translocación a raíces de fuentes de nitrógeno en plántulas de Pinus halepensis Mill. y Quercus ilex L.*" Karla Brighitt Quispe Clemente.
  Calificación: 9.7 (Sobresaliente)
- **Proyecto Fin de Máster** de Restauración de ecosistemas septiembre de 2012 "*Informe sobre la restauración del área del retiro del rio Medellín en la mina de canteras de Girardota, Colombia*" Diana Cuevas Correa.
  Calificación: 9.3 (Sobresaliente)

Seminarios impartidos

- Octubre 2011, Purdue University "*Ecophysiology of nitrogen in Mediterranean plants: strategies of absorption, use of reserves for growth and functional responses*"
- Febrero 2010, Universidad de Alcalá "*Absorción por las raíces y las hojas de diferentes fuentes de N en plántulas de Quercus ilex y Pinus halepensis*"
- Enero 2012, Universidad de Alcalá "*Metodología para la determinación de masa microbiana en suelos*"

GRANDES EQUIPOS QUE UTILIZA O HA UTILIZADO

CLAVE : R= responsable, UA = usuario asiduo, UO = usuario ocasional

EQUIPO: Medidor portátil de contenido de clorofila (chlorophyll content meter CCM-200, Opti-Science, USA)

FECHA:2008 CLAVE:UO

EQUIPO: Molino de bolas (PM100, Retsch, Haan, Germany)

FECHA:2008 CLAVE:UA

EQUIPO: Digestor (Foss tecator DS-40, FOSS, Denmark)

FECHA:2008 CLAVE:UO

EQUIPO: IRGA analizador de gases por infrarrojos (leaf chamber analyser LCA-4, ADC BioScientific Ltd, England)

FECHA:2008 CLAVE:UO

EQUIPO: Conductímetro (conductivity meter 524, CRISON, Spain)

(EC-Meter Basic 30+, CRISON, España)

FECHA:2008 CLAVE:UA

EQUIPO: pHímetro (micropH 2001, CRISON, Spain)

FECHA:2008 CLAVE:UA

EQUIPO: Analizador de imágenes digital (DIAS 1.12, Delta-T Devices LTD, England)

FECHA:2008 CLAVE:UA

EQUIPO: Espectrofotómetro (UV-1800, Shimadzu corporation, Japan)

FECHA:2009 CLAVE:UA

EQUIPO: Bomba de vacio (R-300, BOECO, Alemania)

FECHA:2009 CLAVE:UA

EQUIPO: Destilador (Kjeltec^{TM} 2100, FOSS, Dinamarca)

FECHA:2009 CLAVE:UA

EQUIPO: Titulador (702 titrino, Methrohm, Suiza)

FECHA:2009 CLAVE:UA

Técnicas analíticas que emplea

- Extracción y determinación de pigmentos fotosintéticos (clorofilas, carotenoides y xantófilas) con DMSO y espectrofotometría.
- Determinación de NO_3^-, NH_4^+ y N total en extracto de saturación del suelo por destilación con ácido bórico y titulación con H_2SO_4.
- Determinación de la concentración de N, P y K en tejidos vegetales por digestión ácida.
- Medición de intercambio gaseoso en plantas.
- Utilización de isotopos estables como biomarcadores en la determinación de flujos de elementos en plantas.
- Medición de ^{15}N en biomasa microbiana por el método de fumigación extracción de Vance y captura del nitrógeno en trampas ácidas.
- Determinación de colonización ectomicorrízica.
- Tinción de endomicorrizas por el método de azul Tripan y cuantificación de colonización por el método de Trouvelot.