Universitat de Lleida

Water use patterns and physiological interactions in Submediterranean mixed forests: methodological challenges and implications for ecohydrology

Paula Martín Gómez

http://hdl.handle.net/10803/405511

ADVERTIMENT. L'accés als continguts d'aquesta tesi doctoral i la seva utilització ha de respectar els drets de la persona autora. Pot ser utilitzada per a consulta o estudi personal, així com en activitats o materials d'investigació i docència en els termes establerts a l'art. 32 del Text Refós de la Llei de Propietat Intel·lectual (RDL 1/1996). Per altres utilitzacions es requereix l'autorització prèvia i expressa de la persona autora. En qualsevol cas, en la utilització dels seus continguts caldrà indicar de forma clara el nom i cognoms de la persona autora i el títol de la tesi doctoral. No s'autoritza la seva reproducció o altres formes d'explotació efectuades amb finalitats de lucre ni la seva comunicació pública des d'un lloc aliè al servei TDX. Tampoc s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX (framing). Aquesta reserva de drets afecta tant als continguts de la tesi com als seus resums i índexs.

ADVERTENCIA. El acceso a los contenidos de esta tesis doctoral y su utilización debe respetar los derechos de la persona autora. Puede ser utilizada para consulta o estudio personal, así como en actividades o materiales de investigación y docencia en los términos establecidos en el art. 32 del Texto Refundido de la Ley de Propiedad Intelectual (RDL 1/1996). Para otros usos se requiere la autorización previa y expresa de la persona autora. En cualquier caso, en la utilización de sus contenidos se deberá indicar de forma clara el nombre y apellidos de la persona autora y el título de la tesis doctoral. No se autoriza su reproducción u otras formas de explotación efectuadas con fines lucrativos ni su comunicación pública desde un sitio ajeno al servicio TDR. Tampoco se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR (framing). Esta reserva de derechos afecta tanto al contenido de la tesis como a sus resúmenes e índices.

WARNING. Access to the contents of this doctoral thesis and its use must respect the rights of the author. It can be used for reference or private study, as well as research and learning activities or materials in the terms established by the 32nd article of the Spanish Consolidated Copyright Act (RDL 1/1996). Express and previous authorization of the author is required for any other uses. In any case, when using its content, full name of the author and title of the thesis must be clearly indicated. Reproduction or other forms of for profit use or public communication from outside TDX service is not allowed. Presentation of its content in a window or frame external to TDX (framing) is not authorized either. These rights affect both the content of the thesis and its abstracts and indexes.

Universitat de Lleida

PHD THESIS

Water use patterns and physiological interactions in submediterranean mixed forests: methodological challenges and implications for ecohydrology

Dissertation to obtain the degree of Doctor by

Paula Martín Gómez

Directors
Juan Pedro Ferrio Díaz
Jesús Pemán García

2017

Martín-Gómez, P (2017). PhD thesis. Multifunctional Forest Management Program

This PhD thesis has been carried out at the Laboratory of Silviculture, Departament of Crop and Forest Sciences, University of Lleida, Spain. The author was funded by a PhD FPU fellowship from the Spanish Government (FPU12/00648). The research was supported by the Spanish Government through projects AGL 2012-40039-C02 and AGL 2012 -40151-C03.

Cover design: Lucía Martín. Drawing: Youness El Karkri

Al Boalar, donde nació esta tesis
A Pitter y Youness, por caminar conmigo
A Amín, el mejor de los finales

¡Árboles!
¿Habéis sido flechas
caídas del azul?
¿Qué terribles guerreros os lanzaron?
¿Han sido las estrellas?

Vuestras músicas vienen del alma de los pájaros,
de los ojos de Dios,
de la pasión perfecta.

¡Árboles!
¿Conocerán vuestras raíces toscas
mi corazón en tierra?

Federico García Lorca

TABLE OF CONTENTS

List of publications

This thesis is based on the work contained in the following papers:

I. Martín-Gómez P, Barbeta A, Voltas J, Peñuelas J, Dennis K, Palacio S, Dawson TE, Ferrio JP (2015) Isotope-ratio infrared spectroscopy: a reliable tool for the investigation of plant-water sources? New Phytol 207:914–927, doi: 10.1111/nph.13376.

II. Martín-Gómez P, Serrano L, Ferrio JP (2016) Short-term dynamics of evaporative enrichment of xylem water in woody stems: implications for ecohydrology. Tree Physiol, doi: 10.1093/treephys/tpw115.

III. Martín-Gómez P, Aguilera M. Pemán J, Gil-Pelegrín E, Ferrio JP (2017) Contrasting ecophysiological strategies related to drought: the case of a mixed stand of Scots pine (*Pinus sylvestris*) and a submediterranean oak (*Quercus subpyrenaica*). Under review in Tree Physiology.

IV. Martín-Gómez P, Rodríguez-Robles U, Ferrio JP (2017) Soil- or plant-driven fractionation cause isotopic uncoupling between soil and xylem water during drought and recovery. Manuscript.

SUMMARY

Submediterranean forests are considered an ecotone between Mediterranean and Eurosiberian ecosystems. These transition zones are very sensitive to global change and models on future climatic scenarios predict dramatic changes in their species composition. Indeed, a decline of Scots pine (*Pinus sylvestris*) and a related expansion of *Quercus* species have been already reported in the Spanish Pre-Pyrenees. In this thesis, with the aim of bringing insight into the physiological mechanisms anticipating this selective decline, we studied the physiological behaviour and water use patterns of Scots pine and marcescent submediterranean oaks (*Q.subpyrenaica* and *Q.faginea*) in relation to drought under field and laboratory conditions. We based our work on water stable isotopes as ecological tracers, in combination with other physiological tools (water potential, tree sap flow and leaf gas exchange) and more integrative measures (tree-ring width and remote sensing). Our preliminary results pointed towards to a general uncoupling of soil water isotopic composition in respect to precipitation and irrigation water and also to xylem water.

Thereof, an assessment of the processes behind this uncoupling was needed in order to guarantee that the observed variance in isotopic results between species was truly caused by differences in ecohydrological behaviour, rather than an accumulation of fractionation effects. In this regard, we analysed the interference of xylem and soil organic contaminants (methanol, ethanol) on water isotopic composition analysed with Isotope-Ratio Infrared Spectroscopy (IRIS). We proved that the post-processing correction designed by Picarro, together with the sample pre-treatment through the Micro-Combustion Module (MCM), improved significantly the performance of IRIS in soil and xylem samples when the main (and mostly unique) contaminant detected was methanol. Otherwise, if there were other contaminants such as ethanol only the post-processing method showed acceptable results. Furthermore, in our experiments, we reported evidence of fast and reversible evaporative enrichment in metabolically active stems as a consequence of a temporal decline in sap flow rates. Hence, xylem water isotopic composition did not only reflect source water, but also stem hydraulic processes. In addition, despite we confirmed a generalized divergence between precipitation/irrigation and soil water in the field and under controlled conditions, which pointed towards the existence of non-well mixed pools in the soil, we could not straightforward associate the changes in xylem to it, due to the confounding effect of evaporative enrichment during drought cycles. In this sense, we proposed that the “two water worlds” hypothesis is over-simplified in field conditions and should be replaced by a “high complexity of soil water pools”.

Finally, once the methodological frame was established and the fractionation effects in isotopic water composition were taken into account, we evaluated the main reasons to the pine-oak differential decline. In this sense, we found contrasting ecohydrological strategies and ability to cope with drought between the studied species. The marcescent oaks were able to maintain transpiration and physiological activity under drier soil conditions than Scots pine. This conservative strategy relied on the access to more stable soil water pools (groundwater, deep soil layers or tightly-bound water) in comparison to pines, which developed a more opportunistic strategy of fast use of precipitation water, based in water uptake of top soil layers water. In addition to hydraulic constraint in pines, stomatal limitations to photosynthesis reduced their physiological activity during the first stages of drought, determining negative consequences in carbon assimilation and storage. In our pot experiments, pines recovered faster after drought due to their tight stomatal control. However, under field conditions with longer and more severe drought periods, the greater vulnerability to xylem embolism in pines led to more limited recovery of transpiration flow. Differentially, oaks recovered faster, being benefited from a deep root system. On this account, inter-specific differences in the short-term response to drought were translated into consequences in the long term performance; pines showed higher variations in tree growth and far more pronounced decreasing trend with time, indicating that increasing drought events in the near future may induce long-term physiological decay in pines growing in the submediterranean ecotone.

RESUMEN

Los bosques submediterráneos se consideran un ecotono entre los ecosistemas mediterráneos y eurosiberianos. Por ser zonas de transición son muy sensibles al cambio global, y los modelos de cambio climático predicen en el futuro modificaciones dramáticas en su composición específica. De hecho, en el Prepirineo español, ya puede observarse en la actualidad un declive del pino silvestre (*Pinus sylvestris*), asociado a una expansión de especies del género *Quercus*. En esta tesis, con objeto de elucidar los mecanismos fisiológicos detrás de este declive selectivo, hemos estudiado a partir de muestreos de campo y experimentos de laboratorio, el comportamiento fisiológico y los patrones de captación de agua del pino silvestre y los robles marcescentes submediterráneos (*Quercus subpyrenaica* y *Quercus faginea)* en relación a la sequía. La metodología empleada se basa principalmente en los isótopos estables como trazadores ecológicos, acompañados de otras herramientas fisiológicas (potencial hídrico, flujo de savia e intercambio gaseoso) y medidas más integrativas (estudio de los anillos de crecimiento y sensores remotos). Los resultados preliminares de esta tesis apuntaron hacia la existencia de un desacople general en términos de composición isotópica entre el agua de suelo y el agua de precipitación (o riego), así como entre el suelo y el agua del xilema.

Bajo esta condición, en primer lugar, era necesario caracterizar el desacople para poder garantizar que la variación de los resultados isotópicos entre especies se debía realmente a diferencias en su comportamiento ecohidrológico, y no a una acumulación de los efectos de fraccionamiento. En este sentido, analizamos la interferencia de los contaminantes orgánicos (metanol y etanol), presentes naturalmente en el suelo y el xilema, sobre la composición isotópica del agua cuando empleamos espectroscopía de infrarrojos (IRIS). En nuestros resultados damos prueba de que la corrección post-procesado de Picarro y el pre-tratamiento de muestras con el módulo de micro combustión (MCM) mejoran significativamente la precisión de los resultados obtenidos con IRIS, siempre y cuando el principal (y casi único) contaminante detectado sea metanol. En cambio, si existen otros contaminantes (etanol) únicamente la corrección post-procesado ofrece resultados aceptables. Además, en nuestros experimentos ofrecemos evidencia de la existencia de un enriquecimiento evaporativo rápido y reversible en ramas metabólicamente activas, como consecuencia de una reducción temporal en las tasas de flujo. De este modo, la composición isotópica del agua del xilema no sólo refleja la fuente de agua, si no también los procesos hidráulicos que ocurren en la planta. Por otro lado, aunque confirmamos un desacople general entre los valores de precipitación/irrigación y suelo en campo y en condiciones experimentales; lo que apunta hacia la hipótesis de la existencia de diferentes fuentes de agua no mezcladas en el suelo, no pudimos asociar por completo los cambios isotópicos observados en el xilema a este hecho, debido a que en momentos de sequía el efecto del

enriquecimiento evaporativo en el xilema ocurría en el mismo sentido que el cambio en la fuente de agua. De acuerdo con esto, proponemos que la hipótesis conocida como “dos mundos de agua en el suelo” sobre-simplifica la realidad y que sería más adecuado emplear una como “la elevada complejidad en las fuentes de agua en el suelo”.

Por último, una vez establecido el marco metodológico y los efectos del fraccionamiento isotópico considerados, evaluamos los principales motivos para el decaimiento diferencial de pinos y robles. En este sentido, los robles marcescentes fueron capaces de mantener su actividad fisiológica y transpiración en condiciones hídricas más limitantes que el pino silvestre. Esta estrategia más conservativa en los robles se basaba en la capacidad de acceso a fuentes de agua más estables (agua subterránea, de profundidades del suelo mayores o fuertemente adherida a las partículas del suelo) en comparación con los pinos; éstos, por el contrario, desarrollaron una estrategia mucho más oportunista de uso rápido del agua de precipitación en la superficie del suelo. En los pinos, a parte de las restricciones hidráulicas, las limitaciones estomáticas hicieron que redujeran su actividad fisiológica desde los primeros estadios de sequía, generando problemas en cuanto a la asimilación y almacenamiento de carbono. En los experimentos en maceta, los pinos fueron capaces de recuperarse antes que los robles, debido a su rápido control estomático. Sin embargo, en condiciones de campo, con periodos de sequía más largos y severos, la mayor vulnerabilidad de los pinos a desarrollar embolismo en el xilema les limitó en la recuperación de la transpiración una vez finalizada la sequía. En cambio, los robles se recuperaron mejor, beneficiados particularmente por un sistema radicular profundo. Además, estas diferencias inter-específicas en respuesta a la sequía se tradujeron en consecuencias a largo plazo; los pinos mostraron mayores variaciones y una reducción más pronunciada en el crecimiento radial con el tiempo, indicando que la predicción del incremento de la sequía en un futuro cercano podría inducir un decaimiento fisiológico a largo plazo, comprometiendo la supervivencia del pino silvestre en el ecotono submediterráneo.

RESUM

Els boscos submediterranis estan considerats com un ecotò entre els ecosistemes mediterranis i eurosiberians. Aquestes zones de transició són molt sensibles al canvi global, així doncs, els models d'escenaris climàtics futurs prediuen modificacions importants en la seva composició específica. En els darrers anys, al Pre-Pirineu espanyol s'ha observat un declivi del pi roig (*Pinus sylvestris*), lligat a l'expansió de les espècies de *Quercus*. Amb l'objectiu d'elucidar els mecanismes fisiològics que anticipen el declivi selectiu, hem estudiat el comportament fisiològic i els patrons de l'ús de l'aigua en relació a la sequera en pi roig i roures marcescents submediterranis (*Quercus subpyrenaica* i *Quercus faginea)*, tant en experiments de laboratori com en condicions de camp. La metodologia utilitzada es basa, principalment, en la anàlisi d'isòtops estables d'aigua, en combinació amb altres eines fisiològiques (potencial hídric, flux de saba i intercanvi de gasos), així com tècniques més integratives (anells de creixement de la fusta i dades de sensors remots). Els resultats preliminars d'aquesta tesi van mostrar un desacoblament general entre la composició isotòpica de l'aigua de precipitació (o reg) i el sòl, així com entre el sòl i l'aigua del xilema.

Tot això evidenciava la necessitat de caracteritzar el desacoblament isotòpic detectat, per tal de garantir que els resultats observats es deuen a diferències en el comportament ecohidrològic entre espècies i no pas a una acumulació d'efectes de fraccionament. En aquest sentit, es va analitzar la interferència que els contaminants orgànics (metanol i etanol) presents a les mostres de sòl i xilema poden exercir sobre la composició isotòpica de l'aigua quan utilitzem analitzadors isotòpics d'espectroscòpia d'infraroig (IRIS). Es va trobar que la correcció post-processat realitzada per Picarro i el pretractament de les mostres amb el mòdul de micro-combustió (MCM) milloraven significativament la precisió dels resultats obtinguts amb IRIS, sempre i quan el principal (i gairebé únic) contaminant detectat fos el metanol. En canvi, en el cas d'existir altres contaminants com l'etanol, únicament la correcció post-processat oferia resultats acceptables. A més a més, els nostres experiments evidenciaven l'existència d'un enriquiment evaporatiu ràpid i reversible com a conseqüència d'un descens temporal en les taxes de flux de saba. D'aquesta manera, la composició isotòpica de l'aigua del xilema no tan sols reflecteix la font d'aigua, sinó que també indica els processos hidràulics de la planta. Per una altra banda, tot i que es confirma un desacoblament general entre els valors de precipitació i el sòl, apuntant cap a la hipòtesi de l'existència de diferents fonts d'aigua no homogeneïtzada al sòl, els canvis isotòpics observat en el xilema no es van poder associar completament a aquest fet, degut a que en moments de sequera l'efecte de l'enriquiment evaporatiu en el xilema tenia lloc paral·lelament al teòric canvi en la font d'aigua. D'acord amb aquests resultats, es proposa que la hipòtesi "dos mons d'aigua al sòl" suposa una sobre-simplificació i que seria més adequada una com "l'elevada complexitat en les fonts d'aigua en el sòl".

Finalment, una vegada establert el marc metodològic i considerats els efectes del fraccionament isotòpic sobre les nostres dades, es van avaluar els principals motius del decaïment diferencial entre pins i roures. En aquest sentit, els roures marcescents van ser capaços de mantenir la seva activitat fisiològica i la transpiració en condicions hídriques més limitants que el pi roig. Aquesta estratègia més conservadora en els roures es basa en l'accés a fonts d'aigua més estables (aigua subterrània, de fondàries de sòl major o fortament adherida a les partícules del sòl) en comparació amb els pins, que pel contrari, van desenvolupar una estratègia molt més oportunista amb un ús ràpid de l'aigua de precipitació en la superfície del sòl. A part de les restriccions hidràuliques en els pins, les limitacions estomàtiques van reduir la seva activitat fisiològica des del primers estadis de sequera, generant problemes en l'assimilació i emmagatzematge de carboni. En els experiments en test, els pins van ser capaços de recuperar-se abans que els roures degut a un major control estomàtic. Però, en condicions de camp, amb períodes de sequera més llargs i severs, la major vulnerabilitat dels pins a desenvolupar embolisme en el xilema, va limitar la recuperació de la transpiració d'aquests una vegada finalitzada la sequera. En canvi, els roures es van recuperar millor, beneficiats pel seu sistema radicular més profund. A més a més, aquestes diferències inter-específiques en resposta a la sequera es van traduir també en conseqüències a llarg termini; així, els pins van mostrar variacions més grans i una reducció més pronunciada en el creixement radial en el temps, indicant que l'increment de la sequera que es projecta per un futur proper podria induir un decaïment fisiològic a llarg termini, comprometent la supervivència del pi roig dins l'ecotò submediterrani.

GENERAL INTRODUCTION

1. Overview

1.1 Why to study forest ecology? Water and carbon cycles

Approximately 30% of the global land surface is covered by forests; *ca.* 42 million km^2 distributed among tropical, temperate, and boreal lands. Forests are extremely important for their role in global biogeochemical cycles and the supply of woody and not woody products (mushrooms, medicinal plants, etc.) but also they offer essential forest ecosystem services, such as climate stabilization, carbon storage, protection of hydrological function, and conservation of habitats for species biodiversity (Lindner et al. 2010). In this sense, there is an increasing awareness that broad-scale future environmental changes are threatening the sustainable provision of these services, and this has forced foresters and forest researchers to concentrate on the effects of global change on the regulation of forest functions and their equilibrium. One crucial aspect of this is the role of forests in carbon and water cycles. Forests represent 45% of terrestrial carbon and 50% of terrestrial net primary production (Bonan 2008); globally, the forest carbon sink is 2.4±0.4 Pg C year^{-1}, although current land-use changes in the tropics reduce the actual net sink flux until 1.1±0.8 Pg C year^{-1} (Pan et al. 2011). These estimations in terms of carbon and ecosystem productivity are as large as values in oceans, which confers forest a key role as regulator of the global carbon cycle, influencing importantly in Earth's climate through the greenhouse effect of carbon dioxide. As well, forests play a key role in the water cycle. Precipitation can be intercepted by the forest canopy, from which it evaporates directly back into the atmosphere; on the contrary, it can reach the forest floor, becoming either part of the surface runoff, evaporate from the soil or infiltrate into the soil. At this point, root water uptake consumes soil water and groundwater and delivers it to the atmosphere by transpiration, reducing the amount of water that infiltrates to streams (Bearup et al. 2014). In this sense, it is reported that 64% of the evapotranspiration flux is returned to the atmosphere via plant transpiration and 27% is intercepted and evaporated from the vegetation cover (Good et al. 2015). Both cycles are tightly inter-connected by the stomatal-regulation of photosynthesis. Plants need to open their stomata in order to absorb CO_2 and produce sugars; however, the opening of the stomata produces the release of water by means of plant transpiration (Cochard 2014). If water availability is limited, the plant will try to minimize water loss by closing totally or partially the stomata, limiting the CO_2 absorption. Hence, changes in the water cycle will impact the carbon cycle and the significance of these changes will depend on species-specific physiological behavior.

1.2 Climate change projections: effects in plant water availability

Global climate change scenarios predict an increase in temperatures of 3-4ºC (Christensen et al. 2007) and a reduction of up to 20% of spring and summer precipitation for the next decades (Bates et al. 2008) in the globe. These effects will result in an increase of aridity, particularly in those locations as the Mediterranean Basin, where the water is a limiting factor in the dry or warm season. Besides, the predicted raise in temperature is presumed to increase 200-300 mm plant transpiration (De Luís et al. 2001, Martínez-Vilalta et al. 2002, Valladares et al. 2004) and also plant respiration (Peñuelas 1996), which would diminish real plant water availability in soil and thus, the ecosystem productivity. Additionally, the elevated year to year variation in temperature and precipitation, together with the fluctuating seasonal distribution that is also expected in many areas, will magnify the effects of water deficit (Rambal and Debussche 1995, Reichstein et al. 2002) complicating plant adaptation to new conditions (Peñuelas et al. 2002, Valladares et al. 2004, Resco de Dios et al. 2007). In this sense, in the Mediterranean basin, water is largely the main limiting factor for plant and tree development; thus, the predicted changes in water availability, even subtle, may affect the ecosystem functioning, modifying the species composition and dynamic of current forests systems (e.g. Martínez-Vilalta et al. 2002, Poyatos et al. 2008, Allen et al. 2010, Granda et al. 2013).

1.3 Specific responses to water availability: tree water use and ecophysiological strategies

Nevertheless, considering that different species or populations of the same species are not equally vulnerable to climate changes (Ramírez-Valiente et al. 2009), future range shifts are expected to have a local character, depending on species plasticity, adaptation and capacity to survive stressful conditions (Benito-Garzón et al. 2011). From a physiological view, the way that trees cope with water loses under drought will determine carbohydrate production, storage and growth patterns, in turn affecting the tree susceptibility to drought and to abiotic factors (McDowell et al. 2008, Zweifel et al. 2009). In this regard, there are two main responses or strategies of trees to drought: isohydric and anysohydric. In the first one, the main objective for the plant is to avoid water losses; thus, they regulate stomatal conductance tightly, closing them as soon as they perceive a slight decrease in xylem conductivity caused by raised soil tension (Tardieu and Simonneau 1998, McDowell et al. 2008). In the second one, the priority is carbon fixation; so, plants show lower regulation of the stomata and usually keep higher transpiration rates under limited soil water availability (Tardieu and Simonneau 1998, McDowell et al. 2008). Physiological strategy is generally linked to species-specific differences in rooting depth (West et al. 2012). Plants with a more conservative strategy in terms of stomatal regulation generally develop deeper rooting system, as they need to access to more reliable water moisture. On the contrary, a more opportunistic strategy will be able to depend on short and dynamic precipitation events; and thus develop a more superficial rooting pattern. In this regard, it is generally assumed that coexisting plant species often adopt contrasting resource use strategies through differences in key traits, such as rooting depth or the phenology of resource use, which leads to spatial and temporal partitioning of limiting resources, such as water (Filella and Peñuelas 2003, Peñuelas et al. 2011). In the case of water, they are often called ecohydrological niches (Moreno-Gutiérrez et al. 2012).

1.4 Forest decline and mortality episodes in drought-limited ecosystems

Tree die-off episodes associated with drought and high temperatures have been reported in all the major forested biomes of the Earth over the last decades (Allen et al. 2010). McDowell et al. (2008), working on Manion model (Manion 1991), developed a hydraulically-based framework to explain the physiological mechanisms that underlie drought-induced tree mortality. They suggested that 3 mechanisms (not exclusive) could lead to tree mortality depending on the environmental conditions and the physiological strategy on stomatal regulation of the species involved: hydraulic failure, carbon starvation and biotic agents. Hydraulic failure is more likely under intense drought and for species without a strict stomatal control to regulate water loss (*i.e.* anisohydric species). In these species, xylem water potentials can reach very low negative values, causing irreversible xylem embolism (Tyree and Zimmermann 2002). On the other hand, carbon starvation is particularly likely for long-lasting droughts in species with a tight stomatal regulation. In these isohydric species, water potentials may not become negative enough to cause severe xylem damage, but long periods of near zero assimilation, due to stomatal closure, and continued demand for carbohydrates may exhaust the plant carbon reserves (McDowell et al. 2008).

1.5 Our focus: submediterranean forests, unequal decline

In Europe, the transition zone between the Mediterranean and Eurosiberian region is generally called submediterranean ecotone (Abadía et al. 1996). The boundary between both regions is mostly gradual and has intermediate climatic characteristics (e.g. Sánchez de Dios et al. 2009). The main forest communities in this region are composed by marcescent oaks (*Quercus faginea*, *Q. humilis*, *Q. pyrenaica* and their hybrid form, *Q. subpyrenaica*) and evergreen conifers, either from natural regeneration or reforested (*Pinus sylvestris*, *P. nigra* subsp. *salzmannii* and *P. halepensis*). Marcescence is defined by the retention of withered or dead leaves at the stems during the non-vegetative season. In this regard, marcescent oaks may be more plastic in their leaf span than purely deciduous oaks (e.g. *Q. petraea* or *robur*), but more cold-tolerant than evergreen oaks *(Q.ilex)*. In Spain, submediterranean territories occupy mainly the northern part of the Peninsula, in particular, low altitude mountains in the pre-Pyrenees region, covering above 18.93 % of all the forested land: 16960 km^2 for submediterranean oak species and 9983 km^2 for submediterranean conifers (Maldonado et al. 2001). Both pines and oaks may appear in the form of pure or mixed stands.

In particular, in the medium-high mountains of the Spanish Pyrenees, one typical association is formed by the marcescent oak *Quercus subpyrenaica* E.H del Villar. and Scots pine (*Pinus sylvestris* L.). These co-ocurring species show different functional traits associated to contrasting water-use strategies. Scots pine, as an evergreen conifer has a tracheid-bearing xylem with low hydraulic conductivity and isohydric behavior; based on a tight stomatal control, limiting transpiration when soil moisture deficit reaches a threshold (e.g. Irvine et al. 1998, Poyatos et al. 2008, Martínez-Vilalta et al. 2009). Meanwhile, *Q. subpyrenaica* as its parental species (*Q. faginea* x *Q. humilis*) is a marcescent oak with ring-porous xylem with high maximum hydraulic conductivity and a typical anisohydric behavior, *i.e.* maintaining higher transpiration rates despite soil drought incidence (Nardini and Pitt 2002, Corcuera et al. 2004, Himrane et al. 2004, Tognetti et al. 2007). Additionally, the different physiological behavior between these species is partly associated to their root system distribution (e.g. Filella and Peñuelas 2003, Moreno-Gutiérrez et al. 2012, West et al. 2012, Barbeta et al. 2015); thus, oaks are able to maintain their continuous physiological activity by relying on stable pools like groundwater or deep soil layers while pines develop an opportunistic strategy of fast use of precipitation water based in a high root density on top soil layers (e.g. Poyatos et al. 2008, Klein et al. 2013, del Castillo et al. 2016, Grossiord et al. 2016). Also, because of the ability of oaks to reach lower water potentials than pines, during dry periods they can have access to more tightly-bound water pools in the soil (Tang and Feng 2001).

Considering its transitional nature, submediterranean ecotones are thought to be very sensitive to global change (Gosz 1992). Sánchez de Dios et al. (2009) predicted a dramatic reduction in the size of submediterranean area within IPCC A2 climatic change scenario for the near future (2020, 2050 and 2080 time horizons). In this regard, there is increasing evidence that extreme drought events are causing extensive tree decline in Scots pine populations, particularly in its southern limit of distribution (e.g. Martínez-Vilalta and Piñol 2002, Gómez-Aparicio et al. 2011, Poyatos et al. 2013, Sánchez-Salguero et al. 2016). Conversely, coexisting deciduous or marcescent oaks generally remain unaffected or even expanded towards higher or fresher locations (e.g. Benito-Garzón et al. 2008, Galiano et al. 2010, Poyatos et al. 2013). However, there are also some prediction models that suggest an important reduction in the distribution area of marcescent oaks (Sánchez de Dios et al. 2009, Benito-Garzón et al. 2011) and a displacement towards higher altitudes, in favor of Mediterranean evergreen oaks; e.g the holm oak, *Q. ilex* (Sánchez de Dios et al. 2009, Carnicer et al. 2013a, Carnicer 2014). In this context, it remains unclear how the different physiological and adaptive responses of pines and oaks could determine the composition and structure of submediterranean forests in a climate-change scenario.

2. Stable isotopes for the study of water uptake

2.1 Stable isotopes: theoretical frame and notation

Isotopes are chemical species of the same element that have different numbers of neutrons and, thus, different atomic mass. The superscript number to the left of an element designation is the mass number and represents the sum of the number of protons and neutrons in the isotope. For example, all isotopes of oxygen have 8 protons; however, an oxygen atom with a mass of 18 (denoted ^{18}O) has 2 more neutrons than an oxygen atom with a mass of 16 (^{16}O). Isotopes can be divided into radioactive and non-radioactive. The former disintegrate spontaneously over time to form other isotopes, whereas the latter do not decay to other isotopes on geologic time scales. These are known as stable isotopes and their natural abundance depends on several variables other than time. This is important because the science of stable isotopes focuses on how natural processes affect the relative abundance of each isotope with respect to the other; *i.e.* the ratio of the heavy isotope with respect to the lighter one (for example, $^{13}C/^{12}C$). Changes in such ratio are the result of biogeochemical processes involving kinetic or thermodynamic effects, and thus quantifying fractionation (the effect of such processes) is a useful way to explore what is occurring within a system. Kinetics effects are due to differences between isotopes in the rate of a given reaction and are generally non-additive; on the contrary, thermodynamic effects reflect divergences in the equilibrium constants of the reaction and are additive. The most abundant elements in the biosphere are carbon (C), hydrogen (H), oxygen (O) and nitrogen (N), being ^{13}C, ^{2}H (D), ^{18}O and ^{15}N the stable isotopes of greater interest in plant physiology. The stable isotope composition of a given sample is determined by mass spectrometry, is usually expressed in differential notation:

$$\delta(‰) = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000 \qquad (1)$$

where δ stands for the isotopic composition, in parts per mil (‰), as referred to an standard (see Table 1). R denotes the ratio of the heavy to light isotope (e.g., $^{13}C/^{12}C$), and R_{sample} and $R_{standard}$ are the ratios in the sample and standard, respectively. A positive δ value means that the isotopic ratio of the sample is higher than that of the standard; a negative δ value means that the isotopic ratio of the sample is lower than that of the standard. For example, a $\delta^{13}C$ value of -28‰ means that the $^{13}C/^{12}C$ of the sample is 28 parts-per-thousand lower than the $^{13}C/^{12}C$ of the standard (Pee-Dee Belemnite limestone).

Table 1. Standards, notation, abundance, typical range in plants and mean analytical error of the stable isotopes most commonly used in plant physiology. Adapted from Sulzman (2008).

Element	**Isotope**	**Abundance (%)**	**Relative mass difference (%)**	**International standard**	**Analytical error (‰)**	**Observed range in plants (‰)**
Hydrogen	^{1}H ^{2}H	99.985 0.0155	100	Viena Standard Mean Ocean Water (VSMOV)	4 to 7	-300 to +20
Carbon	^{12}C ^{13}C	98.892 1.108	8.3	Viena Pee Dee Belemnite (VPDB)	0.1	-35 to +5
Nitrogen	^{14}N ^{15}N	99.635 0.365	7.1	Atmospheric Nitrogen (air)	0.2	-10 to +10
Oxygen	^{16}O ^{17}O ^{18}O	99.759 0.037 0.204	12.5 (^{18}O : ^{16}O)	VSMOW in water, generally VPDB in CO2 or carbonate	0.05 to 0.2	-15 to +35

2.2 Water stable isotopes: applications in ecohydrology

The analysis of the isotopic composition of xylem water has been extensively applied to determine the source of water used by plants, providing an useful insight into many ecohydrological processes (Ehleringer and Dawson 1992). The basis of this approach is that the potential water sources available to plants show contrasting isotopic signatures, which can be traced back from the values in xylem water. For example, the different contribution of seasonal precipitation to soils, streams and groundwater lead to substantial isotopic differences among these water pools (see e.g. Gat 1996, Tang and Feng 2001, Máguas et al. 2011). In this sense, the relationship between $\delta^{18}O$ and $\delta^{2}H$ in the world's precipitation (rain, haze or snow) follows a predictable linear relationship, referred to as the "meteoric water line" (Craig 1961), that is a direct consequence of the fact that fresh waters of warm regions have more enriched (positive) values of hydrogen and oxygen isotopes, whereas cool regions are isotopically depleted for both elements. Such differences are mostly due to fractionation occurring during phase changes in the course of the hydrological cycle (see Fig. 1); light isotopes (^{16}O and ^{1}H) evaporate more rapidly than their heavier counterparts, and thus water vapour is isotopically depleted respect to source water (e.g. ocean water). The opposite occurs during precipitation; in order for water to condense and precipitate from an air mass, the temperature must drop. As temperature decreases, the heavier isotopes are selectively precipitated through distillation. This effect can be enhanced by orographic effects that may cause an abrupt cooling of an air mass as it rises.

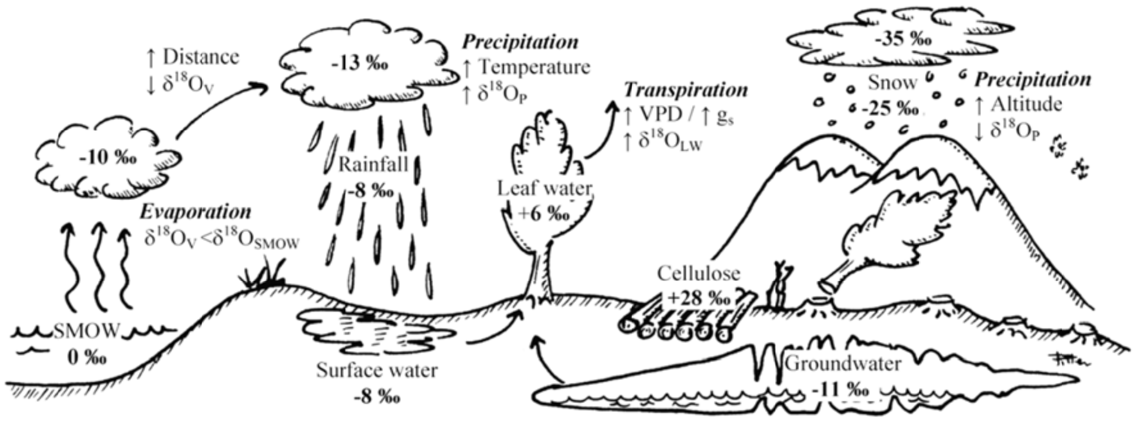

Figure 1. Main fractionation steps and typical values of oxygen isotope composition ($\delta^{18}O$) in a temperate climate. $\delta^{18}O_{SMOW}$, standard mean $\delta^{18}O$ in ocean water; $\delta^{18}O_V$, $\delta^{18}O_P$, $\delta^{18}O_{LW}$, $\delta^{18}O$ in water vapour, precipitation (either rainfall or snow) and leaf water, respectively; VPD, vapour pressure deficit; g_s, stomatal conductance. (Ferrio et al. 2005), original data from IAEA/WMO (2001) and Saurer et al. (1997).

Additionally, the preferential loss of light isotopes during evaporation causes a progressive isotopic enrichment of the liquid phase at the site of evaporation (Craig and Gordon 1965), and creates strong isotopic gradients along the soil profile during dry periods (Allison et al. 1983). The degree of evaporation with respect to the source water can be assessed by comparing the measured values with the Global Meteoric Water Line ($\delta^2H = 8\times \delta^{18}O + 10$) or the Local Meteoric Water Line, which are representative of the relationship between δ^2H and $\delta^{18}O$ that exist for meteoric water in the globe (average values) and a local study place, respectively (Craig 1961) (Fig. 2). Another variable, the Deuterium excess, (*D-excess* = $\delta^2H - 8\times \delta^{18}O$), denotes the divergence from the global meteoric water line, being sensitive to the conditions of evaporation from a surface, particularly near-surface temperature (Dansgaard 1964) but more importantly to relative humidity (Pfahl and Sodemann 2014). Thus, *D-excess* is useful for quantifying the degree of evaporation of water as well as the possible sources of precipitation (Fig. 2).

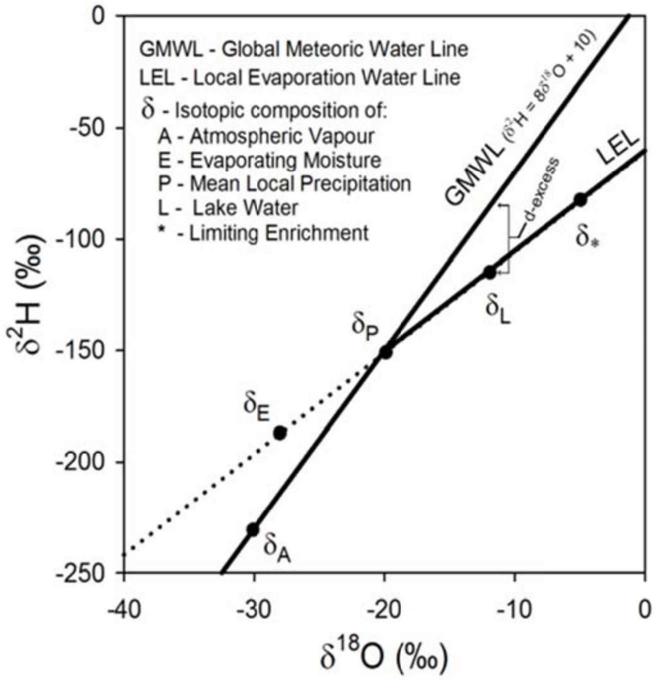

Figure 2. Generalized δ^2H vs δ^{18}O plot showing global meteoric water line and local evaporation line. Adapted from Gibson et al. (1993)

Taking advantage of this variability between water compartments in fresh water, soil and vegetation, isotopic tracing can be used to assess the use of contrasting water sources among adjacent plants, both at the inter-specific (e.g. Sternberg and Swart 1987, Filella and Peñuelas 2004, Máguas et al. 2011) and intra-specific level (Dawson 1993, Voltas et al. 2015). Isotopic tracing of plant water relies on two important working premises (Ehleringer and Dawson 1992). First of all, it is generally accepted that there is no fractionation during the uptake process by roots, except for some xerophytic and halophytic species (Lin et al. 1993, Ellsworth and Williams 2007). The second basic assumption is that there is no fractionation during the transport of water along the xylem, from the roots to the upper-canopy stems, as it is mainly a mass flow movement. Isotopic studies in plants have also shown that water uptake is a highly dynamic process (Brandes et al. 2007, Máguas et al. 2011, Ellsworth and Sternberg 2014, Bertrand et al. 2014), often involving complex ecological interactions like competition (Dawson 1993, Comas et al. 2015) or facilitation through hydraulic redistribution (Filella and Peñuelas 2004, Prieto et al. 2012).

2.3 Ecological limitations of this approach

Water stable isotopes are conceived as very powerful tools for source water and other ecohydrological studies; however, they are not extent of some limitations that are necessary to take into account before determining ecological conclusions.

a. Plant fractionation during water uptake and transport. Effects of leaf water in xylem

As stated before, in land surface and plant models it is generally assumed that no isotopic fractionation occurs during water uptake and transport along the xylem (Ehleringer and Dawson 1992). Nevertheless, there is increasing evidence of significant isotopic changes in the way from the soil to the terminal xylem in the twigs. So far, evidence for direct fractionation associated to water uptake appears to be restricted to xerophytic and halophytic species (Ellsworth and Williams 2007). On the other hand, it is well known that water losses through transpiration in the leaves causes a significant isotopic enrichment of leaf water, which is a function of 1) the humidity gradient between the site of evaporation and the atmosphere and 2) the isotopic composition of atmospheric water vapour and 3) transpiration rates (Dongmann et al. 1974, Farquhar and Lloyd 1993, for a recent review see Cernusak et al. 2016). Similarly, we could expect that if water loss occur through the bark, stems would display an evaporative enrichment of source water proportional to the humidity gradient, and counteracted by the extent of transpiration flow (Dawson and Ehleringer 1993). In this sense, several authors have shown evaporative enrichment in xylem associated to green, unsuberized stems (Dawson and Ehleringer 1993) or to suberized stems during leafless or severe drought periods, when a long-lasting water stagnation in the xylem leads to partial desiccation (Phillips and Ehleringer 1995, Ellsworth and Sternberg 2014, Treydte et al. 2014, Bertrand et al. 2014, del Castillo et al. 2016).

Besides cuticular evaporation of xylem water, some studies suggest that the cause of xylem-soil decoupled observations may be the mixture of xylem water with enriched phloem water from the leaf (Cernusak et al. 2005, Ellsworth and Williams 2007). In fact, xylem-phloem communication has been recently associated to xylem embolism repair (Nardini et al. 2011) by phloem unloading of sugars, inorganic ions, alcohols that increase osmotic potential and, then, facilitate xylem refilling (Secchi and Zwieniecki 2011, Nardini et al. 2011). In line with the effects of leaf water on xylem, there are important evidences of foliar absorption of intercepted rainfall or fog water (Dawson 1998, Burgess and Dawson 2004, Gruber et al. 2012, Breshears et al. 2013, Goldsmith 2013, Hill et al. 2015), but there is a lack of knowledge in the magnitude of this process and in how it is affecting the total plant water and carbon balance.

All this information is crucial to provide a proper interpretation of daily, and even seasonal changes in water uptake patterns, based on isotope measurements, and for the interpretation of ecohydrological processes (see e.g. Filella and Peñuelas 1999, Bertrand et al. 2014, Voltas et al. 2015, Cernusak et al. 2016).

b. Soil distillation methods and separation of different soil water pools

Several water extraction methods have been developed to analyze soil and xylem water isotopes: azeotropic distillation (Revesz and Woods 1990), mechanical squeezing (Böttcher et al. 1997), cryogenic vacuum extraction (West et al. 2006, Orlowski et al. 2013), modified vacuum extraction technique (Koeniger et al. 2011), batch-method for stem water extraction (Vendramini and Sternberg 2007), centrifugation with or without immiscible heavy liquids (Mubarak and Olsen 1976, Peters and Yakir 2008), accelerated solvent extraction (Zhu et al. 2014), liquid-water-vapour equilibrium techniques (Wassenaar et al. 2008, Garvelmann et al. 2011, Volkmann and Weiler 2014), or microwave extraction (Munksgaard et al. 2014). However, despite cryogenic vacuum extraction is the most widely used method in plant-soil studies (Koeniger et al. 2011, Munksgaard et al. 2014) there are several uncertainties about the suitability of this method to obtain a representative isotope value for the water pools in the soil that are actually available to plants.

Firstly, by cryogenic distillation we are expected to recover the complete amount of water in the sample. However, recent studies have revealed that cryogenic extraction conditions, such as the duration (West et al. 2006, Orlowski et al. 2013), the temperature (Walker et al. 1994, Araguás-Araguás et al. 1995, Orlowski et al. 2013) and the vacuum threshold (West et al. 2006, Orlowski et al. 2013) have a significant effect on the isotopic composition of the extracted water. In general, we can overcome this problem by ensuring a complete distillation, using high temperatures and long extraction times (Araguás-Araguás et al. 1995). However, even a complete distillation, which is clearly desirable in plant tissues, is not always the most suitable approach for the soil samples. In particular, one of the main constrains for the determination of plant water sources is to define which soil water fraction (mobile, weakly or tightly bounded) each method captures. Several authors have compared different soil extraction methods obtaining very divergent results (Ingraham and Shadel 1992, Leaney et al. 1993, Walker et al. 1994, Araguás-Araguás et al. 1995, Landon et al. 1999, Koehler et al. 2000, Kelln et al. 2001). The critical point is that, despite the water extraction in cryogenic distillation is complete (and thus be truly representative of total soil water), not all this water may be available for plant consumption. Tang and Feng (2001) demonstrated that different, partially unmixed water pools may co-exist in the soil, each one retained under different matrix potentials, therefore showing different degree of availability to the plants (Fig. 3). Later on, Brooks et al. (2010) and McDonnell (2014) described this as the *two water world hypothesis*, assuming that two independent (decoupled) soil water pools exist in the soil (mobile water-stream vs. tightly bound water-plant water) as a function of soil texture and other properties. For these authors, the immobile water is the one in dead-end pores, thin liquid films around solid particles, and immobile intra-aggregate water, meanwhile mobile water fraction is the water percolating during and shortly after a precipitation event. They assume that "immobile" water corresponds to that used by plants (Evaristo et al. 2015); however, even within this "immobile" water, only one fraction is actually available for plants, and not in the same degree for all species and environmental conditions. Considering that plant's ability to uptake these different soil water pools depends on its physiological status and water-use strategy, this complicates the assessment of plant water uptake patterns.

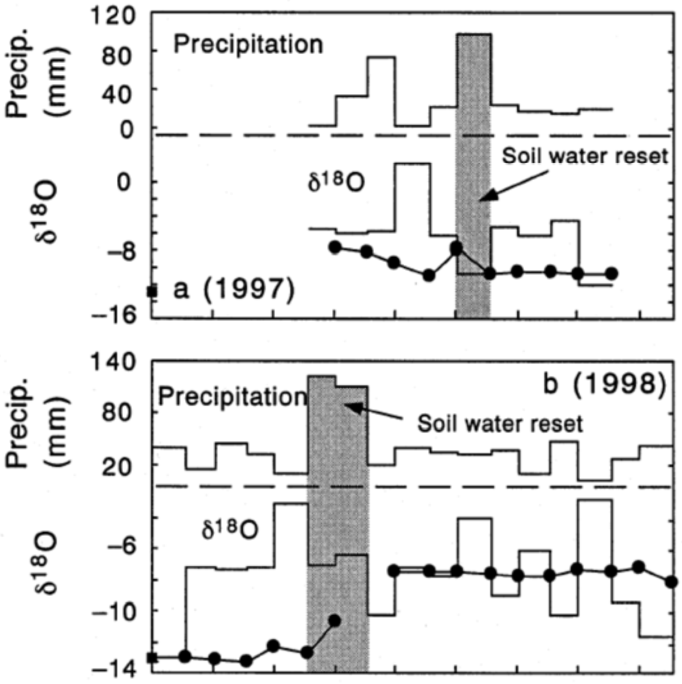

Figure 3. From Tang and Feng (2001). Comparisons of the $\delta^{18}O$ time series of soil water with precipitation. The precipitation samples are represented by a step line. The solid circles represent the $\delta^{18}O$ values of soil water at 50 cm. The shaded areas indicate that the deep soil water was reset during that period because of a large precipitation event. Thus, different soil fractions in soil may exist and only after an important precipitation event can be reset and tightly-bound water have the same isotopic composition as mobile water.

Finally, certain physical and biogeochemical soil properties are considered to have an effect on water isotopic composition, causing potential fractionations on the soil water. In particular, the soil type and properties, such as percentage of clay (Araguás-Araguás et al. 1995, Koeniger et al. 2011, Orlowski et al. 2013, Meißner et al. 2013), gypsum (Palacio et al. 2014) and carbonate content (Meißner et al. 2013), the presence of several cations (Oerter et al. 2014), adsorption to organic matter (Meißner et al. 2013, Chen et al. 2016) or the salinity itself (Sacchi et al. 2001), have been associated to deviations in isotope composition from presumed water source. Also, the water content in soil (Ingraham and Shadel 1992, Araguás-Araguás et al. 1995, Meißner et al. 2013) and microbial activity (Koeniger et al. 2011) have been found to have an important effect, leading to differences between the input water (irrigation/precipitation) and the resulting soil isotopic values.

c. *Analytical error in isotopic analyses: the case of IRIS*

The widespread use of isotope-ratio mass spectrometry (IRMS) technology for measuring water isotopes has been challenged by the development of isotope-ratio infrared spectroscopy (IRIS). IRIS allows the simultaneous measurement of $\delta^{18}O$ and $\delta^{2}H$ with an accuracy comparable to IRMS (Lis et al. 2008, Brand et al. 2009, Gupta et al. 2009, West et al. 2010, 2011) and offers other advantages such as lower cost, easier installation and maintenance, and higher portability (Brand et al. 2009, Gupta et al. 2009, Berman et al. 2009, West et al. 2010, Johnson et al. 2011, Schmidt et al. 2012). However, some organic contaminants, broadly found together with water in plant and soil samples, interfere significantly with the water-isotope spectrum in IRIS analyses (Brand et al. 2009, West et al. 2010, 2011). The magnitude of the error caused by organic interference is not only proportional to the amount of contaminant, but also depends on its spectral properties; e.g. methanol errors become unacceptable starting at very small concentrations (Brand et al. 2009). To solve this problem, IRIS manufacturers have developed software applications that can identify and flag potentially contaminated samples (e.g. ChemCorrect™, Picarro Inc., Santa Clara, CA) or, more recently, Picarro Inc. has developed the Micro-Combustion Module™ (MCM) to remove organic compounds interfering with pure water at concentrations lower than 0.5%. However, the effectiveness of these methods still requires full testing.

3 Other tools for the assessment of tree water status

Even though it has been clearly proved that stable isotopes are a powerful tool to study water tracing on plant, soils and ecosystems, from a physiological to global scale; it is necessary to combine this data with other physiological tools if we want a more complete study of plant water status and plant response and recovery to drought.

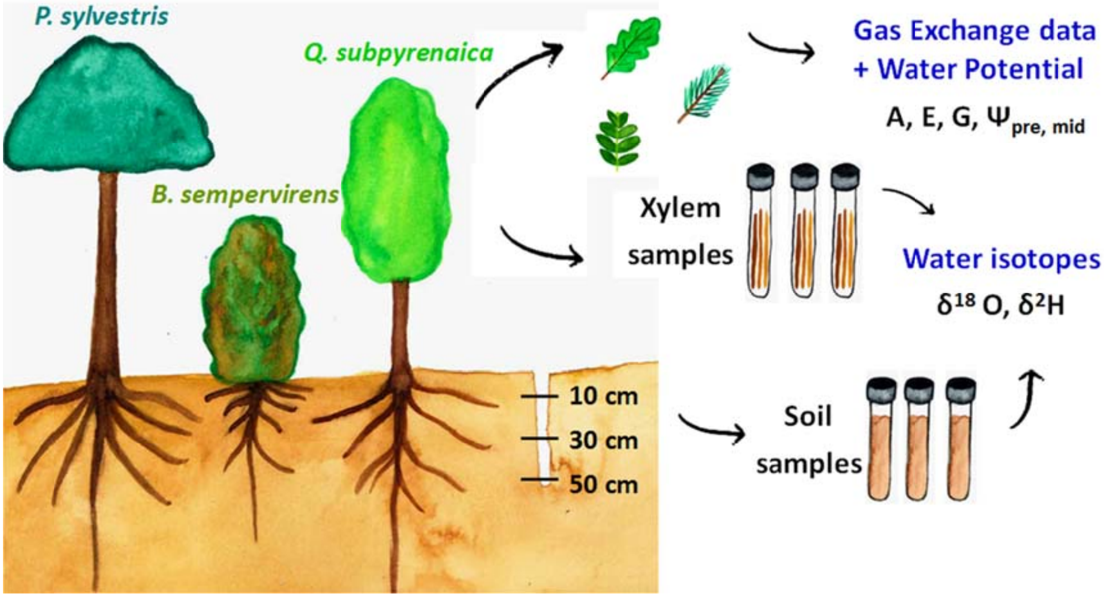

Figure 4 Schema of the sampling measures taken in Chapter III field campaings, where water stable isotopes are accompanied by other measures as leaf gas exchange and xylem water potential. Additionally we took data from sap flow and remote sensing sensors and from carbohydrate in xylem and growth pattern in trunk cores.

3.1 Water potential as a proxy of plant water status

Water potential is defined as the free energy of water per unit of volume (J m^{-3}), and is expressed in units of pressure (MPa). Basically, this value is determined by the energy of free water molecules, and reflects different properties of water. For example, water potential increases with temperature, since it adds energy to the molecules, but decreases with solute concentration, because solutes sequestrate water molecules, limiting the number of free molecules. On the other hand, pressure increases the water potential, by "concentrating" the water molecules (*i.e.* reducing the volume). Conversely, surface tension (*i.e.* negative pressure) in the soil or in evaporative surfaces in the leaf would reduce water potential. The reference value for water potential (0) is pure water under standard conditions of pressure and temperature. For given water pool, different processes may contribute to the total water potential (ψ_w) in different ways, therefore it is generally divided in several additive components, e.g. in plants:

$$\psi_w = \psi_s + \psi_p + \psi_g \qquad (2)$$

where ψ_s ,ψ_p and ψ_g stand for osmotic (solute), pressure and gravity water potential. The latter increases at a rate of about 0.1 MPa every 10 m, therefore it becomes only relevant for very tall trees (Taiz and Zeiger 2010). In plants and soils, water is never pure; therefore the osmotic component is always negative. The pressure component, however, would be positive inside cells due to hydrostatic pressure, but negative under the tension found in the xylem, soil and evaporative surfaces of the leaf mesophyll. For plant physiology, the most interesting point of water potential is its ability to quantify the tendency of water to move from one area to another due to osmosis, gravity, mechanical pressure, or matrix effects such as capillary action. If flow is not restricted, water will move from an area of higher water potential to an area with lower potential, until the difference in potential is equalized or balanced by another water potential factor. Thus, the measurement of xylem/leaf water potential results very useful in studying the movement of water within plants and soil. Generally, plant water potential is measured with a Scholander's pressure chamber, by applying pressure to a cut branch or leaf until it compensates the tension existing in the xylem, which is determined by the extrusion of water from the cutting end (Scholander and Hammel 1965). Although still under debate (Tyree 1997, Angeles et al. 2004), the cohesion-tension theory assumes that the driving force for transpiration is the difference in water potential between the substomatal air space that is generally saturated and the external atmosphere. Water potential can be measured in every time of the day, but it is commonly taken at midday, as a proxy of plant hydric conditions in the driest moment of the day, hence, the most demanding; and at pre-dawn, when plant water content would be stabilized with soil (if drought is not as extreme to prevent plant recovery during night) and then, can be related to soil water potential or soil water content. On that account, it is typical to find a relationship between water isotopes and water potential, as both variables rely on stomatal conductance and plant water strategy (see e.g. Filella and Peñuelas 2003, del Castillo et al. 2016). For this reason, multiple studies (see e.g. Moreno-Gutiérrez et al. 2012, del Castillo et al. 2016) combine data of xylem and soil water isotopes with water potential measurements, in order to make a more precise approximation of real plant water status and behavior in terms of water acquisition and use.

3.2 Leaf gas exchange measurements characterizing tree physiological activity

Gas exchange measures reflect the balance of CO_2 and H_2O between the atmosphere and the leaves. The overall effect depends on the time of day and environmental conditions, such as the light intensity, wind speed or water pressure deficit. Photosynthesis only occurs during the day and it is considered positive when photosynthetic rate is higher than respiration; so, there is a net release of oxygen and a net uptake of carbon dioxide. Stomata play a crucial role, as it is the place where the gas exchange between the leaf and the open air takes place, and depending on atmospheric or the plant conditions, they regulate the processes by opening or closing. The degree of this opening is called stomatal conductance and is a function of plant physiological strategy, together with its water status (Farquhar and Sharkey 1982). Hereof, regarding their response towards water stress, isohydric species will have a more dynamic stomatal regulation, and this in turn would affect photosynthetic activity, in comparison to more anysohydric species, which would maintain higher net carbon gain under drought stress, at the expense of greater water losses. In this sense, the combination of leaf gas exchange measures with isotopic data can help us to understand better the plant hydric behavior against drought conditions (Yakir and Sternberg 2000).

3.3 Carbon isotopes in plant material to describe specific differences in WUE

Carbon isotope composition is described as δ^{13}C, the ratio between ^{13}C and ^{12}C in comparison to Vienna Pee Dee Belemnite (VPDB). Plant organic matter is depleted in ^{13}C compared to source carbon in atmospheric CO_2, due to the discrimination against the heavy isotope by the carbon fixing enzyme RuBisCo (Farquhar and Lloyd 1993). There are several environmental factors that can modify the isotopic composition of plant tissues through their influence on either leaf conductance or photosynthetic rate, or both parameters simultaneously. Changes in irradiance levels, CO_2 concentration, and plant water status are clearly reflected in δ^{13}C variations. In this regard, a close connection has been found between δ^{13}C and intrinsic water use efficiency (WUE_i = net assimilation rate / stomatal conductance) under a variety of conditions (Farquhar et al. 1989, Ehleringer and Vogel 1993). This was explained by the dependency of discrimination on the ratio between leaf internal and ambient CO_2 concentration, c_i/c_a. While c_a is generally respond to atmospheric conditions and it is considered well-mixed and thus, relatively constant for a given place and time; c_i variations depend on stomatal conductance and assimilation rate. Stomatal closure as a response to water deficits reduces c_i, leading to an increase in δ^{13}C. Lowered c_i/c_a ratios by stomatal closure (lower input) mean that Rubisco has less CO_2 available to fix. Because Rubisco preferentially uses the lighter carbon isotope ($^{12}CO_2$) for photosynthesis, the plant is then forced to use (or cannot discriminate against) the heavier isotope ($^{13}CO_2$), thus showing less depletion in ^{13}C for the plant material (never enriched in respect to ambient CO_2). Many studies under growth-chamber and field conditions have shown that plants developed under water stress (stress induced by low soil water content) produced leaves with higher δ^{13}C (see references in Ferrio et al. 2003).

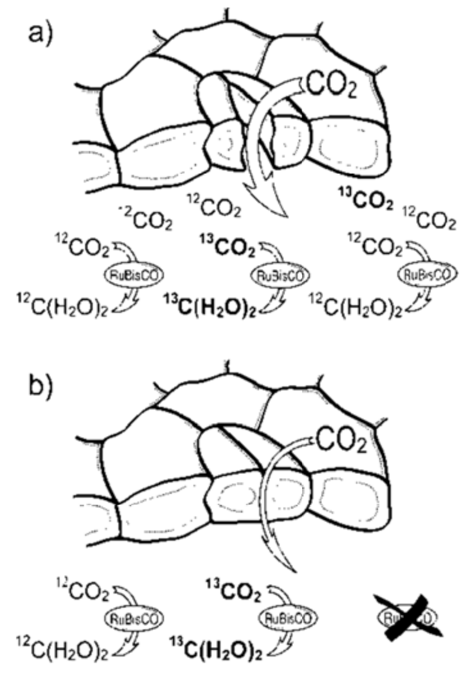

Figure 5. Simplified scheme of the relationship between carbon isotope discrimination ($\Delta^{13}C$) in C_3 plants and stomatal conductance. A) High stomatal conductance, high discrimination: CO_2 diffusses easily into the intercelular space, the activity of the carboxylating enzyme (RuBisCO) is not limited by CO_2 concentration and thus it has more chances to discriminate against ^{13}C. B) Low stomatal conductance, low discrimination: the flux of CO_2 is reduced, and the limiting factor of photosynthesis is stomatal conductance. In this case RuBisCO is forced to fix a higher proportion of ^{13}C. Redrawn from Ferrio et al. (2003).

3.4 Tree sap flow: monitoring tree water use or transpiration

Transpiration rates and water use for whole plants or individual branches can be determined by techniques which measure the rate at which sap ascends stems. Sap flow sensors measure the movement of water in plants due to water potential gradient between soil and atmosphere. Two methods are the most commonly employed, both of them using heat as a tracer for sap movement, allowing for continuous and non-destructive monitoring of whole-tree transpiration. The heat balance, where the stem is monitored with two adjacent sensors, and one of them (downstream) is heated electrically. The temperature difference between them would be lower when the flux of water is higher, due to its cooling effect. The balance is then solved for the amount of heat taken up by the moving sap stream, which is then used to calculate the mass flow of sap in the stem. In another method, the heat-pulse method, rather than using continuous heating, short pulses of heat are applied and the velocity of sap flow is determined from the velocity of the heat pulses moving along the stem. Particularly, sap flow data has been used together with water isotopes in ecosystem evapotranspiration partitioning studies, because of the divergence of isotopic composition between different species transpired-water and soil evaporated water (see e.g. Yepez et al. 2003, Williams et al. 2004). In this sense, isotopes can trace back the water use and sap flow measurements can incorporate the quantification to this data.

3.5 Tree-ring width and basal area increment to reconstruct historical growth patterns

Dendroecology is the science that uses tree rings to study factors that affect forest ecosystems. The main variable to study is the width of the annual rings, matched before to a defined year (dendrochronology). Annual growth is a function of climatic conditions and other factors, such as tree age, trunk diameter, competition or disturbance effects. When tree-ring width is converted into basal area increment (BAI, in mm^2/year), the variations in radial growth attributable to geometric effects of tree size are removed, thus being a more suitable proxy for tree growth. One of the main principles in dendroecology is that the rates of plant processes (as growth) are constrained by the primary environmental variable that is most limiting (e.g. precipitation in arid and semiarid areas). In these regions, tree growth cannot proceed faster than that allowed by the amount of precipitation, causing the width of the rings (*i.e.*, the volume of wood produced) to be a function of precipitation. In this regard, growth is the most sensitive parameter to water stress, followed by photosynthesis, and finally respiration. Initially, when a plant suffers from drought stress the carbohydrate pool in the plant increases, but when photosynthesis starts to decline until a lower rate than respiration the carbohydrate pool start to decline. Besides, if respiration increases with drought and high temperatures, the size of the carbohydrate pool might decline more rapidly and growth might be broadly affected (McDowell 2011). On this account, to include carbohydrate and growth information to plant-source water data might help us to understand long-term drought affection of the studied species and presume which direction of adaptation and survivorship may they follow.

3.6 Vegetation and soil humidity indexes from remote sensors

Vegetation indices are radiometric estimates of the amount of photosynthetically active radiation (PAR) absorbed by the pigments of green leaves as an energy source for photosynthesis. A sensor can gather both the reflected solar radiation from the plant, in order to quantify a proportion of PAR that is used by plants during photosynthesis and the low-energy radiation that increases leaf temperature. The difference between the two reflected radiations provides a very distinctive signature of vegetation, when compared to other earth surface materials (Tucker and Sellers 1986). One of the most typically used indices is the normalized difference radiation index (NDVI), which is calculated as:

$$NDVI = \frac{(\text{Red - Near Infrared})}{(\text{Red + Near Infrared})} \qquad (3)$$

Remote sensors provide some indication of chlorophyll density, which is in turn related to photosynthetic and transpiration rates, rather than just an indicator of the photosynthetic surface (leaf area index or biomass).

Furthermore, some satellites are also capable to capture information on soil humidity in the upper soil layers. Soil moisture can be retrieved from brightness temperature observations. Due to the large dielectric contrast between dry soil and water, the soil emissivity at a particular microwave frequency depends upon the moisture content. These estimations are important for improving short- and medium-term meteorological forecasting, hydrological modelling, monitoring photosynthesis and plant growth, and estimating the terrestrial carbon cycle.

4 Structure of the thesis

The first aim of this thesis was to gain a better understanding of physiological and water uptake strategies of submediterranean pine-oak mixed forests, and the impact of global change on their specific behavior and ecological interactions, by means of water stable isotopes and other physiological methods. In the way to find an answer to this subject, we came across several methodological questions, related mainly to isotopic techniques and to their application in ecohydrological studies. Consequently, the first two chapters of this thesis aimed to establish an adequate methodological frame, while the last two focused on the ecological functioning of submediterranean species. In summary, we developed our specific objectives organized in the following four main chapters:

1. **CHAPTER I:** ***Isotope-Ratio Infrared Spectroscopy: a reliable tool for the investigation of plant-water sources?***
   We analyzed the magnitude of the spectral interference in isotope-ratio infrared spectroscopy (IRIS) methods and examined the performance of two alternative approaches to cope with contaminated samples in IRIS (on-line oxidation and post-processing correction).

2. **CHAPTER II:** ***Short-term dynamics of evaporative enrichment of xylem water in woody stems: implications for ecohydrology***
   We determined the short-term isotopic enrichment of xylem water under conditions of limited sap flow in active, suberized and leaved stems, trying to define the processes behind, e.g. the relative contribution of stem evaporation and back diffusion of leaf water. We also assessed the effect of twig diameter or xylem water content, in order to provide recommendations for future sampling.

3. **CHAPTER III:** ***Contrasting ecophysiological strategies related to drought: the case of a mixed stand of Scots pine (Pinus sylvestris) and a submediterranean oak (Quercus subpyrenaica).***
   In a mixed forest of *P. sylvestris* and *Q. subpyrenaica* in the submediterranean area of the Spanish Pyrenees, we analyzed the species-specific seasonal evolution of water uptake patterns and physiological response during drought and recovery, in terms of water relations and carbon dynamics an in order to understand the mechanisms behind Scots pine decline and the physiological advantages of marcescent oaks in the area.

4. **CHAPTER IV:** ***Soil- or plant-driven fractionation cause isotopic uncoupling between soil and xylem water during drought and recovery***
   We studied the existence of different non-well mixed pools of water in soil and the differential use of them by trees, by precisely controlling the water entering the system and continuously measuring plant physiological status in a pot experiment with submediterranean pines and oaks. We also assessed species-specific differences in ecophysiological strategy during drought and recovery, taking into consideration soil and stem fractionation processes.

REFERENCES

Abadía A, Gil E, Morales F, Montañés L, Montserrat G, Abadía J (1996) Marcescence and senescence in a submediterranean oak (*Quercus subpyrenaica* E.H. del Villar): photosynthetic characteristics and nutrient composition. Plant Cell Environ 19:685–694.

Allen C, Macalady A, Chenchouni H, Bachelet D, Mcdowell N, Vennetier M, Kitzberger T, Rigling A, Breshears D, Hogg E, Gonzales P, Fensham R, Zhang Z, Castro J, Demidova N, Lim J, Running S, Semerci A, Cobb N (2010) A global overview of drought and heat induced tree mortality reveals emerging climate change risk for forests. For Ecol Manage 259:660–684.

Allison GB, Barnes CJ, Hughes MW (1983) The distribution of deuterium and ^{18}O in dry soils 2. Experimental. J Hydrol 64:377–397.

Angeles G, Bond B, Boyer JS, Brodribb T, Brooks JR, Burns MJ, Cavender-Bares J, Clearwater M, Cochard H, Comstock JP, Davis SD, Domec JC, Donovan L, Ewers F, Gartner B, Hacke U, Hinckley T, Holbrook NM, Jones HG, Kavanagh K, Law B, Lopez-Portillo J, Lovisolo C, Martin T, Martinez-Vilalta J, Mayr S, Meinzer FC, Melcher P, M. M, Mulkey S, Nardini A, Neufeld HS, Passioura J, Pockman WT, Pratt RB, Rambal S, Richter H, Sack L, Salleo S, Schubert A, Schulte P, Sparks JP, Sperry J, Teskey R, Tyree M (2004) The Cohesion-Tension theory. New Phytol 163:451–452.

Araguás-Araguás L, Rozanski K, Gonfiantini R, Louvat D (1995) Isotope effects accompanying vacuum extraction of soil water for stable isotope analyses. J Hydrol 168:159–171.

Barbeta A, Mejía-Chang M, Ogaya R, Voltas J, Dawson TE, Peñuelas J (2015) The combined effects of a long-term experimental drought and an extreme drought on the use of plant-water sources in a Mediterranean forest. Glob Chang Biol 21:1213–1225.

Bates BC, Kundzewicz ZW, Wu S, Palutikof JP (2008) Climate Change and Water. IPCC Secretariat (eds), Geneva, 210 p.

Bearup L A, Maxwell RM, Clow DW, Mccray JE (2014) Hydrological effects of forest transpiration loss in bark beetle-impacted watersheds. Nat Clim Chang 4:481–486.

Benito-Garzón M, Alía R, Robson TM, Zavala MA (2011) Intra-specific variability and plasticity influence potential tree species distributions under climate change. Glob Ecol Biogeogr 20:766–778.

Benito-Garzón M, Sánchez de Dios R, Sainz Ollero H (2008) Effects of climate change on the distribution of Iberian tree species. Appl Veg Sci 11:169–178.

Berman ESF, Gupta M, Gabrielli C, Garland T, McDonnell JJ (2009) High-frequency field-deployable isotope analyzer for hydrological applications. Water Resour Res 45:10

Bertrand G, Masini J, Goldscheider N, Meeks J, Lavastre V, Celle-Jeanton H, Gobat J-M, Hunkeler D (2014) Determination of spatiotemporal variability of tree water uptake using stable isotopes (δ^{18}O, δ^{2}H) in an alluvial system supplied by a high-altitude watershed, Pfyn forest, Switzerland. Ecohydrology 7:319–333.

Bonan GB (2008) Forests and climate change: forcings, feedbacks, and the climate benefits of forests. Science 320:1444–1449.

Böttcher G, Brumsack HJ, Heinrichs H, Pohlmann M (1997) A new high-pressure squeezing technique for pore fluid extraction from terrestrial soils. Water Air Soil Pollut 94:289–296.

Brand WA, Geilmann H, Crosson E, Rella C (2009) Cavity ring-down spectroscopy versus high-temperature conversion isotope ratio mass spectrometry; a case study on δ^{2}H and δ^{18}O of pure water samples and alcohol/water mixtures. Rapid Commun Mass Spectrom 23:1879–1884.

Brandes E, Wenninger J, Koeniger P, Schindler D, Rennenberg H, Leibundgut C, Mayer H, Gessler A (2007) Assessing environmental and physiological controls over water relations in a Scots pine (*Pinus sylvestris* L.) stand through analyses of stable isotope composition of water and organic matter. Plant Cell Environ 30:113–127.

Breshears DD, Mcdowell NG, Goddard KL, Dayem KE, Martens SN, Meyer CW, Brown KM, Mcdowell G, Goddard L, Martens N, Dayem E (2013) Foliar absorption of intercepted rainfall improves woody plant water status most during drought. Ecology 89:41–47

Brooks JR, Barnard HR, Coulombe R, McDonnell JJ (2010) Ecohydrologic separation of water between trees and streams in a Mediterranean climate. Nat Geosci 3:100–104.

Burgess SSO, Dawson TE (2004) The contribution of fog to the water relations of *Sequoia sempervirens* (D. Don): foliar uptake and prevention of dehydration. Plant, Cell Environ 27:1023–1034.

del Castillo J, Comas C, Voltas J, Ferrio JP (2016) Dynamics of competition over water in a mixed oak-pine Mediterranean forest: Spatio-temporal and physiological components. For Ecol Manage 382:214–224.

Cernusak LA, Barbour MM, Arndt SK, Cheesman AW, English NB, Feild TS, Helliker BR, Holloway-Phillips MM, Holtum JAM, Kahmen A, McInerney FA, Munksgaard NC, Simonin KA, Song X, Stuart-Williams H, West JB, Farquhar GD (2016) Stable isotopes in leaf water of terrestrial plants. Plant Cell Environ 39:1087–1102.

Cernusak L a, Farquhar GD, Pate JS (2005) Environmental and physiological controls over oxygen and carbon isotope composition of Tasmanian blue gum, *Eucalyptus globulus*. Tree Physiol 25:129–146.

Chen G, Auerswald K, Schnyder H (2016) ^{2}H and ^{18}O depletion of water close to organic surfaces. Biogeosciences 13:3175–3186.

Christensen JH, Hewitson B, Busuioc A, Chen A, Gao X, Held I, Jones R, Kolli RK, Kwon W-T, Laprise R, Rueda VM, Mearns L, Menéndez CG, Räisänen J, Rinke A, Sarr A, Whetton P (2007) Regional climate projections. In: Climate Change 2007: The physical science basis. contribution of working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change (eds), pp 847–940.

Cochard HH (2014) The basics of plant hydraulics. J Plant Hydraul 1: e0001.

Comas C, del Castillo J, Voltas J, Ferrio JP (2015) Point processes statistics of stable isotopes: Analysing water uptake patterns in a mixed stand of Aleppo pine and Holm oak. For Syst 24(1):e009, doi:http://dx.doi.org/10.5424/fs/2015241-05846.

Corcuera L, Camarero JJ, Gil-Pelegrín E (2004) Effects of a severe drought on growth and wood anatomical properties of *Quercus faginea* Iawa Journal 25:185–204.

Craig H (1961) Isotopic variations in meteoric waters. Science 133:1702–1703.

Craig H, Gordon L (1965) Deuterium and oxygen-18 variations in the ocean and the marine atmosphere. In: Proceedings of a conference on stable isotopes in oceanographic studies and palaeotemperatures. E. Tongiorgi (eds). Lischi and Figli, Pisa, pp 9-130.

Dansgaard W (1964) Stable isotopes in precipitation. Tellus A 16: 436-468

Dawson TE (1993) Water sources of plants as determined from xylem-water isotopic composition: Perspectives on plant competition, distribution, and water relations. In: Ehleringer JR, Hall AE, Farquhar GD (eds), Stable isotopes and plant carbon-water relations. Academic Press, Inc., New York, pp 465–496.

Dawson TE (1998) Fog in the California redwood forest: ecosystem inputs and use by plants. Oecologia 117:476–485.

Dawson T, Ehleringer JR (1993) Isotopic enrichment of water in the 'woody' tissues of plants: Implications for plant water source, water uptake, and other studies which use the stable isotopic composition of cellulose. Geochim Cosmochim Acta 57:3487–3492.

Dongmann G, Nürnberg HW, Förstel H, Wagener K (1974) On the enrichment of $H_2{}^{18}O$ in the leaves of transpiring plants. Radiat Environ Biophys 11:41–52.

Ehleringer JR, Dawson TE (1992) Water uptake by plants: perspectives from stable isotope composition. Plant Cell Environ 15:1073–1082.

Ehleringer JR, Vogel JC (1993) Historical aspects of stable isotopes in plant carbon and water relations. In: JR Ehleringer, AE Hall, and GD Farquhar (eds), Stable Isotopes and Plant Carbon/Water Relations. Academic Press, San Diego, pp 9-18.

Ellsworth PZ, Sternberg LSL (2014) Seasonal water use by deciduous and evergreen woody species in a scrub community is based on water availability and root distribution. Ecohydrology 8:538–551.

Ellsworth PZ, Williams DG (2007) Hydrogen isotope fractionation during water uptake by woody xerophytes. Plant Soil 291:93–107.

Evaristo J, Jasechko S, McDonnell JJ (2015) Global separation of plant transpiration from groundwater and streamflow. Nature 525:91–94.

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 40:503–537.

Farquhar GD, Lloyd J (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In: Stable isotopes and plant carbon-water relations.pp 47–70.

Farquhar GD, Sharkey TD (1982) Stomatal conductance and photosynthesis. Annu Rev Plant Physiol 33:317–345.

Ferrio JP, Resco V, Williams DG, Serrano L, Voltas J (2005) Stable isotopes in arid and semi-arid forest systems. Invest Agrar Sist Recur For 14:371–382.

Ferrio JP, Voltas J, Araus JL (2003) Use of carbon isotope composition in monitoring environmental changes. Manag Environ Qual An Int J 14:82–98.

Filella I, Peñuelas J (1999) Altitudinal differences in UV absorbance, UV reflectance and related morphological traits of *Quercus ilex* and *Rhododendron ferrugineum* in the Mediterranean region. Plant Ecol 145:157–165.

Filella I, Peñuelas J (2003) Partitioning of water and nitrogen in co-occurring Mediterranean woody shrub species of different evolutionary history. Oecologia 137: 51–61.

Filella I, Peñuelas J (2004) Indications of hydraulic lift by *Pinus halepensis* and its effects on the water relations of neighbour shrubs. Biol Plant 47:209–214.

Galiano L, Martínez-Vilalta J, Lloret F (2010) Drought-induced multifactor decline of Scots Pine in the Pyrenees and potential vegetation change by the expansion of co-occurring oak species. Ecosystems 13:978–991.

Garvelmann J, Külls C, Weiler M (2011) A porewater – based stable isotope approach for the investigation of subsurface hydrological processes. Hydrol Earth Syst Sci Discuss 8:9089–9112.

Gat J (1996) Oxygen and hydrogen isotopes in the hydrologic cycle. Annu Rev Earth Planet Sci 24:225–262.

Gibson JJ, Edwards TWD, Bursey GG (1993) Estimating evaporation using stable isotopes : quantitative results and sensitivity analysis for two catchments in Northern Canada. Nord Hydrol 24:79–94.

Goldsmith GR (2013) Changing directions : the atmosphere – plant – soil continuum. New Phytol 199:4–6.

Gómez-Aparicio L, García-Valdés R, Ruíz-Benito P, Zavala MA. (2011) Disentangling the relative importance of climate, size and competition on tree growth in Iberian forests: implications for forest management under global change. Glob Chang Biol 17:2400–2414.

Good SP, Noone D, Bowen G (2015) Hydrologic connectivity constrains partitioning of global terrestrial water fluxes. Science 349:175–177.

Gosz JR (1992) Ecological functions in a biome transition zone: translating local responses to broad-scale dynamics. In: Hansen AJ, di Castri F (eds), Landscape boundaries: consequences for biotic diversity and ecological flows. Springer New York, New York, NEW YORK,pp 55–75.

Granda E, Camarero JJ, Gimeno TE, Martínez-Fernández J, Valladares F (2013) Intensity and timing of warming and drought differentially affect growth patterns of co-occurring Mediterranean tree species. Eur J For Res 132:469–480.

Grossiord C, Sevanto S, Dawson TE, Adams HD, Collins AD, Dickman LT, Newman BD, Stockton EA, McDowell NG (2016) Warming combined with more extreme precipitation regimes modifies the water sources used by trees. New Phytol 213:584-596.

Gruber A, Pirkebner D, Florian C, Oberhuber W (2012) No evidence for depletion of carbohydrate pools in Scots pine (*Pinus sylvestris* L.) under drought stress. 14:142–148.

Gupta P, Noone D, Galewsky J, Sweeney C, Vaughn BH (2009) Demonstration of high-precision continuous measurements of water vapor isotopologues in laboratory and remote field deployments using wavelength-scanned cavity ring-down spectroscopy (WS-CRDS) technology. Rapid Commun Mass Spectometry 23:2534–2542.

Hill AJ, Dawson TE, Shelef O, Rachmilevitch S (2015) The role of dew in Negev Desert plants.

Oecologia 178:317–327.

Himrane H, Camarero J, Gil-Pelegrín E (2004) Morphological and ecophysiological variation of the hybrid oak *Quercus subpyrenaica* (*Q. faginea* x *Q. pubescens*). Trees 18:566–575.

IAEA/WMO (2001) Global Network of Isotopes in Precipitation. GNIP Database

Ingraham NL, Shadel C (1992) A comparison of the toluene distillation and vacuum/heat methods for extracting soil water for stable isotopic analysis. J Hydrol 140:371–387.

Irvine J, Perks MP, Magnani F, Grace J (1998) The response of *Pinus sylvestris* to drought: stomatal control of transpiration and hydraulic conductance. Tree Physiol 18:393–402.

Johnson LR, Sharp ZD, Galewsky J, Strong M, Van Pelt AD, Dong F, Noone D (2011) Hydrogen isotope correction for laser instrument measurement bias at low water vapor concentration using conventional isotope analyses: application to measurements from Mauna Loa Observatory, Hawaii. Rapid Commun Mass Spectrom 25:608–616.

Kelln CJ, Wassenaar LI, Hendry MJ (2001) Stable isotopes ($\delta^{18}O$, $\delta^{2}H$) of pore waters in clay-rich aquitards: A comparison and evaluation of measurement techniques. Gr Water Monit Remediat 21:108–116.

Klein T, Shpringer I, Fikler B, Elbaz G, Cohen S, Yakir D (2013) Relationships between stomatal regulation, water-use, and water-use efficiency of two coexisting key Mediterranean tree species. For Ecol Manage 302:34–42.

Koehler G, Wassenaar LI, Hendry MJ (2000) An automated technique for measuring δD and $\delta^{18}O$ values of porewater by direct CO_2 and H_2 equilibration. Anal Chem 72:5659–5664.

Koeniger P, Marshall JD, Link T, Mulch A (2011) An inexpensive, fast, and reliable method for vacuum extraction of soil and plant water for stable isotope analyses by mass spectrometry. Rapid Commun Mass Spectrom 25:3041–3048.

Landon MK, Delin GN, Komor SC, Regan CP (1999) Comparison of the stable-isotopic composition of soil water collected from suction lysimeters, wick samplers, and cores in a sandy unsaturated zone. J Hydrol 224:45–54.

Leaney FW, Smettem KRJ, Chittleborough DJ (1993) Estimating the contribution of preferential flow to subsurface runoff from a hillslope using deuterium and chloride. J Hydrol 147:83–103.

Lin GH, Sternberg LDL, Ehleringer JR, Hall AE, Farquhar GD (1993) Hydrogen isotopic fractionation by plant roots during water uptake in coastal wetland plants. In: Ehleringer, JR, Hall, AE, Farquhar, GD (eds), Stable isotopes and plant carbon–water relations. Academic Press Inc., San Diego, pp 497–510.

Lindner M, Maroschek M, Netherer S, Kremer A, Barbati A, García-Gonzalo J, Seidl R, Delzon S, Corona P, Kolström M, Lexer MJ, Marchetti M (2010) Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. For Ecol Manage 259:698–709.

Lis G, Wassenaar LI, Hendry MJ (2008) High-precision laser spectroscopy D/H and $^{18}O/^{16}O$ measurements of microliter natural water samples. Anal Chem 80:287–293.

De Luís M, Francisca García-Cano M, Cortina J, Raventós J, Carlos González-Hidalgo J, Rafael Sánchez J (2001) Climatic trends, disturbances and short-term vegetation dynamics in a Mediterranean shrubland. For Ecol Manage 147:25–37.

Máguas C, Rascher KG, Martins-Loução A, Carvalho P, Pinho P, Ramos M, Correia O, Werner C (2011) Responses of woody species to spatial and temporal ground water changes in coastal sand dune systems. Biogeosciences 8:3823–3832.

Maldonado F, Sainz Ollero H, Sánchez de Dios R, Xandri P (2001) Distribución y estado de conservación de los bosques españoles: un análisis de las carencias en la red de territorios protegidos. Camprodon y Plana (eds). Conservación de la biodiversidad y gestión forestal. Edicions de la Universitat de Barcelona, pp: 101-117.

Manion P (1991) Tree disease concepts. 2nd ed. Prentice Hall. New Jersey. 422 p.

Martínez-Vilalta J, Cochard H, Mencuccini M, Sterck F, Herrero A, Korhonen JFJ, Llorens P, Nikinmaa E, Nolè A, Poyatos R, Ripullone F, Sass-Klaassen U, Zweifel R (2009) Hydraulic adjustment of Scots pine across Europe. New Phytol 184:353–64.

Martínez-Vilalta J, Piñol J (2002) Drought-induced mortality and hydraulic architecture in pine populations of the NE Iberian Peninsula. For Ecol Manage 161:247–256.

Martínez-Vilalta J, Piñol J, Beven K (2002) A hydraulic model to predict drought-induced mortality in woody plants: An application to climate change in the Mediterranean. Ecol Modell 155:127–147.

McDonnell JJ (2014) The two water worlds hypothesis: ecohydrological separation of water between streams and trees? WIREs Water 2014, 1: 323-329.

McDowell NG (2011) Mechanisms linking dought, hydraulics, carbon metabolism, and vegetation mortality. Plant Physiol 155:1051–1059.

McDowell N, Pockman WT, Allen CD, Breshears DD, Cobb N, Kolb T, Plaut J, Sperry J, West A, Williams DG, Yepez E a (2008) Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? New Phytol 178:719–39.

Meißner M, Köhler M, Schwendenmann L, Hölscher D, Dyckmans J (2013) Soil water uptake by trees using water stable isotopes (δ^2H and δ^{18}O)−a method test regarding soil moisture, texture and carbonate. Plant Soil 376:327–335.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI (2012) Isotopes reveal contrasting water use strategies among coexisting plant species in a mediterranean ecosystem. New Phytol 196:489–496.

Mubarak A, Olsen RA (1976) Immiscible displacement of the soili solution by centrifugation. Soil Sci Soc Am J 40:329–331.

Munksgaard NC, Cheesman AW, Wurster CM, Cernusak LA, Bird MI (2014) Microwave extraction-isotope ratio infrared spectroscopy (ME-IRIS): A novel technique for rapid extraction and in-line analysis of δ^{18}O and δ^2H values of water in plants, soils and insects. Rapid Commun Mass Spectrom 28:2151–2161.

Nardini A, Pitt F (2002) Drought resistance of *Quercus pubescens* as a function of root hydraulic conductance, xylem embolism and hydraulic architecture. New Phytol 143: 485–493.

Nardini A, Salleo S, Jansen S (2011) More than just a vulnerable pipeline: xylem physiology in the light of ion-mediated regulation of plant water transport. J Exp Bot 62:4701–4718.

Oerter E, Finstad K, Schaefer J, Goldsmith GR, Dawson T, Amundson R (2014) Oxygen isotope fractionation effects in soil water via interaction with cations (Mg, Ca, K, Na) adsorbed to phyllosilicate clay minerals. J Hydrol 515:1–9.

Orlowski N, Frede H-G, Brüggemann N, Breuer L (2013) Validation and application of a cryogenic vacuum extraction system for soil and plant water extraction for isotope analysis. J Sensors Sens Syst 2:179–193.

Palacio S, Azorín J, Montserrat-Martí G, Ferrio JP (2014) The crystallization water of gypsum rocks is a relevant water source for plants. Nat Commun 5:4660.

Pan Y, Birdsey RA, Fang J, Houghton R, Kauppi PE, Kurz WA, Phillips OL, Shvidenko A, Lewis SL, Canadell JG, Ciais P, Jackson RB, Pacala SW, McGuire AD, Piao S, Rautiainen A, Sitch S, Hayes D (2011) A large and persistent carbon sink in the world's forests. Science 333:988–93.

Peñuelas J (1996) Overview on current and past global changes in the Mediterranean ecosystems. Orsis Org i Sist 11:165–175.

Peñuelas J, Filella I, Comas P (2002) Changed plant and animal life cycles from 1952 to 2000 in the Mediterranean region. Glob Chang Biol 8:531–544.

Peñuelas J, Terradas J, Lloret F (2011) Solving the conundrum of plant species coexistence: Water in space and time matters most. New Phytol 189:5–8.

Peters LI, Yakir D (2008) A direct and rapid leaf water extraction method for isotopic analysis. Rapid Commun Mass Spectrom 22:2929–2936.

Pfahl S, Sodemann H (2014) What controls deuterium excess in global precipitation? Clim Past 10:771–781.

Phillips SL, Ehleringer JR (1995) Limited uptake of summer precipitation by bigtooth maple (*Acer grandidentatum* Nutt) and Gambel's oak (*Quercus gambelii* Nutt). Trees Struct Funct 9:214–219.

Poyatos R, Aguadé D, Galiano L, Mencuccini M, Martínez-Vilalta J (2013) Drought-induced defoliation and long periods of near-zero gas exchange play a key role in accentuating metabolic decline of Scots pine. New Phytol 200:388–401.

Poyatos R, Llorens P, Piñol J, Rubio C (2008) Response of Scots pine (*Pinus sylvestris* L.) and pubescent oak (*Quercus pubescens* Willd.) to soil and atmospheric water deficits under Mediterranean mountain climate. Ann For Sci 65:306.

Prieto I, Armas C, Pugnaire FI (2012) Water release through plant roots: New insights into its consequences at the plant and ecosystem level. New Phytol 193:830–841.

Rambal S, Debussche G (1995) Water Balance of Mediterranean Ecosystems Under a Changing Climate. In: JM Moreno and WC Oechel (eds), Global Change and Mediterranean-Type Ecosystems. Springer-Verlag, New York, pp 386–407.

Ramírez-Valiente JA, Lorenzo Z, Soto A, Valladares F, Gil L, Aranda I (2009) Elucidating the role of genetic drift and natural selection in cork oak differentiation regarding drought tolerance. Mol Ecol 18:3803–3815.

Reichstein M, Tenhunen JD, Roupsard O, Ourcival JM, Rambal S, Dore S, Valentini R (2002) Ecosystem respiration in two Mediterranean evergreen Holm Oak forests: Drought effects and decomposition dynamics. Funct Ecol 16:27–39.

Resco de Dios V, Fischer C, Colinas C (2007) Climate change effects on mediterranean forests and preventive measures. New For 33:29–40.

Revesz K, Woods PH (1990) A method to extract soil water for stable isotope analysis. J Hydrol 115:397–406.

Sacchi E, Riva Roveda C, Facchinelli A, Defilippi A, Magnoni M, E A (2001) Geochemical and mineralogical evidence of the recent trophic evolution of a small peri-alpine lake (Lake Sirio, Ivrea, Northern Italy). 10th International Symposium on Water-Rock Interaction, Billasimius, Italy.

Sánchez-Salguero R, Linares JC, Camarero JJ, Madrigal-González J, Hevia A, Sánchez-Miranda A, Ballesteros-Cánovas JA, Alfaro-Sánchez R, García-Cervigón AI, Bigler C, Rigling A (2016) Disentangling the effects of competition and climate on individual tree growth: A retrospective and dynamic approach in Scots pine. J For Sci 358:53–64.

Sánchez de Dios R, Benito-Garzón M, Sainz-Ollero H (2009) Present and future extension of the Iberian submediterranean territories as determined from the distribution of marcescent oaks. Plant Ecol 204:189–205.

Saurer M, Aellen K, Siegwolf R (1997) Correlating δ^{13}C and δ^{18}O in cellulose of trees. Plant Cell Environ 20:1543–1550.

Schmidt M, Maseyk K, Lett C, Biron P, Richard P, Bariac T, Seibt U (2012) Reducing and correcting for contamination of ecosystem water stable isotopes measured by isotope ratio infrared spectroscopy. Rapid Commun Mass Spectrom 26:141–153.

Scholander PF, Hammel HT (1965) Sap pressure in vascular plants. Science 148:339–346.

Secchi F, Zwieniecki MA (2011) Sensing embolism in xylem vessels: The role of sucrose as a trigger for refilling. Plant, Cell Environ 34:514–524.

Sternberg LDSL, Swart PK (1987) Utilization of freshwater and ocean water by coastal plants of southern Florida. Ecology 68:1898–1905.

Sulzman EW (2008) Stable Isotope Chemistry and Measurement: A Primer. In: Michener RH, Lajtha K, (eds). Stable isotopes in ecology and environmental science. Malden, USA, pp 1–21.

Taiz L, Zeiger E (2010) Plant Physiology, Fifth Edition. Sinauer Associates Inc., Sunderland, 782 p.

Tang K, Feng X (2001) The effect of soil hydrology on the oxygen and hydrogen isotopic compositions of plants' source water. Earth Planet Sci Lett 185:355–367.

Tardieu F, Simonneau T (1998) Variability among species of stomatal control under fluctuating soil water status and evaporative demand: modelling isohydric and anisohydric behaviours. J Exp Bot 49:419–432.

Tognetti R, Cherubini P, Marchi S, Raschi A (2007) Leaf traits and tree rings suggest different water-use and carbon assimilation strategies by two co-occurring *Quercus* species in a Mediterranean mixed-forest stand in Tuscany, Italy. Tree Physiol 27:1741–51.

Treydte K, Boda S, Graf Pannatier E, Fonti P, Frank D, Ullrich B, Saurer M, Siegwolf R, Battipaglia G, Werner W, Gessler A (2014) Seasonal transfer of oxygen isotopes from precipitation and soil to the tree ring: Source water versus needle water enrichment. New Phytol 202:772–783.

Tucker CJ, Sellers PJ (1986) Satellite remote-sensing of primary production. Int J Remote Sens 7:1395–1416.

Tyree MT (1997) The Cohesion-Tension theory of sap ascent: current controversies. J Exp Bot 48:1753–1765.

Tyree MT, Zimmermann MH (2002) Xylem structure and the ascent of sap. In: TE Timell (eds), Springer Series in Wood Science, 2nd Ed. Springer-Verlag, Berlin, Germany.

Valladares F, Vilagrosa A, Peñuelas J, Ogaya R, Julio J, Corcuera L, Sisó S (2004) Estrés hídrico: ecofisiología y escalas de la sequía. In: Valladares F (eds). Ecología del bosque mediterráneo en un mundo cambiante. Naturaleza y Parques Nacionales. Ministerio de Medio Ambiente. Madrid, pp 165-192.

Vendramini PF, Sternberg LDSL (2007) A faster plant stem-water extraction method. Rapid Commun Mass Spectrom 21:164–168.

Volkmann THM, Weiler M (2014) Continual in situ monitoring of pore water stable isotopes in the subsurface. Hydrol Earth Syst Sci 18:1819–1833.

Voltas J, Lucabaugh D, Chambel MR, Ferrio JP (2015) Intraspecific variation in the use of water sources by the circum-Mediterranean conifer *Pinus halepensis*. New Phytol 208:1031–41.

Walker GR, Woods PH, Allison GB, Orlowski N, Frede H-G, Brüggemann N, Breuer L (1994) Interlaboratory comparison of methods to determine the stable isotope composition of soil water. Chem Geol 111:297–306.

Wassenaar LI, Hendry MJ, Chostner VL, Lis GP (2008) High resolution pore water δ^2H and δ^{18}O measurements by H_2O (liquid) − H_2O (vapor) equilibration laser spectroscopy. Environ Sci Technol 42:9262–9267.

West AG, Dawson TE, February EC, Midgley GF, Bond WJ, Aston TL (2012) Diverse functional responses to drought in a Mediterranean-type shrubland in South Africa. New Phytol 195:396–407.

West A, Goldsmith G, Brooks P, Dawson T (2010) Discrepancies between isotope ratio infrared spectroscopy and isotope ratio mass spectrometry for the stable isotope analysis of plant and soil waters. Rapid Commun Mass Spectometry 24:1948–1954.

West AG, Goldsmith GR, Matimati I, Dawson TE (2011) Spectral analysis software improves confidence in plant and soil water stable isotope analyses performed by isotope ratio infrared spectroscopy (IRIS). Rapid Commun Mass Spectrom 25:2268–2274.

West AG, Patrickson SJ, Ehleringer JR (2006) Water extraction times for plant and soil materials used in stable isotope analysis. Rapid Commun Mass Spectrom 20:1317–1321.

Williams DG, Cable W, Hultine K, Hoedjes JCB, Yepez EA, Simonneaux V, Er-Raki S, Boulet G, De Bruin HAR, Chehbouni A, Hartogensis OK, Timouk F (2004) Evapotranspiration components determined by stable isotope, sap flow and eddy covariance techniques. Agric For Meteorol 125:241–258.

Yakir D, Sternberg LSL (2000) The use of stable isotopes to study ecosystem gas exchange. Oecologia 123:297–311.

Yepez EA, Williams DG, Scott RL, Lin G (2003) Partitioning overstory and understory evapotranspiration in a semiarid savanna woodland from the isotopic composition of water vapor. Agric For Meteorol 119:53–68.

Zhu QZ, Sun Q, Su ZG, Xie MM, Song JY, Shan YB, Wang N, Chu GQ (2014) A soil water extraction method with accelerated solvent extraction technique for stable isotope analysis. Chinese J Anal Chem 42:1270–1275.

Zweifel R, Rigling A, Dobbertin M (2009) Species-specific stomatal response of trees to drought - A link to vegetation dynamics? J Veg Sci 20:442–454.

OBJECTIVES

The specific objectives of this thesis can be grouped in two main blocks:

1. **To analyze the effect of contaminants and isotopic uncoupling between precipitation, soil and xylem water, as the main methodological limitations in ecohydrological studies based on stable isotopes:**

    1.1. To assess the interference of organic contaminants in water isotopic composition analysed with the commonly used Isotope-Ratio Infrared Spectroscopy (IRIS). Particularly, we aimed to test how the post-processing correction and the sample pre-treatment through the micro-combustion module (MCM) improved the performance of IRIS in soil and xylem samples, and establish a protocol of analysis and correction when contaminated samples.

    1.2. To determine whether fast and reversible evaporative enrichment in metabolically active stems can occur as a consequence of a temporal decline in sap flow rates, and to analyze the possible causes (evaporation from stem, feedback effect of leaf water) of the changes in xylem isotopic composition.

    1.3. To evaluate the causes for a generalized uncoupling between soil water and previous precipitation or irrigation event, pointing towards to the existence of different non-well mixed water pools in soil. Also, to try to define which soil water fraction (mobile, weakly or tightly bounded) each soil extraction method captures and which fraction can be used by plants.

2. **To analyze the ecohydrology and ecophysiology of submediterranean forests in a climate change context:**

    2.1. To assess species-specific physiological behaviour in terms of hydraulic and carbon assimilation limitations during drought conditions

    2.2. To relate differences in species-specific physiological strategies to water uptake patterns analyzed by water stable isotopes.

    2.3. To analyze the response of these species in the recovery after drought events, focusing on the vulnerability to xylem embolism.

    2.4. To determine the long term performance of these species in the area in terms of growth patterns and the effects of climate change projections in their decay or survivorship.

CHAPTER I.

Isotope-Ratio Infrared Spectroscopy: a reliable tool for the investigation of plant-water sources?

Paula Martín-Gómez[1]; Adrià Barbeta[2,3]; Jordi Voltas[1]; Josep Peñuelas[2,3]; Kate Dennis[4]; Sara Palacio[5]; Todd E Dawson[6]; Juan Pedro Ferrio[1]

[1]Dept. Crop and Forest Sciences-AGROTECNIO Center, Universitat de Lleida, E-25198 Lleida, Spain; [2]CSIC, Global Ecology Unit CREAF-CSIC-UAB, E-08193 Cerdanyola del Valles (Catalonia), Spain; [3]CREAF, E-08193 Cerdanyola del Vallès (Catalonia), Spain; [4]Product Manager for Isotopic Water, Picarro Inc., Santa Clara, California 95054, USA; [5]Instituto Pirenaico de Ecología (IPE-CSIC), E-22700 Jaca, Spain; [6]Department of Integrative Biology, University of California, Berkeley, California 94720 USA

SUMMARY

Stable isotopes are extensively used as tracers for the study of plant-water sources. Isotope-ratio infrared spectroscopy (IRIS) offers a cheaper alternative to isotope-ratio mass spectroscopy (IRMS), but its use in plant and soil water is limited by the spectral interference caused by organic contaminants. Here, we examine two approaches to cope with contaminated samples in IRIS: on-line oxidation of organic compounds (MCM) and post-processing correction. We assessed these methods compared to IRMS across 136 samples of xylem and soil water and a set of ethanol- and methanol-water mixtures. A post-processing correction improved significantly IRIS accuracy in both natural samples and alcohol dilutions, being effective with concentrations up to 8% of ethanol and 0.4% of methanol. MCM outperformed the post-processing correction in removing methanol interference, but was not effective for high concentrations of ethanol. By using both approaches IRIS can overcome with reasonable accuracy the analytical uncertainties associated to most organic contaminants found in soil and xylem water. We recommend the post-processing correction as the first choice for the analysis of samples of unknown contamination. Nevertheless, MCM can be more effective for samples containing contaminants responsible of strong spectral interferences from small concentrations, such as methanol.

Keywords:

CRDS, ecohydrology, $\delta^{18}O$, $\delta^{2}H$, IRIS, IRMS, soil, xylem

INTRODUCTION

The stable isotope composition of oxygen ($\delta^{18}O$) and hydrogen ($\delta^{2}H$) in xylem water is widely used as a tracer for the study of plants and fungi water uptake and redistribution (Ehleringer and Dawson 1992, Dawson 1996, Warren et al. 2008, Lilleskov et al. 2009, Dawson and Simonin 2011, Moreno-Gutiérrez et al. 2012, Prieto et al. 2012, Palacio, Azorín, et al. 2014, Treydte et al. 2014). Recently, the widespread use of isotope-ratio mass spectrometry (IRMS) technology for measuring water isotopes has been challenged by the development of isotope-ratio infrared spectroscopy (IRIS). IRIS methods provide isotopic compositions of water samples by spectroscopy, taking advantage of the different absorption spectra of water isotopologues in the gaseous phase (Lis et al. 2008, Gupta et al. 2009). This allows the simultaneous measurement of $^{1}H_2{}^{16}O$, $^{1}H_2{}^{18}O$, and $^{1}H^{2}H^{16}O$ with an accuracy comparable to IRMS, at least when analysing pure water (Lis et al. 2008, Brand et al. 2009, Gupta et al. 2009, West et al. 2010, 2011). IRIS, unlike IRMS, does not need the prior chemical equilibration or conversion into elemental constituents that often limits precision (Brand et al. 2009, Schultz et al. 2011, Schmidt et al. 2012). IRIS also offers other advantages such as lower cost, easier installation and maintenance, and higher portability (Brand et al. 2009, Gupta et al. 2009, Berman et al. 2009, West et al. 2010, Johnson et al. 2011, Schmidt et al. 2012).

However, some organic contaminants significantly interfere with the water-isotope spectrum in IRIS analyses (Brand et al. 2009, West et al. 2010, 2011). Organics are broadly found together with water in plant and soil samples, and cryogenic distillation, which is the most common method for extracting water from plant and soil matrices, frequently co-distils them. The magnitude of the error caused by organic interference is not only proportional to the amount of contaminant, but also depends on its spectral properties; for some compounds, the associated analytical errors may become unacceptable starting at very small concentrations (e.g. <0.1% for methanol (Brand et al. 2009)). In contrast, the magnitude of the errors associated with contaminants in IRMS depends on the mass-balance contribution of the contaminant to the pool of H and O atoms in the sample. Hence, substantial errors can only be expected for IRMS if the concentration of the contaminant is high and/or the isotopic composition of the organic compound differs strongly from that of water (Brand et al. 2009, West et al. 2010).

IRIS manufacturers have developed software applications that can identify and flag potentially contaminated samples (e.g. Spectral Contamination Identifier, Los Gatos Research, Inc., Mountain View, CA; ChemCorrect™, Picarro Inc., Santa Clara, CA). In the case of Picarro's, firstly ChemCorrect™ compares the measured spectral profile of the sample with that of small molecules such as methane and methanol contained in its library. If the features match, the compound concentration is calculated. Afterwards, the software uses a set of quantitative spectral indicators (mainly spectral baseline and slope) to generate information of larger organics, such as ethanol and other alcohols, included in a 'C_{2+} alcohols' pool (Picarro 2010, Richman et al. 2010). Thus, a set of organic-corrected spectra is currently available for post-processing correction in the raw data files of L2110-*i* and L2120-*i* models. Later Picarro models (L2130-*i* and L2140-*i* analysers) do not include this information in the raw data files, but a new post-fit correction for these models is expected to be released in the future (Picarro development team, personal communication). The protocol for deriving corrected isotopic values from the spectral data is available for registered users in the Picarro forum. However, it has not been extensively validated due to limited accessibility and, therefore, not widely used to date.

More recently, Picarro Inc. has developed the Micro-Combustion Module™ (MCM) to remove organic compounds interfering with pure water; the MCM uses high-temperature oxidation to eliminate problematic contaminants in the water sample (Picarro 2012, Saad et al. 2013). Briefly, once a sample is evaporated the entire gaseous phase is swept in a carrier gas across the heated metal catalyst in which oxidation efficiently converts the organics into minute quantities of CO_2 and nascent water. This procedure is expected to eliminate most common alcohols and other plant contaminants of low molecular weight, including multicomponent mixtures of alcohols and terpenes, and green-leaf volatiles. Its optimal efficacy is claimed to be achieved for samples containing total organics at concentrations lower than 0.5%, with a complete elimination for higher concentrations not entirely guaranteed. However, the effectiveness of the MCM pre-treatment and post-processing corrections in soil and xylem samples still requires full testing.

We present here the first evaluation of the performance of MCM using an array of soil and xylem samples from a wide range of sites and species; these results are compared with standard IRIS analyses without the MCM installed. We also present the first validation of a post-processing method, based on ChemCorrect^{TM} post-fit spectral information, to reduce the effects of organic contamination on water isotopic analysis. Both MCM and post-processed values are validated in field-collected samples against IRMS, and further tested by using a set of standard dilutions of two representative contaminants (methanol, MeOH, and ethanol, EtOH).

MATERIALS AND METHODS

Sample collection and water extraction

We tested 136 samples from 26 species (xylem samples) and 8 sites (soil samples). The samples were collected from a range of Mediterranean-type ecosystems in Spain and the USA (Table 1) following the same standard procedure (Moisture Isotopes in the Biosphere and Atmosphere -MIBA- protocol from International Atomic Energy Agency -IAEA-, available at http://www-naweb.iaea.org/napc/ih/IHS_resources_miba.html). The species belonged to 13 families, which were subsequently used as main taxonomic units. Sunlit twigs were harvested near midday, bark and phloem were removed, and the xylem was immediately sealed in glass vials (air-tight tubes, Duran GL-18). Soil samples from different depths were simultaneously collected and were also rapidly sealed in glass vials. The samples were placed on dry ice in the field and kept frozen until processing.

The extraction of water from the soil and xylem samples was performed by cryogenic vacuum distillation (Dawson and Ehleringer 1993). Samples from the Iberian Peninsula were processed at the Dept. of Crop and Forest Sciences, Universitat de Lleida (Spain). The extraction system consisted of 10 sample tubes connected with Ultra-Torr™ fittings (Swagelok Company, Solon, Ohio, USA) to 10 U-shaped collection tubes specifically designed for this system. The sample tubes were submerged in mineral oil at a constant temperature (110-120ºC) to evaporate water and the U-tubes were cooled with liquid nitrogen to condense the water vapour. The extraction system was connected to a vacuum pump (model RV3; Edwards, Bolton, UK) to guarantee the flow of water vapour from the sample tubes to the collection tubes and to prevent contamination with atmospheric water vapour. The entire system maintained constant vacuum pressures of *ca.* 10^{-2} mbar. Distillation of sample collected in the USA was conducted at the Center for Stable Isotope Biogeochemistry at the University of California, Berkeley, CA, USA, using the same procedure but with a slightly different design (Goldsmith et al. 2012).

Ethanol- and methanol-water mixtures

To determine the influence that organic contaminants may have on the analysis of isotope ratios of water using either IRIS or IRMS, we prepared a set of mixtures with different concentrations of two organic compounds, EtOH and MeOH, representative of broadband (baseline) and narrowband spectral interference, respectively (Schultz et al. 2011, Leen et al. 2012). The mixtures were used as known “reference” samples by mixing EtOH or MeOH with water of known isotopic composition ($\delta^{18}O$ = -9.48‰, $\delta^{2}H$ = -65.05‰). EtOH was mixed with water at concentrations of 0.5, 1, 2, 4, and 8%

(vol./vol.) and MeOH at 0.1, 0.2, 0.4, 0.8, and 1.6% (vol./vol.). An additional set of dilutions was prepared combining both compounds at two concentrations (2 and 8% EtOH with 0.4 and 1.6% MeOH). The same set of mixtures was used to fit linear regressions for 1) predicting the error associated with varying contaminant concentration, as proposed in earlier studies (Brand et al. 2009, Schultz et al. 2011, Leen et al. 2012), and 2) estimating MeOH-equivalent and EtOH-equivalent concentrations in natural samples. For this purpose, we took the unitless contaminant levels determined by ChemCorrect™ and identified as 'ORGANIC_MEOH_AMPL' (for MeOH) and 'ORGANIC_BASE' (for EtOH) in the raw output files (.csv).

Isotopic analyses

We analysed the water isotopes of the xylem and soil samples and of the standard dilutions by IRMS and IRIS. Isotopic ratios were expressed relative to international standard (VSMOW, Vienna Standard Mean Ocean Water) in per mil notation (‰) (*i.e.* isotopic composition):

$$\delta^{18}\mathrm{O} \text{ or } \delta^{2}\mathrm{H} = \left(R_{\mathrm{sample}} / R_{\mathrm{standard}} - 1\right) \times 1000 \qquad (1)$$

where R_{sample} and R_{standard} are the heavy to light isotopic ratios (^{2}H/H and ^{18}O/^{16}O) of the sample and the standard, respectively.

IRMS methods

We used three different methods for IRMS analysis: (1) δ^{18}O and δ^{2}H by high temperature pyrolysis (labelled as TCEA), conducted at the Paul Scherrer Institute (Villigen, Switzerland); (2) δ^{18}O by CO_2 headspace equilibration using a GasBench II system (labelled as GB; Thermo Finnigan, Bremen); and (3) δ^{2}H by reduction over chromium using an H/Device (labelled as HDEV; Thermo Finnigan, Bremen). The latter two methods were applied at the Center for Stable Isotope Biogeochemistry (Berkeley, CA, USA). For determining δ^{18}O and δ^{2}H by high-temperature pyrolysis (1), a 0.6 µl aliquot of the water sample was injected into a High Temperature Combustion Elemental Analyzer (TC/EA, Thermo Finnigan, Bremen). The water was reduced at 1450ºC on glassy carbon to H_2 and CO, and these components were then carried in a helium stream to the mass spectrometer (Delta plus XP, Thermo Finnigan, Bremen). The hydrogen isotope ratio was determined from the ^{2}H/^{1}H ratio of the H_2 molecule, and the oxygen isotope ratio was determined from the ^{12}C^{18}O/^{12}C^{16}O ratio of the CO molecule. The precision of this method (± standard error of replicates of reference samples) was estimated to be <0.2‰ for δ^{18}O and <1.0‰ for δ^{2}H. In the GasBench method (2), water samples were equilibrated with a 0.2% CO_2 headspace in Helium for

48 h at 21-23ºC and later inserted into the GasBench II system connected to the Delta Plus XL mass spectrometer, which measured the $^{18}O/^{16}O$ ratio from the CO_2. The precision was about 0.12‰ for $\delta^{18}O$. In the chromium combustion method (3), microlitre quantities of water were injected into the H/Device and reduced to H_2 gas. The $^{2}H/H$ ratio of this gas was measured by the coupled Delta Plus mass spectrometer. The precision for this method was about 0.80‰ for $\delta^{2}H$.

IRIS methods

The IRIS analyses used L2120-*i* and L1102-*i* isotopic water analysers (Picarro Inc., Sunnyvale, CA, USA) available at the Serveis Científico-Tècnics of the Universitat de Lleida (Lleida, Spain) and at the Center for Stable Isotope Biogeochemistry of the University of California (Berkeley, USA) respectively. The L2120-*i* was coupled to an A0211 high-precision vaporiser, and the L1102-*i* was coupled to a V1102-*i* vaporisation module. One microlitre of water was injected into a vaporisation chamber, and the vapour was then passed into an infrared absorbance cavity. The hydrogen and oxygen isotope ratios were calculated by measuring the decay time of laser light at specific wavelengths on the cavity and by reference to the absorption peaks of the three most abundant isotopologues of water ($H_2{}^{16}O$, $HD^{16}O$, and $H_2{}^{18}O$) (Cavity Ring-Down Spectroscopy – CRDS (Gupta et al. 2009)). The estimated precision for the L2120-*i*, based on the repeated analysis of 4 reference water samples was 0.10‰ and 0.40‰, for $\delta^{18}O$ and $\delta^{2}H$, respectively. The long-term external precision for the L1102-*i* is 0.14‰ for $\delta^{18}O$ and 1.0‰ for $\delta^{2}H$.

Micro-Combustion Module

After the analysis of water samples with both L2120-*i* and L1102-*i* set with default settings, the MCM was installed to reanalyse a subset of 79 samples representing most plant species and soil samples. The MCM was integrated in-line between the Picarro vaporiser and the L2120-*i* water-isotope analyser at the Serveis Científico-Tècnics of the Universitat de Lleida. Samples with a small amount of water available after long-term storage were discarded to avoid potential fractionation effects.

Spectral analysis of IRIS data: ChemCorrect™ and post-processing correction

A first quality assessment of the spectral IRIS data was made by running the PostProcess ChemCorrect™, version 1.2.0 (Picarro Inc., Santa Clara, CA) with "chemcorrect_inst avg_orgeval_06.csv" instruction file. This software does not perform corrections on contaminated data but assigns metrics describing the magnitude of the contamination and its potential source. The software also includes flagging indicating the degree of potential contamination by a colour code: green for uncontaminated samples, yellow for possibly contaminated samples (*i.e.* warranting further attention), and red for very contaminated samples (*i.e.* designating unreliable results).

As described in Picarro's forum (link only available for registered users: http://www.picarro.com/community/picarro_community/applying_corrections_to_contaminated_water_isotope_measurements_using_ch#comment-964) the raw output files (.csv extension) from the Picarro analysers also provide values of $H_2{}^{18}O$, $HD^{16}O$, and $H_2{}^{16}O$ peaks filtered by the spectral features of organic compounds (columns 'ORGANIC_77', 'ORGANIC_82' and 'SPLINEMAX', respectively). The values of the filtered peaks can be converted to organic-corrected $\delta^{18}O$ and δ^2H by applying unit-specific factory calibration settings (*slope* and *offset*) as:

$$\delta^2\mathrm{H} = slope \times \left(\mathrm{HD^{16}O} / \mathrm{H_2{}^{16}O}\right) + offset \tag{2}$$

$$\delta^{18}\mathrm{O} = slope \times \left(\mathrm{H_2{}^{18}O} / \mathrm{H_2{}^{16}O}\right) + offset \tag{3}$$

The values for *slope* and *offset* can be found in the file "Picarrocrds.ini" for Picarro L11xx-*i* units, and the files "InstrCal_Air.ini" and "InstrCal_N2.ini" (measuring in air and N_2, respectively) for L21xx-*i* units. After including these formulae in a custom-made Excel spreadsheet, we ended up with two columns with the pre-existing uncorrected values (labelled as 'd(18_16)Mean' and 'd(D_H)Mean' in the original .csv file), plus two new columns of post-processed values. In both cases, calibration was then performed by fitting a linear regression to two sets of three internal laboratory standards included in each batch, using the same custom-made Excel spreadsheet. It should be noted that we did not use the results from the calibration procedure included in ChemCorrect™, since this could only be applied to the original, uncorrected values.

Data analysis

Differences between IRMS, uncorrected IRIS and post-processed IRIS measurements were estimated using mixed models based on Restricted Maximum Likelihood (REML) estimations for both δ^{18}O and δ^{2}H ($\alpha = 0.05$). Type of analysis (IRMS, IRIS uncorrected, IRIS post-processed, MCM and MCM plus post-processing), type of sample (plant family or soil) and their interaction were considered as fixed factors, while species within family and sample ID were taken as random factors. The effectiveness of the different methods in field-collected samples was assessed by the determination coefficient (R^2) of the linear regression between IRIS and IRMS values, and the root mean square error (RMSE), calculated as follows:

$$\mathrm{RMSE} = \sqrt{\left(Y_{\mathrm{IRIS}} - Y_{\mathrm{IRMS}}\right)^2 / N} \quad (4)$$

Where Y_{IRIS} and Y_{IRMS} stand for measured IRIS and IRMS, respectively, and N is the number of samples. Hence, we assumed that IRMS provided “true” values and also uniform results across IRMS methods. Indeed, a previous study (West et al. 2010) reported very consistent isotopic ratios among IRMS methods (HDEV, TCEA, and GB), with discrepancies lower than the range of long-term instrument precision. To assess the capacity of ChemCorrect™ to flag contaminated samples and to better understand the limitations of each method, we plotted the differences between IRIS and IRMS values by plant family and ChemCorrect™ category.

For the batch of standard dilutions of MeOH and EtOH, the error was directly calculated as the difference between the measured value of each dilution (both IRIS and IRMS) and that of pure water analysed by IRMS. As a broad quality threshold for method comparison, we adopted the values of the maximum accepted bias (MAB) applied in the most recent proficiency test for the analysis of water isotopes coordinated by the Isotope Hydrology Section of the IAEA (±0.8‰ and ±6‰ for δ^{18}O and δ^{2}H, respectively; http://nucleus.iaea.org/rpst/ReferenceProducts/Proficiency_Tests/IAEA-TEL-2011-01/index.htm, M. Groening, pers. comm.). Other studies have proposed narrower limits for accuracy on hydrological studies (e.g. ±0.2‰ for δ^{18}O and ±2‰ for δ^{2}H, as used in the IAEA inter-laboratory test WICO2011, see Wassenaar et al. 2012 or ±0.15‰ for δ^{18}O and ±1‰ for δ^{2}H in Wassenaar et al. 2014). However, since in our study we compared different methods and different laboratories among them, and not against a reference value, we considered more informative to use a broader threshold as primary assessment. In any case, in order to overcome the limitations associated to the use of arbitrary thresholds to identify a proper methodology we also assessed the distribution of errors among the samples using a histogram with 0.2‰ and 2‰ classes for $\delta^{18}O$ and $\delta^{2}H$, respectively. Statistical analyses were performed with JMP Pro 11 (SAS Inc., Cary, NC, USA).

RESULTS

Effect of contaminants on water isotopic composition and correction methods: ethanol- and methanol-water mixtures

Table 2 shows the range of deviations of IRIS and IRMS values from IRMS analysis of pure water (used as reference value) for the set of mixtures. The errors associated with mixtures at different MeOH and EtOH concentrations are shown in Fig. 1. MeOH/water mixtures analysed by IRIS differed substantially from the reference value starting at the lowest contaminant concentration (0.1% MeOH), with maximum discrepancies between the uncorrected IRIS and the IRMS reference value as large as -142.96 and -1077‰ for $\delta^{18}O$ and $\delta^{2}H$, respectively. In contrast, EtOH did not interfere as strongly with pure water, even at very high concentrations (up to 8%). Maximum differences were -0.39‰ for $\delta^{18}O$ and -10.76‰ for $\delta^{2}H$. The error exceeded the established maximum bias for $\delta^{2}H$ only at concentrations of 8%. Similarly, the interferences caused by MeOH and EtOH mixtures on water isotopic signatures were mostly due to MeOH, as any particular combination of EtOH and MeOH produced a deviation in isotopic signatures similar to that using MeOH alone. The maximum errors for EtOH and MeOH mixtures (-147.06‰ for $\delta^{18}O$ and -1104.64‰ for $\delta^{2}H$ at 1.6% MeOH and 2% EtOH) were thus comparable to the error for the highest MeOH concentration (1.6% MeOH). In contrast, MeOH caused negligible effects on IRMS values within the range of concentrations used, whereas we found larger errors for IRMS than for IRIS with EtOH concentrations starting at 4% for $\delta^{18}O$ and 1% for $\delta^{2}H$.

The post-processing correction of contaminant interference for L2110-*i* and L2120-*i* reallocated the IRIS values within threshold limits (±0.8 and ±6‰ for $\delta^{18}O$ and $\delta^{2}H$, respectively) for MeOH concentrations below 0.8% for $\delta^{18}O$ and 0.2% for $\delta^{2}H$. For EtOH, the correction always increased analytical accuracy, even though uncorrected values were usually within MAB limits (Fig. 1). The removal of organic interferences by the MCM improved the accuracy of IRIS values for both isotopes in the MeOH dilutions for contaminant concentrations up to 0.8%, but for EtOH dilutions the MCM tended to produce larger errors than the non-treated IRIS for concentrations ≥2%. In mixed dilutions, the MCM was clearly influenced by the quantity of EtOH at equal MeOH concentrations (Fig. 1). The effect of small amounts of residual MeOH after MCM pre-treatment in the highest concentration levels was generally corrected by post-processing, but the treatment of EtOH produced overcorrected values (Fig. 1).

Effect of contaminants on water isotopic composition and correction methods: natural samples

We found significant differences in isotopic compositions between uncorrected IRIS and IRMS values for the complete set of 136 samples analysed (Table 3). The maximum discrepancies between methods were -17.25‰ ($\delta^{18}O$) and -78.08‰ ($\delta^{2}H$) for soil samples, and -8.34‰ ($\delta^{18}O$) and -92.19‰ ($\delta^{2}H$) for xylem samples. In particular, 20% ($\delta^{18}O$) and 22% ($\delta^{2}H$) of the samples fell outside the limits of the MAB, and about 10% showed very strong negative deviations (below -2‰ and -20‰, for $\delta^{18}O$ and $\delta^{2}H$, respectively, see Fig. 2a,b). After post-processing, differences in isotopic compositions between IRIS and IRMS values were still significant for $\delta^{18}O$ but became non-significant for $\delta^{2}H$ (Table 3). The maximum differences were -1.79‰ for $\delta^{18}O$ and +26.74‰ for $\delta^{2}H$ in soil samples and +1.76‰ for $\delta^{18}O$ and +8.55‰ for $\delta^{2}H$ in xylem samples. Overall, the number of samples outside the MAB decreased to 7% ($\delta^{18}O$) and 4% ($\delta^{2}H$). Deviations from IRMS values produced a slight (although within MAB limits) positive bias (Fig. 2c,d).

Considering only the subset of 79 samples reanalysed with the MCM, we also found significant differences in isotopic compositions between uncorrected IRIS and IRMS values (Table 3). Within this subset, 29% ($\delta^{18}O$) and 27% ($\delta^{2}H$) of the samples were originally outside the threshold values of the MAB. This percentage decreased to 9% ($\delta^{18}O$) and 4% ($\delta^{2}H$) after post-processing correction and to 5% ($\delta^{18}O$) and 6% ($\delta^{2}H$) with MCM pre-treatment (IRIS plus MCM, Table 2). In this regard, there were no significant differences between the pre-treatment and the software correction methods, both being statistically equivalent to IRMS. Besides, we also did not find significant differences between post-processing correction after MCM operation (IRIS plus MCM post-processed) and the other combinations (IRIS post-processed and IRIS plus MCM alone) (Table 3). However, the MCM pre-treatment produced a larger number of samples having systematic positive errors (although still within MAB limits) than the post-processing correction (Fig. 2e,f), resulting in a histogram clearly biased to positive values, particularly for $\delta^{18}O$. For the MCM with post-processing, differences in isotopic compositions between IRIS and IRMS were slightly higher than those without post-processing, but in the range of the other two combinations, with 10% ($\delta^{18}O$) and 6% ($\delta^{2}H$) of samples outside the MAB and similar positive bias (Fig. 2g,h).

The correction based on linear regression of the water mixtures was less successful than the post-processing correction or the removal of organics by MCM. For the MeOH concentration only ('ORGANIC_MEOH_AMPL' column in the raw Picarro output files), the regression-based correction placed 13% ($\delta^{18}O$) and 26% ($\delta^{2}H$) of collected samples outside the MAB. Adding a second correction based on EtOH concentration ('ORGANIC_BASE' column) produced very similar results (data not shown).

Relationships between IRMS- and IRIS-based approaches for stable isotopes in water

Fig. 3a,b compares IRMS and IRIS values (before and after post-processing correction) for the entire dataset (N=136). Goodness-of-fit statistics (R^2 and root mean square error, RMSE) of the linear regressions between IRIS and IRMS indicated that the post-processing correction eliminated most discrepancies due to organic interference, even for highly contaminated samples.

Table 4 shows the statistics of the linear regressions between IRIS and IRMS values for the subset of samples analysed with the MCM for each category of ChemCorrect™ contamination. An important improvement in R^2 and a concomitant decrease in the RMSE were observed after activating the MCM, indicating an effective removal of interferences caused by contamination with organics. Considering the ChemCorrect™ categories, R^2 increased from 0.06 to 0.89 ($\delta^{18}O$) and from 0 to 0.88 (δ^2H) for the red-flagged samples. For the yellow-flagged samples, R^2 increased from 0.69 to 1 ($\delta^{18}O$) and from 0.69 to 0.99 (δ^2H). Moreover, 43 and 83% of the samples first flagged as yellow and red, respectively, were classified as green after MCM operation.

Elimination of contaminants by the MCM

We found a strong correspondence between known alcohol concentrations and ChemCorrect™ quantification values for MeOH (R^2 = 0.99) and EtOH (R^2 = 0.99). In our set of mixtures, 1% MeOH corresponded to approximately 0.1 units in the ‘ORGANIC_MEOH_AMPL’ column and 1% EtOH corresponded to approximately 245 units in the ‘ORGANIC_BASE’ column. We applied these equivalences in order to compare the effectiveness of the MCM to remove contaminants in alcohol-water mixtures and natural samples. Mean values for equivalent MeOH and EtOH (%) concentration for each family and ChemCorrect™ flagging category are shown in Fig. 4. Equivalent MeOH concentrations in samples ranged from 0 to 0.06% for xylem water, and from 0 to 0.32% for soil samples. Equivalent EtOH concentrations ranged from 0 to 6.7% in the xylem, and from 0 to 0.03% for soil samples. These values were within the range of the set of standard dilutions (0.1-1.6% for MeOH and 0.5-8% for EtOH). MeOH was nearly completely eliminated by the MCM in both the natural samples and the set of mixtures; the estimated maximum residual concentration was 0.01% for samples and up to 0.09% for 1.6% MetOH dilutions. In contrast, the MCM was more effective at removing the EtOH from the artificial mixtures than at eliminating 'C_{2+} alcohols' in soil and xylem samples. Despite having higher initial concentrations, the residual EtOH-equivalent concentration was about one order of magnitude lower in the mixtures (mean, 0.016%; maximum, 0.03%) than in the samples (mean, 0.17%; maximum, 3.9%; see Fig. 4). The higher residual concentrations in samples, however, did not produce higher deviations from IRMS values (compare Fig.1 for artificial mixtures with Figs. 5 and 6 for the natural samples).

Contaminant effects among plant families

Figs. 5a,b,c,d and 6a,b,c,d illustrate the differences in isotopic compositions ($\delta^{18}O$ and $\delta^{2}H$, respectively) between IRIS and IRMS values (δ_{IRIS}- δ_{IRMS}) among plant families. These differences were generally negative in the most contaminated samples (see Fig. 4). Both MCM operation and post-processing correction increased the agreement between IRIS and IRMS and reallocated most samples within the established MAB threshold. The MCM, however, produced a systematic positive bias in δ_{IRIS}-δ_{IRMS} differences in almost all plant families.

DISCUSSION

A simple IRIS post-processing reduces contaminant interference

As previously reported, the isotopic composition of some natural samples analysed by IRIS showed strong negative deviations from IRMS values (Brand et al. 2009, West et al. 2010, 2011, Zhao et al. 2011, Schmidt et al. 2012). Differences were particularly high for soil samples as compared to other studies (West et al. 2010, Zhao et al. 2011), being in the range of previously published values for xylem samples (West et al. 2010, 2011, Zhao et al. 2011, Schmidt et al. 2012). Nevertheless, it should be noted that the most contaminated samples (differences < -2‰ in $\delta^{18}O$) corresponded to downhill and valley-bottom soils in a gypsum-rich area, characterized by the accumulation of solutes and mineral nutrients, contrasting with the limited nutrient availability in the top of the hills (Guerrero-Campo et al. 1999, Palacio et al. 2014). Hence, the potential interference of electrolytes in soils with IRIS measurements may require a more detailed assessment.

The post-processing correction proposed by Picarro strongly reduced the effects of contamination, even in cases of heavily contaminated samples. As expected, the correction limits for MeOH were relatively low due to its strong spectral interference (deviations were within MAB up to concentrations of 0.4% and 0.1% MetOH for $\delta^{18}O$ and $\delta^{2}H$, respectively). Conversely, the deviation of corrected values was below the MAB even at the highest tested concentration of EtOH (8%). For xylem and soil samples, the post-processing correction reduced the discrepancies between IRIS and IRMS from RMSEs of 2.42‰ to 0.42‰ for $\delta^{18}O$ and of 18.46‰ to 3.95‰ for $\delta^{2}H$, and reallocated more than 70% of highly deviating samples within MAB limits. Nevertheless, a closer look at the error distribution (Fig. 2c,d) reveals a positive error bias of about 0.2‰ for $\delta^{18}O$ and 2‰ for $\delta^{2}H$. This bias, however, is in the range of the expected additive effect of laboratory uncertainties and potential sample alteration during transport and storage. In fact, we also found slightly positive differences between IRIS and IRMS for the pure water samples used for the set of alcohol-water mixtures (up to +0.36‰ for $\delta^{18}O$, and +0.89‰ for $\delta^{2}H$, see Fig. 1). In this regard, our results show the potential of post-processing correction methods as a way to solve contaminant issues for IRIS, but also encourage a more exhaustive assessment of their accuracy, e.g. following the robust procedures of global inter-laboratory tests.

MCM: effectiveness and limitations

An overall reduction in the maximum differences between IRIS and IRMS values in the natural samples was obtained with the MCM in operation, even for highly contaminated samples. The RMSE decreased to 0.54‰ for $\delta^{18}O$ and 3.52‰ for $\delta^{2}H$. More than 75% of samples initially placed outside the MAB fell within this threshold after using the MCM. The post-processing correction and the MCM were generally equally effective for $\delta^{18}O$ analysis in the presence of MeOH contamination, but the post-processing correction was less precise for $\delta^{2}H$ (Table 4). Indeed, when methanol was the main contaminant (as in methanol-water mixtures and in contaminated soil samples; Fig. 4a), MCM seemed to outperform the post-processing correction (Fig. 1, Fig. 5b,c, and Fig. 6b,c). In contrast, the post-processing correction was consistently more effective than the MCM at removing errors associated with C_{2+} alcohols such as EtOH (see Fig. 1). Furthermore, using the MCM a substantial proportion of samples showed positive deviations between 0.4‰ and 0.8‰ for $\delta^{18}O$, and between 2‰ and 6‰ for $\delta^{2}H$ (Fig. 2e,f). This positive bias is likely to be a collateral effect of the contaminant removal. MCM oxidation converts organic compounds into CO_2 and nascent water by using an air carrier gas supported by ambient O_2. For each EtOH molecule, the MCM generates three water molecules that mix with the water in the sample. In this reaction the hydrogen atoms originate from the alcohol, whereas the oxygen mostly comes from the carrier gas ($\delta^{18}O_{air} = +23.8 \pm 0.3‰$ (Coplen et al. 2002)). If the alcohol content in the sample is sizeable, the MCM significantly alters the isotopic signature of the water proportionally to (i) the relative mass contribution of the hydrogen and oxygen atoms of the sample water and that of the water formed through chemical oxidation of alcohols, and (ii) their corresponding isotopic signatures. Our results were consistent with this expectation, with more biased values for $\delta^{18}O$ than for $\delta^{2}H$ analysed by the MCM in comparison to IRMS, due to the very positive oxygen isotopic composition of air. Large errors can consequently be generated at high concentrations of organic contaminants in the samples because the oxidation process adds new water molecules to the water pool, despite effectively reducing spectral interference. We would thus recommend post-processing correction instead of MCM operation when analysing samples of unknown composition or with expectedly high concentrations of EtOH and longer-chain alcohols.

The MCM nearly completely eliminated MeOH and EtOH from the artificial mixtures, but was less effective at removing the C_{2+} alcohols pool from natural samples (presumably including ethanol derived from anaerobic metabolism, terpenols and other volatiles, see refs. in (Niinemets U. and Monson R.K. (eds) 2013). Despite this, the artificial mixtures still produced divergences beyond the MAB for concentrations above 0.4% MeOH and 2% EtOH, due to the side-effects of the oxidation process. Conversely, we could not establish a clear threshold for natural samples based on estimated contaminant concentration (Fig. 4). Although the concentrations of C_{2+} alcohols remaining after MCM operation were higher in the natural samples than in the mixtures, the spectral interference was significantly lower in the samples. This could be attributed to a limited interference of other C_{2+} alcohols as compared to EtOH.

We also tested the possibility of applying post-processing correction to samples previously treated by micro-combustion to improve the performance of the MCM. The post-processing correction, however, apparently overcorrected the isotopic values, with the exception of highly contaminated samples with residual MeOH (see Fig. 2g,h). The MCM was designed to remove the spectral interference of organic compounds at low concentrations. In samples with high concentrations of contaminants (e.g. EtOH dilutions) the organic interference is effectively removed by the MCM, but at the expense of altering the isotope composition of water. Hence, although post-processing may still correct the spectral interferences caused by remaining alcohols after MCM operation, the resulting "corrected" values will be those of the isotopically-altered water. The development of an integrated post-processing correction would thus be advisable (e.g. considering spectral information before and after MCM operation) as a way to account for changes in water isotope composition caused by the MCM.

Spectral post-processing outperforms previously proposed empirical corrections

Previous studies have proposed correction curves as a function of the degree of contaminant concentration (Brand et al. 2009, Schultz et al. 2011, Leen et al. 2012). Schultz et al. (2011) eliminated (for $\delta^{18}O$) or reduced (for $\delta^{2}H$) the discrepancies between IRIS and IRMS results using an LGR Liquid Water Isotope Analyzer (Los Gatos Research Inc., Mountain View, CA). The recommended curves, however, did not match those provided by the manufacturer, so the authors suggested that every analyser could require a customized correction. Brand et al. (2009) also performed regressions of δ-values and contaminant concentrations for a set of standard dilutions and concluded that corrections of isotopic values are feasible provided the alcohol content in the samples is known. We consequently corrected the isotopic values by using linear regressions to predict IRIS δ-errors for pure water as a function of contaminant concentration according to the CH_3OH and C_{2+} alcohol outputs from ChemCorrect™. The precision however, was lower than that obtained through post-processing correction, and the most contaminated samples were usually extremely overcorrected, resulting in very high isotopic values. Post-processing correction based on peaks filtered by ChemCorrect™ seems thus a more suitable alternative than the correction based on organic concentrations in samples.

In spite of this, corrections based on estimated MeOH concentrations still improved the accuracy of isotopic records, but the calibrations performed with the EtOH dilutions did not work well for natural samples. This could be due to the fact that MeOH concentration can be specifically quantified based on a well-defined peak, whereas EtOH produces mainly a baseline drift in the spectra, and is measured together with a pool of long-chain alcohols. Similarly, Brand et al. (2009) found no relationship between EtOH concentration and δ-value in wine due to interference from contaminants such as MeOH, phenols, or organic acids.

CONCLUSIONS

According to our results, the post-processing correction of isotope values based on spectral analyses improves significantly the performance of IRIS in soil and xylem samples, thus allowing detailed ecohydrological studies at a reasonable cost. In particular, differences between IRMS and IRIS-corrected values fell within reasonable limits in most field-collected samples (>90%). According to our dilution tests, interferences associated to organic contaminants can be successfully removed with concentrations up to 8% and 0.4% for EtOH and MeOH, respectively. Sample pre-treatment through the MCM slightly outperforms post-processing correction in removing MeOH interference. Nevertheless, for heavily MeOH-contaminated samples, the best results would be obtained combining both methods, which together may be able to correct samples with up to 1.6% MeOH contamination. In contrast, the MCM was not effective in removing EtOH interference: with high concentrations of contaminant the module causes significant changes in the isotope composition of water (particularly strong for $\delta^{18}O$). Hence, for contaminated samples we generally recommend to adopt post-processing correction in isotopic analyses, and only when the main (and mostly unique) contaminant detected is MeOH (as in our soil samples), the use of MCM (eventually combined with post-processing correction).

ACKNOWLEDGEMENTS

This research was supported by the Spanish Government projects CGL2013-48074-P, AGL 2012-40039-C02 and AGL 2012-40151-C03, the Catalan Government project SGR 2014-274 and the European Research Council Synergy grant ERC-2013-SyG-610028 IMBALANCE-P. The Spanish Government funded the FPU predoctoral fellowship to P.M.G., the FPI predoctoral fellowship and travel grant to A.B., and the Ramón y Cajal Programme to J.P.F. (RYC-2008-02050). We thank Gregor Hsiao for sharing the post-processing correction, and for his useful advice. We thank Pilar Sopeña, Mireia Oromí, Paul Brooks, Rolf Siegwolf and Matthias Saurer for their technical support with isotope analyses. We also thank the useful comments from Margaret Barbour and three anonymous referees, who made a substantial contribution to the revised manuscript.

Author contributions

P.M.G., J.P.F., A.B. and J.V. planned and designed the research; P.M.G., J.P.F., A.B. and S.P. collected the data; P.M.G., J.P.F., A.B. and J.V. analysed the data and P.M.G., J.P.F., A.B., J.V., J.P., S.P., T.E.D. and K.D. wrote the manuscript.

REFERENCES

Berman ESF, Gupta M, Gabrielli C, Garland T, McDonnell JJ (2009) High-frequency field-deployable isotope analyzer for hydrological applications. Water Resour Res 45:10.

Brand WA, Geilmann H, Crosson E, Rella C (2009) Cavity ring-down spectroscopy versus high-temperature conversion isotope ratio mass spectrometry; a case study on δ^2H and δ^{18}O of pure water samples and alcohol/water mixtures. Rapid Commun Mass Spectrom 23:1879–1884.

Coplen TB, Bohlke JK, De Bievre P, Ding T, Holden NE, Hopple JA, Krouse HR, Lamberty A, Peiser HS, Revesz K, Rieder SE, Rosman KJR, Roth E, Taylor PDP, Vocke RD, Xiao YK (2002) Isotope-abundance variations of selected elements (IUPAC Technical Report). Pure Appl Chem 74:1987–2017.

Dawson TE (1996) Determining water use by trees and forests from isotopic, energy balance and transpiration analyses: the roles of tree size and hydraulic lift. Tree Physiol 16:263–272.

Dawson TE, Ehleringer JR (1993) Isotopic enrichment of water in the ‘woody’ tissues of plants: Implications for plant water source, water uptake, and other studies which use the stable isotopic composition of cellulose. Geochim Cosmochim Acta 57:3487–3492.

Dawson TE, Simonin KA (2011) The roles of stable isotopes in forest hydrology and biogeochemistry: synthesis of research and future directions. In: Levis D, Carlyle-Moses D, Tanaka T (eds), Forest hydrology and biogeochemistry, Ecological. Springer Netherlands, pp 137–161.

Ehleringer JR, Dawson TE (1992) Water uptake by plants: perspectives from stable isotope composition. Plant, Cell Environ 15:1073–1082.

Goldsmith GR, Muñoz-Villers LE, Holwerda F, Mcdonnell JJ, Asbjornsen H, Dawson TE (2012) Stable isotopes reveal linkages among ecohydrological processes in a seasonally dry tropical montane cloud forest. Ecohydrology 5:779–790.

Guerrero-Campo J, Alberto F, Hodgson J, García-Ruiz JM, Montserrat-Martí G (1999) Plant community patterns in a gypsum area of NE Spain. I. Interactions with topographic factors and soil erosion. J Arid Environ 41:401–410.

Gupta P, Noone D, Galewsky J, Sweeney C, Vaughn BH (2009) Demonstration of high-precision continuous measurements of water vapor isotopologues in laboratory and remote field deployments using wavelength-scanned cavity ring-down spectroscopy (WS-CRDS) technology. Rapid Commun Mass Spectometry 23:2534–2542.

Johnson LR, Sharp ZD, Galewsky J, Strong M, Van Pelt AD, Dong F, Noone D (2011) Hydrogen isotope correction for laser instrument measurement bias at low water vapor concentration using conventional isotope analyses: application to measurements from Mauna Loa Observatory, Hawaii. Rapid Commun Mass Spectrom 25:608–616.

Leen JB, Berman ESF, Liebson L, Gupta M (2012) Spectral contaminant identifier for off-axis integrated cavity output spectroscopy measurements of liquid water isotopes. Rev Sci Instrum 83:0445305.

Lilleskov EA, Bruns TD, Dawson TE, Camacho FJ (2009) Water sources and controls on water-loss rates of epigeous ectomycorrhizal fungal sporocarps during summer drought. New Phytol 182:483–494.

Lis G, Wassenaar LI, Hendry MJ (2008) High-precision laser spectroscopy D/H and ^{18}O/^{16}O measurements of microliter natural water samples. Anal Chem 80:287–293.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI (2012) Isotopes reveal contrasting water use strategies among coexisting plant species in a mediterranean ecosystem. New Phytol 196:489–496.

Niinemets U. and Monson R.K. (eds) (2013) Biology, controls and models of tree volatile organic compound emissions, Tree Physi. Springer ScienceBusiness.

Palacio S, Azorín J, Montserrat-Martí G, Ferrio JP (2014a) The crystallization water of gypsum rocks is a relevant water source for plants. Nat Commun 5:4660.

Palacio S, Maestro M, Montserrat-Martí G (2014b) Differential nitrogen cycling in semiarid sub-shrubs with contrasting leaf habit. PLoS One 9:e93184.

Picarro (2010) ChemCorrectTM - Solving the Problem of Chemical Contaminants in H_2O Stable Isotope Research. White Pap:2–4.

Picarro (2012) Micro-Combustion Module^{TM} (MCM): Elimination of organics datasheet.

Prieto I, Armas C, Pugnaire FI (2012) Water release through plant roots: New insights into its consequences at the plant and ecosystem level. New Phytol 193:830–841.

Richman BA, Hsiao GS, Rella C (2010) Detecting and Eliminating Interfering Organic Compounds in Waters Analyzed for Isotopic Composition by CRDS. AGU Fall Meet Abstr 1:379.

Saad N, Hsiao G, Chapellet-Volpini L, Vu D (2013) Two-pronged approach to overcome spectroscopically interfering organic compounds with isotopic water analysis. EGU Gen Assem Conf Abstr 15:13296.

Schmidt M, Maseyk K, Lett C, Biron P, Richard P, Bariac T, Seibt U (2012) Reducing and correcting for contamination of ecosystem water stable isotopes measured by isotope ratio infrared spectroscopy. Rapid Commun Mass Spectrom 26:141–153.

Schultz NM, Griffis TJ, Lee X, Baker JM (2011) Identification and correction of spectral contamination in $^{2}H/^{1}H$ and $^{18}O/^{16}O$ measured in leaf, stem, and soil water. Rapid Commun Mass Spectrom 25:3360–3368.

Treydte K, Boda S, Graf Pannatier E, Fonti P, Frank D, Ullrich B, Saurer M, Siegwolf R, Battipaglia G, Werner W, Gessler A (2014) Seasonal transfer of oxygen isotopes from precipitation and soil to the tree ring: Source water versus needle water enrichment. New Phytol 202:772–783.

Warren JM, Brooks JR, Meinzer FC, Eberhart JL (2008) Hydraulic redistribution of water from Pinus ponderosa trees to seedlings: Evidence for an ectomycorrhizal pathway. New Phytol 178:382–394.

Wassenaar LI, Ahmad M, Aggarwal P, van Duren M, Pöltenstein L, Araguas L, Kurttas T (2012) Worldwide proficiency test for routine analysis of $\delta^{2}H$ and $\delta^{18}O$ in water by isotope-ratio mass spectrometry and laser absorption spectroscopy. Rapid Commun mass Spectrom 26:1641–1648.

Wassenaar LI, Coplen TB, Aggarwal PK (2014) Approaches for achieving long-term accuracy and precision of $\delta^{18}O$ and $\delta^{2}H$ for waters analyzed using laser absorption spectrometers. Environ Sci Technol 48:1123–1131.

West A, Goldsmith G, Brooks P, Dawson T (2010) Discrepancies between isotope ratio infrared spectroscopy and isotope ratio mass spectrometry for the stable isotope analysis of plant and soil waters. Rapid Commun Mass Spectometry 24:1948–1954.

West AG, Goldsmith GR, Matimati I, Dawson TE (2011) Spectral analysis software improves confidence in plant and soil water stable isotope analyses performed by isotope ratio infrared spectroscopy (IRIS). Rapid Commun Mass Spectrom 25:2268–2274.

Zhao L, Xiao H, Zhou J, Wang L, Cheng G, Zhou M, Yin L, McCabe MF (2011) Detailed assessment of isotope ratio infrared spectroscopy and isotope ratio mass spectrometry for the stable isotope analysis of plant and soil waters. Rapid Commun Mass Spectrom 25:3071–3082.

TABLES AND FIGURES

Table 1. Description of the plant species and soil samples used in this study. Soil description according to FAO classification.

Species/Soil type	Family	Season	Origin	No. of samples	No. of samples in the MCM subset
Arbutus unedo L.	Ericaceae	Fall, winter & summer	Catalonia	5	3
Artemisia herba-alba Asso	Asteraceae	Spring	Aragon	1	1
Baccharis pilularis DC.	Asteraceae	Fall	California	7	5
Buxus sempervirens L.	Buxaceae	Summer	Aragon	3	3
Cistus clusii Dunal	Cistaceae	Fall	Murcia	3	0
Erica arborea L.	Ericaceae	Winter & summer	Catalonia	4	2
Erica multiflora L.	Ericaceae	Fall, winter & summer	Valencia	5	1
Fagus sylvatica L.	Fagaceae	Summer	Catalonia	1	1
Helianthemum squamatum (L.) Dum. Cours.	Cistaceae	Spring	Aragon	4	4
Lepidium subulatum L.	Brassicaceae	Spring	Aragon	3	3
Linum suffruticosum L.	Linaceae	Spring	Aragon	1	1
Stipa tenacissima L.	Poaceae	Fall & spring	Andalusia	3	2
Phillyrea latifolia L.	Oleaceae	Fall, winter & summer	Catalonia	4	1
Phlomis purpurea sub. *almeriensis* Pau	Lamiaceae	Fall	Andalusia	2	0
Pinus halepensis Mill.	Pinaceae	Fall	Murcia	2	0
Pinus sylvestris L.	Pinaceae	Summer	Aragon	4	4
Pistacia lentiscus L.	Chenopodiaceae	Winter	Catalonia	2	1
Quercus agrifolia Née	Fagaceae	Fall	California	3	3
Quercus coccifera L.	Fagaceae	Winter	Catalonia	1	0
Quercus douglasii Hook. & Arn.	Fagaceae	Fall	California	3	1
Quercus ilex L.	Fagaceae	All	Catalonia	13	6
Quercus kelloggii Newb.	Fagaceae	Fall	California	9	6
Quercus lobata Née	Fagaceae	Fall	California	14	7
Quercus subpyrenaica Villar	Fagaceae	Summer	Aragon	4	4
Suaeda pruinosa Lange	Chenopodiaceae	Spring	Aragon	1	1
Umbellularia californica Hook. & Arn.	Lauraceae	Fall	California	5	4
TOTAL xylem				**107**	**64**
Calcaric Leptosol		Winter & summer	Catalonia	5	3
Calcic Cambisol		Summer	Aragon	2	2
Dystric Cambisol		Spring	Catalonia	5	0
Dystric Leptosol		Spring & summer	Catalonia	7	0
Gypsiric Regosol		Spring	Aragon	4	4
Gypsisol/Solonchak		Spring	Aragon	6	6
TOTAL soil				**29**	**15**
TOTAL				136	79

Table 2. Range (minimum value; maximum value) of δ^{18}O and δ^{2}H discrepancies between IRIS and IRMS, and number of samples within the maximum accepted bias (MAB) used in the last proficiency test of the International Atomic Energy Agency (±0.8‰ for δ^{18}O and ±6‰ for δ^{2}H, IAEA-TEL-2011-01, see text for details) for IRIS, IRIS plus post-processing correction, and IRIS plus the MCM (either uncorrected or post-processed) in standard dilutions and the subset (N=79) of xylem and soil water samples.

IRIS

		Uncorrected				Post-processed			
Sample type	No. total samples	Error δ^{18}O (‰)	No. within MAB	Error δ^{2}H (‰)	No. within MAB	Error δ^{18}O (‰)	No. within MAB	Error δ^{2}H (‰)	No. within MAB
Standard dilutions									
MeOH	5	(-142.96; -8.64)	0	(-1077.00; -64.58)	0	(-2.15; -0.01)	3	(3.35; 44.39)	1
EtOH	5	(-0.39; 0.20)	5	(-10.76; 0.49)	4	(0.13; 0.46)	5	(-4.38; 0.56)	5
MeOH+EtOH	4	(-147.06; -39.65)	0	(-1104.64; -298.72)	0	(-2.29; -0.38)	2	(6.98; 45.39)	0
Collected samples									
Xylem	64	(-8.34; 0.85)	48	(-92.19; 6.17)	50	(-0.33; 1.43)	60	(-1.01; 8.55)	62
Soil	15	(-17.25; 0.24)	8	(-78.08; 4.99)	8	(-1.79; 0.49)	12	(0.13; 6.66)	14

IRIS plus MCM

		Uncorrected				Post-processed			
Sample type	No. total samples	Error δ^{18}O (‰)	No. within MAB	Error δ^{2}H (‰)	No. within MAB	Error δ^{18}O (‰)	No. within MAB	Error δ^{2}H (‰)	No. within MAB
Standard dilutions									
MeOH	5	(-8.61; 0.41)	3	(-77.01; 1.21)	3	(0.31; 0.90)	4	(-2.96; 1.90)	5
EtOH	5	(0.22; 2.08)	3	(-19.45; -0.59)	3	(0.41; 2.48)	3	(-17.24; -0.07)	3
MeOH+EtOH	4	(0.31; 2.44)	2	(-23.08; -10.27)	0	(1.11; 3.08)	0	(-19.38; -4.64)	1
Collected samples									
Xylem	64	(-0.92; 1.21)	60	(-8.97; 8.78)	60	(0.01; 1.36)	56	(0.57; 8.91)	60
Soil	15	(-0.09; 0.79)	15	(0.16; 6.5)	14	(-0.09; 0.8)	15	(0.4; 7.45)	14

Table 3. *P*-values and *F*-ratio of the statistical comparisons ($\alpha = 0.05$, mixed models based on Restricted Maximum Likelihood, REML) between correction methods for the complete dataset ($N = 136$) and a subset of natural samples ($N = 79$) for both $\delta^{18}O$ and $\delta^{2}H$.

		$\delta^{18}O$		$\delta^{2}H$	
		F-ratio	*P*-value	*F*-ratio	*P*-value
Complete dataset *N*=136	IRMS vs. IRIS uncorrected	9.6875	0.0061*	4.5324	0.0390 *
	IRIS post-processed vs. IRIS uncorrected	29.4845	<0.0001*	8.2688	0.0062 *
	IRMS vs. IRIS post-processed	5.3707	0.0327 *	0.5574	0.4593
Subset *N*=79	IRMS vs. IRIS uncorrected	4.2368	0.0443 *	4.7005	0.0341 *
	IRIS post-processed vs. IRIS uncorrected	6.6844	0.0124 *	8.3796	0.0053 *
	IRIS+MCM vs. IRIS uncorrected	8.7293	0.0046 *	8.0996	0.0060 *
	IRMS vs. IRIS post-processed	0.2778	0.6002	0.5281	0.4702
	IRMS vs. IRIS+MCM	0.8032	0.374	0.4596	0.5004
	IRIS post-processed vs. IRIS+MCM	0.1363	0.7134	0.0024	0.9613
	IRIS+MCM vs. IRIS+MCM post-processed	0.1213	0.7289	0.1488	0.7010

Table 4. Summary statistics of the relationship between IRMS and IRIS values for the subset of natural samples analysed with the MCM (*N*=79) within each ChemCorrect™ contamination categories. *N*, Number of samples, R^2 coefficient of determination for the linear regression between IRIS and IRMS, RMSE (‰); Root mean square error of the difference between IRMS and IRIS values.

	ChemCorrect Category	Green			Yellow			Red			All		
	IRMS-IRIS Linear regression	*N*	R^2	RMSE	*N*	R^2	RMSE	*N*	R^2	RMSE	*N*	R^2	RMSE
$\delta^{18}O$	IRIS	37	0.97	0.53	13	0.69	1.43	29	0.06	5.09	79	0.31	3.16
	IRIS post-processed		0.99	0.27		0.97	0.54		0.92	0.56		0.97	0.44
	IRIS+MCM	56	0.99	0.54	11	1.00	0.58	12	0.89	0.50	79	0.98	0.54
	IRIS+MCM post-processed		0.99	0.58		1.00	0.62		0.98	0.75		0.99	0.62
$\delta^{2}H$	IRIS	37	0.89	3.33	13	0.69	5.87	29	0.00	38.34	79	0.05	23.46
	IRIS post-processed		0.98	2.45		0.96	2.50		0.95	3.60		0.96	2.93
	IRIS+MCM	56	0.97	3.55	11	0.99	2.62	12	0.88	4.05	79	0.93	3.52
	IRIS+MCM post-processed		0.97	4.00		0.98	3.06		0.99	3.55		0.97	3.82

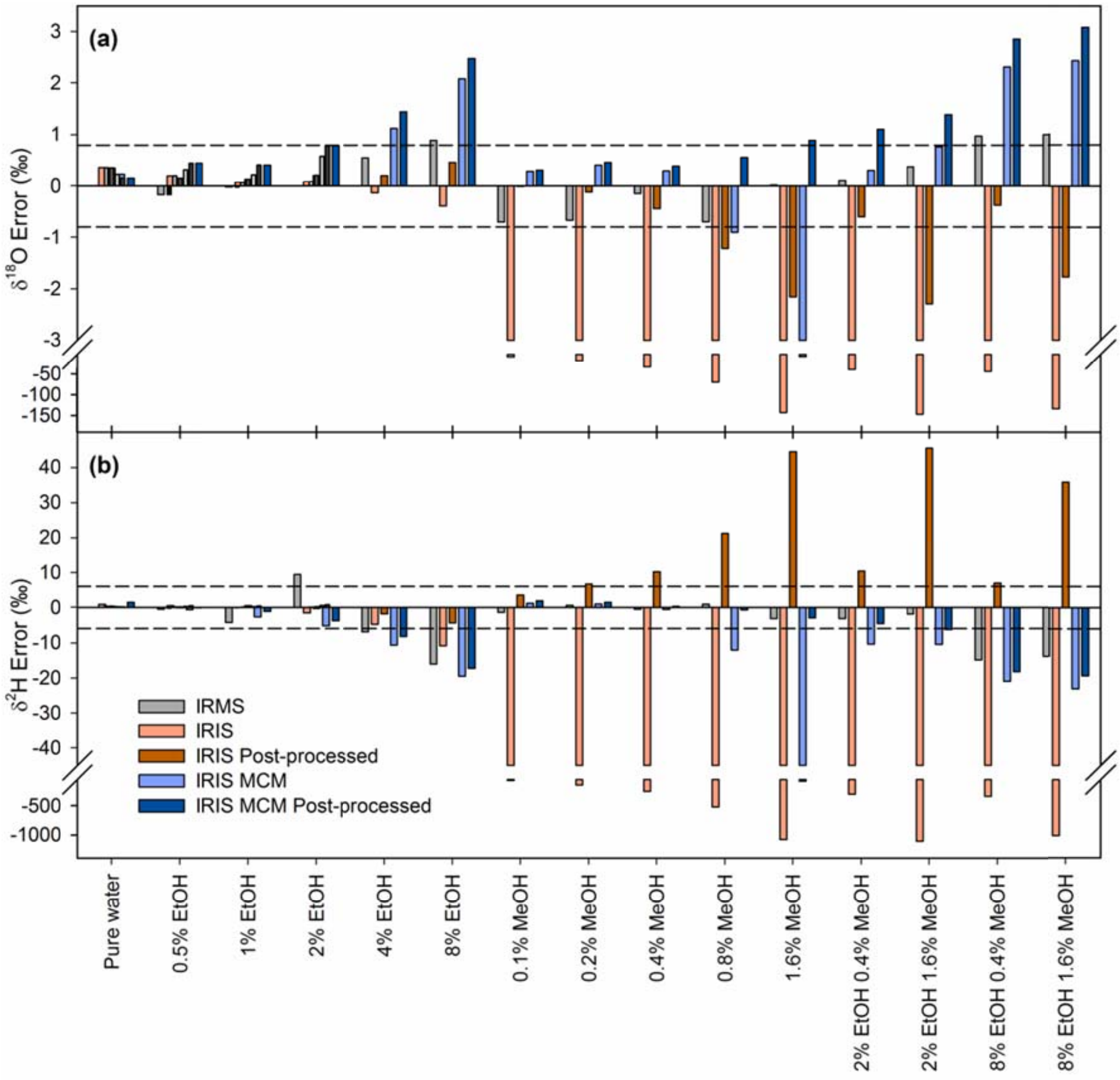

Figure 1. Errors for $\delta^{18}O$ (a) and $\delta^{2}H$ (b) in ‰, associated with various methanol (MeOH) and ethanol (EtOH) concentrations in the alcohol-water mixtures. The errors have been calculated as the differences between the dilutions and pure water analysed by IRMS. Dashed lines represent the accuracy thresholds based on the maximum accepted bias (MAB) established by the International Agency of Atomic Energy.

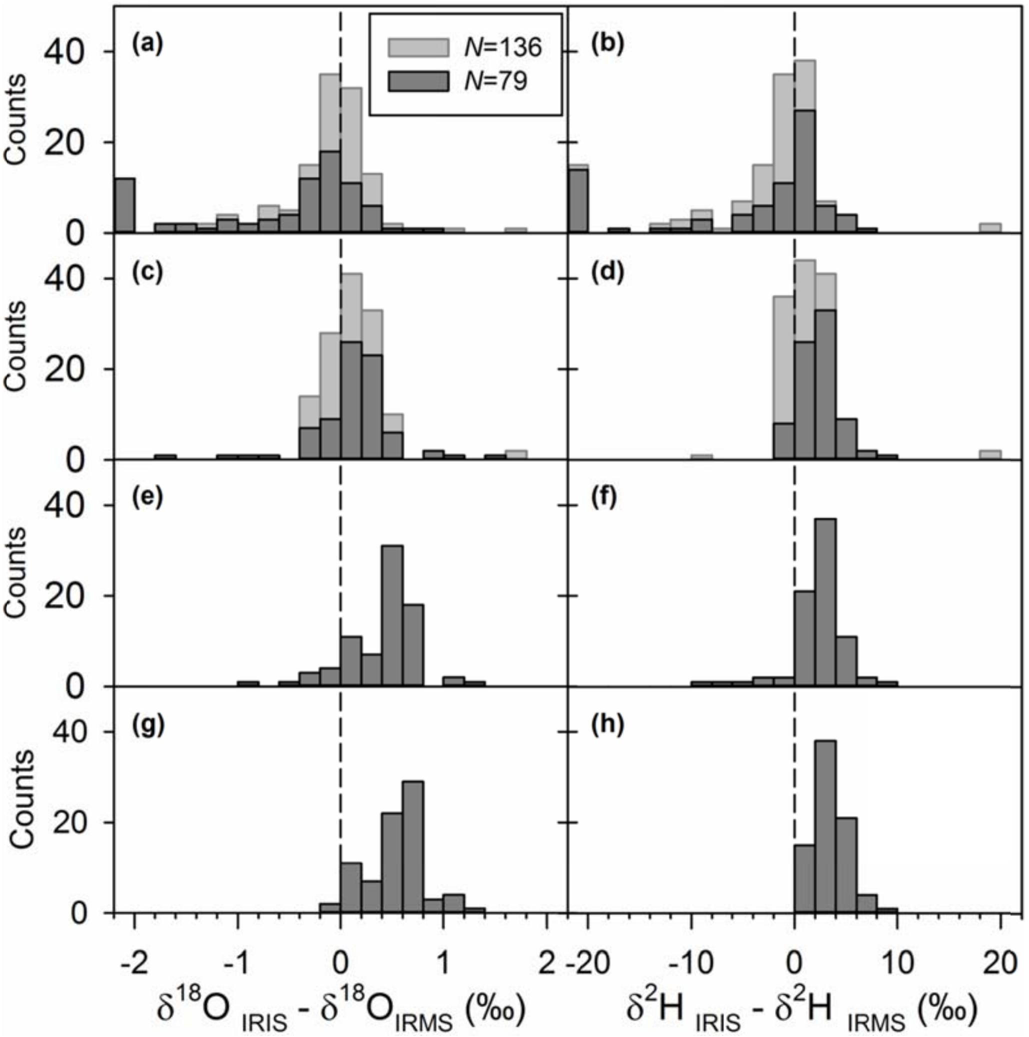

Figure 2. Histogram representing the distribution of deviations between IRIS and IRMS for $\delta^{18}O$ (left panels) and $\delta^{2}H$ (right panels). (a,b) IRIS uncorrected; (c,d) IRIS post-processed; (e,f) IRIS plus MCM; (g,h) IRIS plus MCM plus post-processing. The samples were grouped into 0.2‰ and 2‰ intervals, for $\delta^{18}O$ and $\delta^{2}H$, respectively. For simplicity, heavily deviated samples (outside the [-2‰ +2‰] range for $\delta^{18}O$ and the [-20‰ +20‰] range for $\delta^{2}H$) were included in a single bin. Light grey: whole dataset. Dark grey: subsample used for the assessment of the MCM.

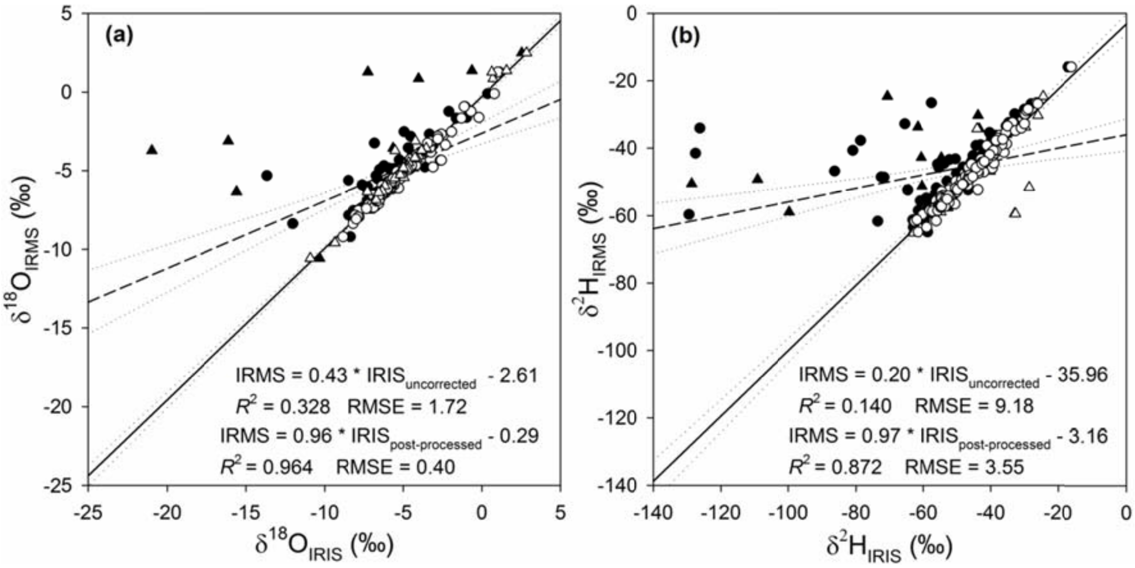

Figure 3. Comparison between IRIS and IRMS for uncorrected (filled symbols) and post-processed (empty symbols) $\delta^{18}O$ values (a) and $\delta^{2}H$ values (b) using 136 field samples. Linear regression equations, R^2 and RMSEs are also presented. Dotted lines represent 95% confidence intervals. Circles, xylem samples; Triangles, soil samples.

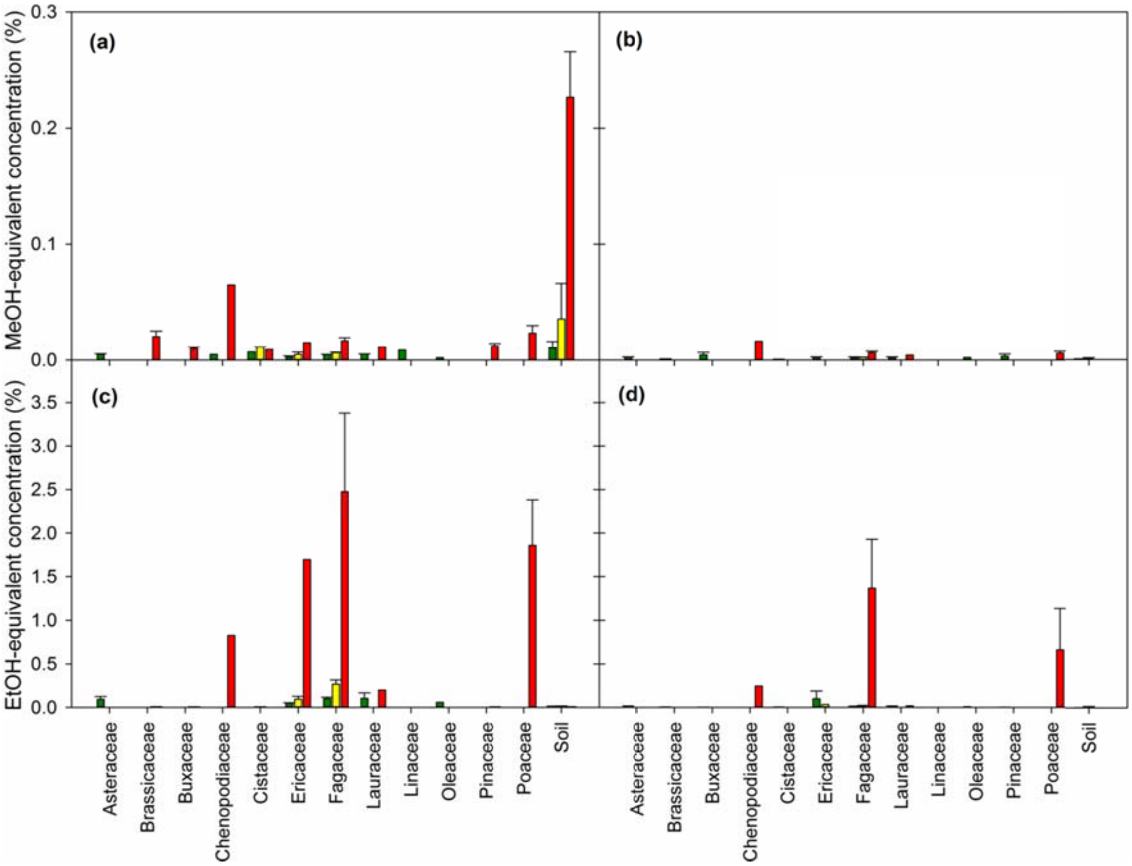

Figure 4. Estimated equivalent concentrations (%) of methanol (MeOH, upper panels, a-b) and ethanol (EtOH, lower panels, c-d) by plant family, determined by fitting the spectral information provided by the Chemcorrect^{TM} (respectively, 'ORGANIC_MEOH_AMPL' and 'ORGANIC_BASE' columns in the raw output files) against known values of MeOH and EtOH in the alcohol-water mixtures. Within each family, three coloured bars indicate the flagging categories of ChemCorrect™. Left (a, c) and right (b,d) panels show raw sample concentrations and concentrations after MCM pre-treatment, respectively.

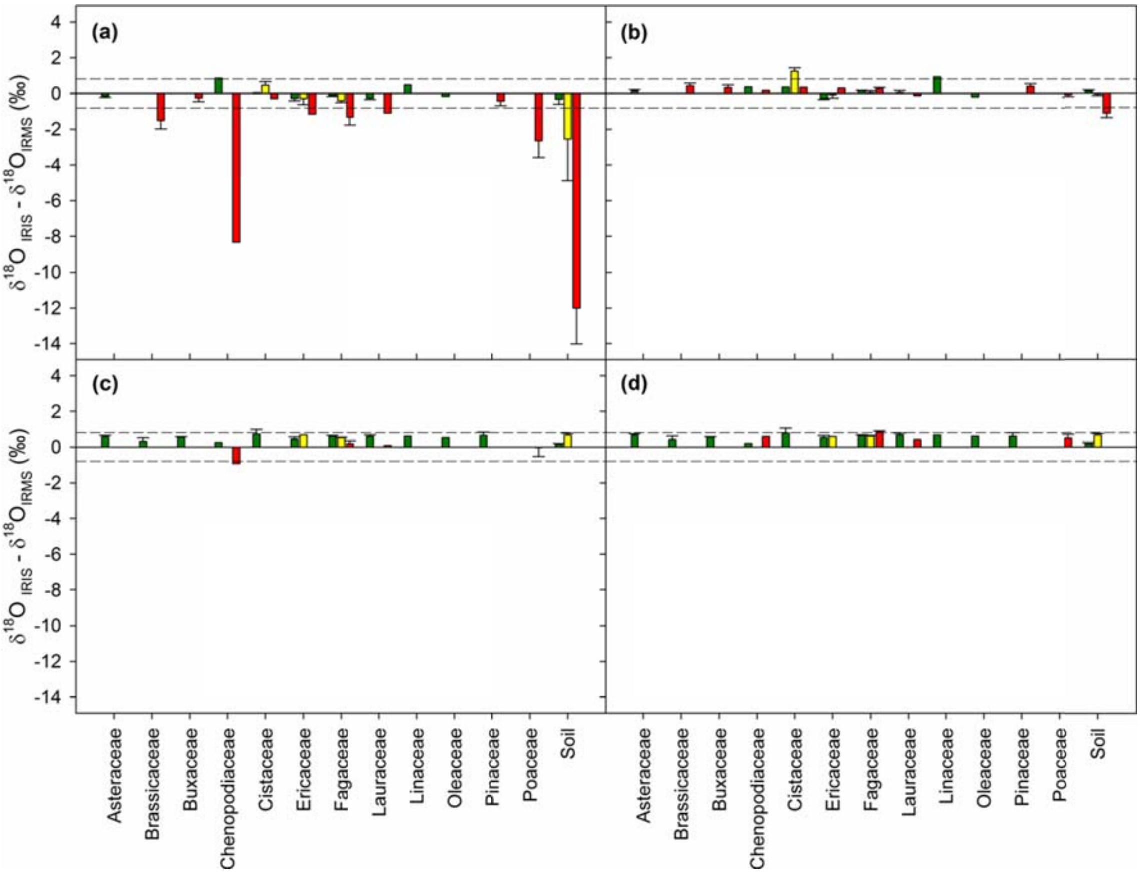

Figure 5. Differences in δ^{18}O values between IRIS and IRMS by plant family. IRIS uncorrected (a), IRIS post-processed (b), IRIS plus MCM (c) and IRIS plus MCM plus post-processing (d). Error bars are standard errors. Dashed lines identify the maximum accepted bias (MAB) established by the International Agency of Atomic Energy. Colour codes represent the flagging categories of ChemCorrect™.

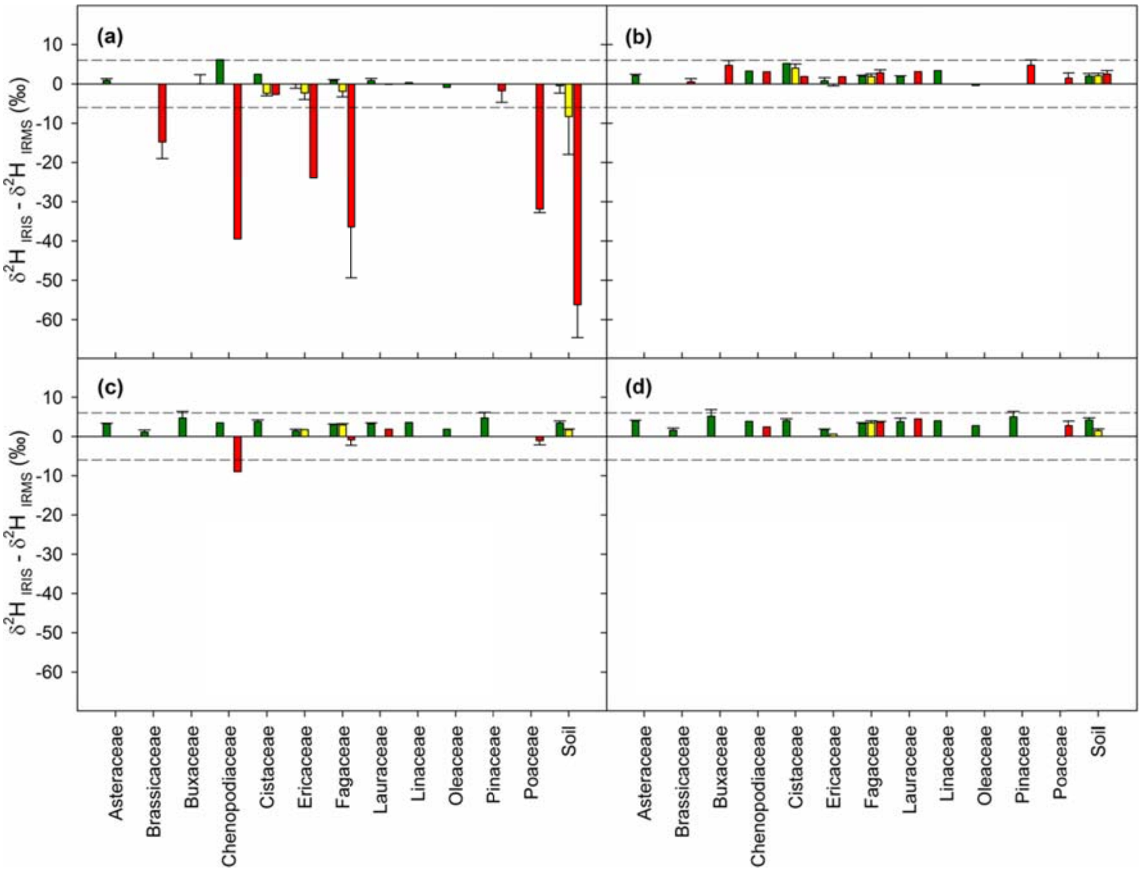

Figure 6. Differences in δ^2H values between IRIS and IRMS by plant family. IRIS uncorrected (a), IRIS post-processed (b), IRIS plus MCM (c) and IRIS plus MCM plus post-processing (d). Error bars are standard errors. Dashed lines identify the maximum accepted bias (MAB) established by the International Agency of Atomic Energy. Colour codes represent the flagging categories of ChemCorrect™.

CHAPTER II.

Short-term dynamics of evaporative enrichment of xylem water in woody stems: implications for ecohydrology

Paula Martín-Gómez[1], Luis Serrano[1], Juan Pedro Ferrio[1,2]

[1] Department of Crop and Forest Sciences - AGROTECNIO Center, Universitat de Lleida, Avda. Rovira Roure 191, E-25198 Lleida (Spain), [2] Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Casilla 160-C, Concepción, Chile.

SUMMARY

In ecohydrology, it is generally assumed that xylem water reflects the water source used by plants. Several studies have reported isotopic enrichment within woody tissues, particularly during dormancy periods or after long periods of inactivity. However, little is known about the short-term dynamics of this process. Here we assessed the magnitude and dynamics of xylem isotopic enrichment in suberized twigs of pines and oaks. We performed a series of laboratory experiments, in which we monitored hourly changes in water content and isotopic composition under two contrasting scenarios of sap flow restriction. Firstly, we simulated the effect of extreme hydraulic failure by excising twigs to restrict sap flow, while sealing the wounds to ensure that water loss took place only through the leaves or bark, as would be the case of evaporation in attached stems. Secondly, we studied the effect of reduced leaf transpiration by darkening with aluminium foil all the leaves of healthy, well-watered saplings growing in pot conditions. We found evidence of fast evaporative enrichment in metabolically-active stems, as a consequence of a temporal decline in sap flow rates, and not necessarily linked to a traceable decline in stem water content. The excision experiments showed significant isotopic changes (*ca.* +1 ‰ in oxygen) appearing in less than one hour. Similarly, the pot experiment showed a progressive increase in isotope composition (up to +8‰ in oxygen in three days-cycle) when the leaves were covered, and a rapid recovery to initial values when sap flow rates were re-established (Fig. 4). We conclude that evaporative enrichment of xylem water in stems is a highly dynamic process that may cause significant effects even during short periods of restricted water flow. This has important implications for the study of plant water uptake, as well as for ecosystem- and global-scale hydrological models.

Keywords: deuterium excess, drought, evaporative enrichment, humidity, oxygen isotope composition, sap flow, transpiration, source water.

INTRODUCTION

The analysis of the isotopic composition of xylem water has been extensively applied to determine the source of water used by plants, providing an useful insight into many ecohydrological processes (Ehleringer and Dawson 1992). The basis of this approach is that the potential water sources available to plants show contrasting isotopic signatures, which can be traced back from the values in xylem water. For example, the different contribution of seasonal precipitation to soils, streams and groundwater lead to substantial isotopic differences among these water pools (see e.g. Gat 1996, Tang and Feng 2001, Máguas et al. 2011). Additionally, the preferential loss of light isotopes during evaporation causes a progressive isotopic enrichment of the liquid phase at the site of evaporation (Craig and Gordon 1965), and creates strong isotopic gradients along the soil profile during dry periods (Allison et al. 1983). Taking advantage of this variability, isotopic tracing has revealed the use of contrasting water sources among adjacent plants, both at the inter-specific (e.g. Sternberg and Swart 1987, Filella and Peñuelas 2004, Máguas et al. 2011) and intra-specific level (Dawson 1993, Voltas et al. 2015). Isotopic studies have also shown that water uptake is a highly dynamic process (Brandes et al. 2007, Máguas et al. 2011, Ellsworth and Sternberg 2014, Bertrand et al. 2014), often involving complex ecological interactions like competition (Dawson 1993, Comas et al. 2015) or facilitation through hydraulic redistribution (Filella and Peñuelas 2004, Prieto et al. 2012).

Isotopic tracing of plant water relies on two important working premises (Ehleringer and Dawson 1992). First of all, it is generally accepted that there is no fractionation during the uptake process by roots, except for some xerophytic and halophytic species (Lin et al. 1993, Ellsworth and Williams 2007). The second basic assumption is that there is no fractionation during the transport of water along the xylem, from the roots to the upper-canopy stems, as it is mainly a mass flow movement. In the leaves, the magnitude of isotopic enrichment is a function of 1) the humidity gradient between the site of evaporation and the atmosphere and 2) the isotopic composition of atmospheric water vapour and 3) transpiration rates (Dongmann et al. 1974, Farquhar and Lloyd 1993, for a recent review see Cernusak et al. 2016). Similarly, if water loss occur through the bark, stems would display an evaporative enrichment of source water proportional to the humidity gradient, and counteracted by the extent of transpiration flow (Dawson and Ehleringer 1993). In this sense, Dawson and Ehleringer (1993) clearly proved the existence of xylem isotopic enrichment for green, unsuberized stems. Similarly, some authors have also described evaporative enrichment in suberized stems of deciduous plants during leafless periods, when a long-lasting water stagnation in the xylem leads to partial desiccation (Phillips and Ehleringer 1995, Treydte et al. 2014, Bertrand et al. 2014, del Castillo et al. 2016). Only recently, Ellsworth and Sternberg (2014) tested the effect of manipulative defoliation in the evergreen oak *Quercus virginiana*: they observed a significant enrichment in xylem water 4 weeks after defoliation, diverging from soil values until the new leaves were formed. However, the short-term (hourly) dynamic of evaporative enrichment in active, leaved branches is still unknown. This information is crucial to provide a proper interpretation of daily, and even seasonal changes in water uptake patterns, based on isotope measurements (see e.g. Filella and Peñuelas 1999, Bertrand et al. 2014, Cernusak et al. 2016, Voltas et al. 2015).

Besides bark transpiration, it has been suggested that an additional cause for xylem enrichment and xylem-soil decoupled observations may be the mixture of xylem water with enriched water from the leaf (Brandes et al. 2007, Ellsworth and Williams 2007). This may occur directly through the back-diffusion of enriched water from the leaf veins to the twig xylem (Dawson and Ehleringer 1993, Farquhar and Lloyd 1993), or by means of water exchange between xylem and phloem tissues (Cernusak et al. 2005, Brandes et al. 2007). In this regard, manipulative experiments in leaved branches are necessary to characterize not only the short-term dynamic of evaporative enrichment during the growing cycle, but also to disentangle the role of leaves in stem isotopic enrichment.

The general aim of the current study was to determine the short-term dynamics of isotopic enrichment of xylem water under conditions of limited sap flow in active, suberized and leaved stems. For this purpose, we conducted a series of laboratory experiments, representative of two alternative scenarios of limited sap flow. On the one hand, twig excision experiments were used to emulate the effect of severe hydraulic failure (*i.e.* source-limited), therefore associated to progressive branch dehydration. On the other, we tested the effect of sap flow restrictions under stomatal-limited transpiration (*i.e.* shadowed leaves or drought limitation), by temporarily covering the leaves of well-watered saplings. Three species were selected as representative of conifer and broadleaves: the Scots pine (*Pinus sylvestris*), and two closely-related species of marcescent oak (*Quercus faginea* and *Quercus subpyrenaica*). Our first objective was to determine the magnitude and timing of the isotopic enrichment in suberized stems. Considering stem water flow as a highly dynamic process, we predicted evaporative enrichment to fluctuate rapidly following a short-term sap flow restriction. Our second objective was to define the processes behind isotopic enrichment in the stem, particularly the relative contribution of stem evaporation and back diffusion of leaf water. Finally, we aimed to provide recommendations for future sampling, assessing the effect of different variables that could affect isotopic enrichment, such as twig diameter or xylem water content, and to discuss the implications of evaporative enrichment of xylem water for the interpretation of ecohydrological processes.

MATERIALS AND METHODS

Twig excision experiments

In order to assess evaporative enrichment under hydraulic limitation of sap flow, we monitored the evolution of isotope composition in excised twigs. After cutting the twigs, the input of water is instantly restricted, leading to a progressive dehydration of the xylem tissues, with the subsequent loss of conductivity and downregulation of stomatal conductance (Sperry et al. 2002). Directly after excision, the cut was immediately sealed with silicon to prevent water loss through the wound. In this way, we aimed to emulate the effect of severe hydraulic failure in attached stems, while allowing to register weight losses and changes in water content.

Twig samples were collected in summer 2013 and 2014 from adult trees growing in a pine-oak mixed stand located in the Spanish Pre-Pyrenees (Boalar Forest - Jaca - Huesca, 30T 693606 4714041). The species sampled were Scots pine (*Pinus sylvestris* L.) and a Mediterranean marcescent oak (*Quercus subpyrenaica* Villar). After sealing the cut, twigs were left drying under ambient conditions for different periods of time, and then a piece of xylem (1.75 ± 0.43 mm in diameter) was sampled as described below (see section "sample collection and water extraction"). In 2013 (from now on referred as "medium-term excision experiment") the drying process ranged from 1.5 to 68 hours, whereas in 2014 ("short-term excision experiment") we focused on the early stages of hydraulic limitation (from 0.5 to 8.75 hours). In order to assess the potential effect of back-diffused leaf water on xylem isotopic enrichment, we applied three different treatments to the twigs in the medium-term experiment: 1) twigs with uncovered leaves; 2) twigs with leaves covered with aluminium foil to restrict transpiration and 3) defoliated twigs. In the latter, leaf scars were sealed with silicon immediately after defoliation. In the short-term experiment, we only applied the two first treatments (leaves covered and leaves uncovered), in order to increase the level of replication. Percentage of water loss (% weight loss) was calculated as the difference between the initial fresh weight, *i.e.* after completing the treatment preparation, and the fresh weight at the time of xylem sampling. During the experiments, temperature and relative humidity were continuously monitored using EL-USB-2 datalogger from EasyLog (Lascar Electronics Ltd).

Pot experiment

The effect of restricted sap flow rates as a consequence of limited transpiration was assessed during summer 2014 in a pot experiment located at the Experimental Fields of the Universitat de Lleida. Commercial saplings of Scots pine and a marcescent oak

(*Quercus faginea* L.) were used for this experiment. Three saplings per species (3 and 5 years old, for oaks and pines, respectively) were originally cultivated in nursery containers with standard substrate. Six months before the experiment, they were transplanted to 20-liter pots filled with a forest loamy soil, collected in the same area of the field campaigns. To minimize isotopic fractionation due to soil evaporative enrichment, the pots were kept near field capacity throughout the experiment, and the soil surface was covered with aluminium foil. A separate test with defoliated trees (one per species) in aluminium-covered pots showed negligible water losses after 12 consecutive days without watering (less than 0.8% weight loss). Well-watered, leaved trees kept under the same environmental conditions showed *ca.* 2-3% daily water loss, *i.e.* 30-45 fold larger than in defoliated trees. Tap water with nearly constant isotopic signature was used for irrigation (-9.59 ± 0.19 for $\delta^{18}O$ and -65.92 ± 1.52 for $\delta^{2}H$, measured from June to September 2014). During the experiment, we followed a 3-day cycle. In the first cycle (reference), plants remained uncovered until the third night, when all the leaves were covered with aluminium foil. The leaves remained covered until the third night, when the aluminium foil was removed. After this, sampling continued for three days (recovery). Twigs were sampled twice everyday (at pre-dawn and midday), leaves were sampled simultaneously from the last reference day to the end of the experiment. Soil cores (15 mm diameter × *ca.* 200 mm height) were sampled during the last day of each experimental phase, pooling the soil from the whole profile, after removing the upper 2 cm. Throughout the experiment, sap flow was monitored with "baby gauges" SF62, coupled to the Sap flow meter T4.2 (EMS Brno, Brno, Czech Republic). These data were downloaded and analyzed with Mini32 software ver.403.34 (EMS Brno, Brno, Czech Republic). Xylem diameter (without bark and phloem) was about 1.50 ± 0.59 mm. Climatic data (temperature and relative humidity) was obtained from a nearby (14 km) public meteorological station (Raimat - Lleida).

Sample collection and water extraction

For xylem sampling, bark and phloem were removed and the peeled xylem was immediately placed in air-tight glass tubes (Duran GL-18). The tubes were frozen on dry ice (twig excision experiment) or liquid nitrogen (pot experiment) directly after sampling, and kept frozen until processing. Water extraction was performed by cryogenic vacuum distillation (Dawson and Ehleringer 1993) at the Dept. of Crop and Forest Sciences, Universitat de Lleida (Spain). Xylem and soil water was extracted by cryogenic vacuum distillation (Ehleringer and Dawson 1992) at the Dept. of Crop and Forest Sciences of the Universitat de Lleida. Sample tubes were placed in a heated silicone oil bath (120°C), and connected with Ultra-Torr^{TM} unions (Swagelok Company, Solon, Ohio, USA) to a vacuum system (*ca.* 10^{-2} mbar), in series with U-shaped collector tubes, cooled with liquid N_2. After an extraction time of 2 h (soil) and 1.5 h (xylem), trapped water was transferred into 2 ml vials, and stored at 4°C until analysis. Preliminary recovery tests were performed to ensure complete distillation (see e.g.

Martín-Gómez et al. 2014; Palacio et al. 2014, Orlowski et al. 2016). All xylem samples were weighted before and after distillation, in order to calculate xylem water content (Xylem WC), and measured to determine mean twig diameter. In a preliminary test with 13 soil and 69 xylem samples, we compared the weight just after distillation, and after oven-drying at 60ºC for 24h. 93% of the samples gained weight in the oven-drier, indicating that they were dry enough to rehydrate under such conditions, with an average significant increase of 1.33±0.18% (P<0.0001) in xylem samples, and a marginally significant gain in soils (0.24±0.11%; P=0.062). For a subset of the soil samples used in this study, we also tested alternative distillation times, showing no significant differences in WC between the samples distilled at C for 2h and 5h (13.0±1.4% and 13.3±2.6%, respectively; N=9).

Isotopic analyses

We analysed the isotope composition of water samples by Cavity Ring-Down Spectroscopy (CRDS) in a Picarro L2120-*i* isotopic water analyser (Picarro Inc., Sunnyvale, CA, USA) at the Serveis Científico-Tècnics of the Universitat de Lleida (Lleida, Spain). The analyser was coupled to a high-precision vaporiser (A0211) through a Micro-Combustion Module^{TM} (MCM), integrated in-line between the vaporiser and the analyser. The MCM removes the contaminants through oxidation, in a way that only pure water arrives to the analyser (Picarro 2012). For each sample, six replicates of 1 μl were injected into the vaporizer, keeping the last three injections for calculation, which showed negligible memory effects and rather homogeneous values (average within-sample standard deviation was 0.16‰ for δ^{18}O and 0.67‰ for δ^{2}H). The estimated precision for the L2120-*i*, based on the repeated analysis of 4 reference water samples was 0.10‰ and 0.40‰, for δ^{18}O and δ^{2}H, respectively. After calibration with three internal standards, isotope composition was expressed in per mil notation (δ^{18}O and δ^{2}H, for oxygen and hydrogen, respectively), relative to VSMOW (Vienna Standard Mean Ocean Water). Deuterium excess (D-ex) was calculated as D-ex = δ^{2}H – 8 × δ^{18}O, and it reflects the slower movement of the $H_2{}^{18}O$ molecule during diffusion, leading to a relative enrichment of the H^2HO molecules in the less strongly bound phase (e.g. in the gas phase during the evaporation of water) and thus, it can be considered as a good indicator of evaporative processes in water (Craig and Gordon 1965, Gat 1996).

As described in Martín-Gómez et al. (2015), residual organic compounds in the distilled water can interfere with the analysis of plant and soil samples using CRDS, but it is possible to overcome this with a post-processing correction. As a quality assessment of the level of contamination among our samples, we compared the results with and without post-processing correction (δ_{corr} and δ_{raw}, respectively). We found a very strong correlation between corrected and uncorrected values ($\delta^{18}O_{raw}=-0.33+0.9997\times\delta^{18}O_{corr}$, $R^2=0.998$; $\delta^{2}H_{raw}=-2.60+1.0035\times\delta^{2}H_{corr}$, $R^2=0.989$ for δ^{2}H; N=369). The offset between raw and corrected values was significant, but small, and the slope of the relationship between raw and corrected values did not differ significantly from unity. Due to the low level of contamination, and for consistency we used module raw results for all samples.

Statistical analyses

In the two excision experiments, differences in isotope composition, % weight loss and xylem WC were initially assessed with full factorial models of time, species and treatment, again including xylem diameter as a covariable. After discarding treatment effects, we further assessed changes over time with a simplified model for each species, including initial values (*i.e.* before treatment). Subsequently, % weight loss and xylem WC were included as covariables in a full factorial of species and time, in order to assess their contribution to changes in isotope composition. In the pot experiment, the relationship between xylem isotope composition and either transpiration rates or leaf water isotopic composition was assessed by means of pair-wise Pearson correlations and linear regressions. At this point, it should be noted that we found two outlier values for pine xylem WC, likely associated to weighting errors, in the medium-term excision experiment (time = 7.75 hours). These values caused a highly unbalanced error distribution, and increased dramatically the error term in the models. Therefore, we decided to exclude the 7.75 hours-time in all the statistical analyses in which xylem WC was involved.

In all cases, generalized linear mixed models were based on Restricted Maximum Likelihood - REML ($\alpha = 0.05$). Differences among levels of a given factor were tested by Least Square Mean (LSM) contrast ($\alpha = 0.05$). All the statistical analyses were performed with JMP Pro 11 (SAS Inc., Cary, NC, USA).

RESULTS

General trends in isotopic values

As shown in Fig. 1, we found isotopic enrichment in xylem water both under hydraulic and stomatal-limited sap flow (excision experiments and pot experiment, respectively). All samples followed a similar evaporative water line, which varied slightly depending on the species and the particular environmental conditions (temperature and relative humidity). The three experiments were conducted during summer, under warm and relatively dry conditions. However, in the excision experiments the conditions were less variable, and on average slightly warmer and drier (Fig. 1a,b) than in the pot experiment (Fig. 1c). Mean temperature was of 27.8ºC and 24.5ºC, and relative humidity of 45.9% and 54.0%, for medium-term and short-term experiments, respectively. In the pot experiment, temperature ranged from 13.0 to 32.0 ºC (mean: 22.3ºC) and humidity from 34.0 to 96.0% (mean: 69.9%). The magnitude of evaporative enrichment was greater at the day scale (δ^{18}O changes up to +7.4‰ for pine and +12.2‰ for oak in the medium-term excision experiment, Fig. 2a,c) but was also significant at the hourly scale (δ^{18}O changes up to 0.92‰ for pine and 1.98‰ for oak in short-term excision experiment, Fig. 2b,d). In the long-term experiment, first significant changes emerged after 7.75h for δ^{18}O (Fig. 2b) in both species, but after 28.25h for δ^{2}H (Fig. 2a). In the short-term experiment, the pines only showed a significant enrichment for δ^{18}O after 8.75h (Fig. 2d), whereas in the oaks first significant differences appeared after 0.5h (Fig. 2b,d).

Water loss and isotopic changes under hydraulic limitation of sap flow

In the excision experiments, isotopic enrichment was largely explained by time after cut (Table 1). Xylem diameter was not significant as a co-variable for isotopic results (δ^{18}O, δ^{2}H, D-ex) but it explained part of the variability in % weight loss and xylem WC in the short-term excision experiment (Table 1). We found significant differences between species in isotopic values, % weight loss and xylem WC. In contrast, we only found a significant effect of the treatment for % weight loss (Table 1). In the medium-term experiment, LSM contrasts revealed significant changes in δ^{18}O from time=7.75 h, and in δ^{2}H from time=28.75 h, regardless of the species. In the short-term experiment, significant differences in δ^{18}O and δ^{2}H appeared after 0.5 hours in oaks and (only for δ^{18}O) after 8.75 hours in pines. There were also significant changes in % weight loss with time, up to 31 % for pines and 43 % for oaks in the medium-term excision experiment (Fig. 3a), and up to 10 % in the short-term experiment (Fig. 3b). Statistical contrasts showed significant differences in % weight loss in the medium-term experiment from 50.25 and 28.25 hours, for pine and oak, respectively (Fig. 3a), and from 1.25 hours in the short-term experiment (Fig. 3b). Xylem WC content was initially higher in pines than in oaks, and showed larger changes in the medium-term (Fig. 3c) than in the short-term experiment (Fig. 3d).

Table 2 shows the output of the models explaining isotopic composition by means of % weight loss and xylem WC in the two excision experiments. We did not find significant effects of xylem WC and % weight loss on $\delta^{18}O$ and $\delta^{2}H$. However, we found that xylem WC partially explained the observed changes in % weight loss.

Sap flow and isotopic changes under stomatal-limited transpiration

In the pot experiment, the reduction in sap flow was gradual, reaching its minimum on the third day after the leaves were covered (Fig. 4a,b), whereas xylem isotopic enrichment was already visible from 6 hours after covering (Fig. 4c-f). Un-covering produced a gradual recovery of sap flow and isotopic values, with first visible changes appearing after 12 hours, and reaching initial isotopic values in 36 hours. It should be noted that, due to the progressive reduction in total leaf area with time (as a consequence of twig sampling) and the changing environmental conditions, we did not observe a complete recovery of initial daily sap flow values after un-covering. In uncovered periods, we generally observe more enriched values at pre-dawn than at midday (Fig. 4c-f). Unlike for the excision experiments, in the pot experiment we did not find significant changes in xylem WC, remaining stable throughout the experiment (60.3 ±0.62 % for pines, 49.6 ±0.64 % for oaks). Besides, in relation to leaf water, after covering the leaves with aluminium foil, a depletion in $\delta^{18}O$ and $\delta^{2}H$ in leaf water is seen (Fig. 4c-f). To test whether isotope mixing between xylem and leaf water could explain this pattern, we compared xylem and leaf isotopic values during the covered period. Isotopic changes in leaf water were not significantly correlated with those of xylem water either for $\delta^{18}O$ (R^2=-0.47, P=0.079 for pine and R^2=-0.30, P=0.272 for oak, N=15) or $\delta^{2}H$ (R^2=-0.28, P=0.272 for pine and R^2= +0.31, P=0.265 for oak, N=15). Conversely, the decline in sap flow rates from initial values to the end of the covered period was significantly associated with an enrichment in the isotopic composition of xylem water for both species (Fig. 5).

DISCUSSION

Experimental evidence of fast evaporative enrichment

Our results show that evaporative enrichment of xylem water takes place in fully-developed, suberized stems shortly after sap flow is limited, regardless of the cause of this limitation. Firstly, the excision experiments showed that the isotopic composition of xylem water increases over time directly after the cut, with significant isotopic changes appearing in less than one hour. Secondly, the pot experiment showed that isotopic enrichment in xylem water could also appear in healthy, well-watered trees when stem flow is limited by leaf-level stomatal closure (e.g. in response to shadowing or low humidity), but evaporative demand is high enough to keep a significant stem transpiration. Similar to our results, Ellsworth and Sternberg (2014) also found a departure from original values in the xylem of suberized stems after artificial defoliation to emulate sap flow limitation in deciduous species during leafless periods. Notably, the magnitude of the change observed in our pot experiment after only three days of sap flow restriction was in the range of that found by Ellsworth and Sternberg (2014) about one month after defoliation (*ca.* +4‰ in $\delta^{18}O$; +20‰ in $\delta^{2}H$). They interpreted that evaporation from the stem was minimal, but the period between defoliation and the first sampling was long enough to cause significant effects on the xylem water. However, our results indicate that this process is much faster than originally expected. Ellsworth and Sternberg (2014) also reported a rather slow recovery of the initial values (>50 days), linked to the development of new leaves. In line with this, Phillips and Ehleringer (1995) realised that the xylem water of winter-deciduous trees departed from the meteoric water line during leafless periods, returning to it only after complete leaf flushing. In our case, after uncovering the leaves, the xylem recovered to non-evaporated soil values in a few hours, proving that in fully active plants evaporative enrichment in the xylem is a reversible, highly dynamic process.

Mechanisms causing isotopic enrichment in the xylem

It is generally assumed that water loss through the stem surface in suberized stems is minimal (see e.g. Schönherr 1982). However, some studies have measured significant bark and lenticular transpiration (e.g. Stöhr and Lösch 2004, Catinon et al. 2012) pointing to the need to test this assumption for an extensive amount of species and environmental conditions. The outcome of the three experiments strongly supports a major effect of stem transpiration on the isotopic enrichment of xylem water. When leaf transpiration is limited, the gradient in water potential between the stem and the leaves is smaller, and hydraulic flow decreases (Sperry et al. 1993); as a consequence, water storage may increase to mitigate xylem cavitation. Under such conditions, the limited stem flow increases water turnover time, reducing the input of fresh, unenriched xylem water, and allowing for accumulative evaporative enrichment. The progressive enrichment in xylem water followed a typical evaporation line, *i.e.* with flatter slope in the $\delta^{18}O/\delta^{2}H$ bi-plot than the meteoric water line (see e.g. Craig and Gordon 1965, Gat 1996). In all cases, the slope of the evaporative line fell within the expected range for soil evaporation in the area (*ca.* 3-4; see e.g. Gibson et al. 2008), hence being compatible with evaporation in a porous media (Allison et al. 1983). Evaporative enrichment would also increase with time, until a limiting threshold when further evaporation no longer results in isotope enrichment of the remaining water (Skrzypek et al. 2015). In our study, the magnitude of the deviation of δ-values from original, non-evaporated, xylem water was a function of the time since transpiration was stopped/limited in the two excision experiments, as well as in the pot experiment.

Unlike in the excision experiments, the strong changes in isotope composition in the pot experiment were not associated to a net loss of water in the xylem. Apparently, covering with aluminium foil strongly reduced leaf transpiration, but kept a significant residual flow, presumably through stem transpiration. Estimated daily water use after covering the leaves was still 20 ± 4.5% and 30 ± 9.5% of that with uncovered leaves, for pines and oaks, respectively. Hence, even a short, moderate limitation of the transpiration flow (e.g. under mild drought stress, or during cloudy days) may be enough to create significant evaporative gradients within the stem, without causing a measurable dehydration. Although absolute sap flow rates in saplings should be taken with caution due to the strong effect of temperature gradients on heat balance sensors, particularly under limited flow (Do and Rocheteau 2002), our findings support previous works suggesting that water losses through the stem surface are not negligible (Stöhr and Lösch 2004, Catinon et al. 2012).

Isotopic enrichment in the stem might be also explained through the contribution of enriched leaf water (either through backward diffusion, or indirectly through the phloem) to changes in the xylem (Dawson and Ehleringer 1993, Bertrand et al. 2014). However, we did not find significant differences between defoliated and non-defoliated stems in the excision experiment, suggesting a minor effect of leaf processes on xylem enrichment. Furthermore, in the pot experiment, leaf and xylem water were not significantly correlated during the covering period, contrary to what would be expected if back-diffusion of leaf water were the main source for changes in the xylem. On the other hand, phloem water is also enriched (see e.g. Adar et al. 1995, Cernusak et al. 2005), and is known to exchange with xylem water under certain conditions (Nardini et al. 2011). However, due to the smaller amount of water in the phloem, xylem-phloem exchange is likely to have greater effect on phloem values than on the xylem (Cernusak et al. 2005).

Defining the most suitable sampling strategy

Ellsworth and Williams (2007) observed that mature stems with fully developed bark had a lower proportion of water in the phloem, and explained the difference between young and old stems through changing proportions of xylem, phloem and bark water. Unlike in this work (and others, e.g. Dawson and Ehleringer 1993, Phillips and Ehleringer 1995), we peeled the branches before sampling, in order to avoid direct contamination from phloem water. Despite this, if the relative proportion of water in the phloem decreases with age, sampling older branches would reduce the potential effect of xylem-phloem exchange on xylem water. Besides, increasing branch diameter would also minimize the effect of stem evaporation due to the lower surface/volume ratio. In our experiments, twig diameter explained part of the variability in stem water loss, but was not associated with isotopic composition. Regarding the sampling time, Dawson and Ehleringer (1993) reported that evaporative enrichment was still present at pre-dawn but was lower than at midday, so they proposed to collect xylem at pre-dawn to avoid evaporation. However, our results highlight that pre-dawn xylem water might suffer substantial evaporative enrichment due to temporary water stagnation. In our pot experiment, after uncovering the leaves, xylem water remained unchanged (*i.e.* enriched) at pre-dawn, and did not reflect the input of new source water until midday. This suggests that pre-dawn sampling, which in principle implies more limited sap flow rates, may not be appropriate if night-time or previous-day conditions favoured evaporative losses (see e.g. Resco de Dios et al. 2013). Therefore, we recommend to sample medium-size twigs at the time of maximum transpiration, avoiding pre-dawn measurements and afternoon depression of stomatal conductance during drought stress.

CONCLUSIONS

In our study, we report evidence of fast evaporative enrichment in metabolically active stems as a consequence of a temporal decline in sap flow rates. In other words, xylem water isotopic composition does not only reflect source water, but also stem hydraulic processes. Hence, observed seasonal fluctuations, e.g. in response to drought (Bertrand et al. 2014, Voltas et al. 2015), or even daily variations (Filella and Peñuelas 1999) might not necessarily reflect changes in source water, as previously assumed, but a confounding effect of xylem evaporative enrichment under limited sap flow (see e.g. del Castillo et al. 2016). Although stem evaporative enrichment can be seen as a handicap for water-sourcing studies, once evaporative effects are constrained (e.g. through the use of ^{17}O-excess; Landais et al. 2006) it could provide a new insight into xylem water dynamics. Beyond the tree scale, our findings also highlight the need to assess the contribution of stem transpiration to tree water balance, and its potential effect on the isotopic partition of water fluxes at the ecosystem level (Wang and Yakir 2000, Dubbert et al. 2013).

Evaporation through the stem surface appears to be the main driver of xylem isotopic enrichment during periods of limited sap flow. In particular, we did not find evidence of a feedback effect of leaf water on stem values. On the other hand, the dynamics of isotopic enrichment were similar, regardless of the original cause for sap flow reductions (*i.e.* limited leaf transpiration or stem hydraulic restrictions), further supporting a physical rather than a physiological regulation of this process. Nevertheless, additional studies addressing specifically isotopic variations in leaf xylem and phloem water might help to disentangle the potential role of leaf-derived water pools in evaporative enrichment.

ACKNOWLEDGEMENTS

This research was supported by the Spanish Government through projects AGL 2012-40039-C02 and AGL 2012-40151-C03, the PhD fellowship to P.M.G. (FPU12/00648) and *Ramón y Cajal* contract to J.P.F. (RYC-2008-02050). We thank Pilar Sopeña, Maria Josep Pau and Mireia Oromí for laboratory assistance on water isotope analyses; *Instituto de Formación Agroambiental de Jaca*, *Unidad de Salud de los Bosques de Aragón*, Miguel Ángel Lázaro, Jesús Pemán, Mònica Aguilera and Jorge del Castillo for their support in field campaigns; Youness El Karkri for experimental assistance and Eustaquio Gil-Pelegrín for borrowing laboratory equipment. We also thank Víctor Resco de Dios and two anonymous referees for their useful comments on the manuscript.

Author contributions

P.M.G., J.P.F. and L.S. planned and designed the research, collected and analysed the data, and wrote the manuscript.

REFERENCES

Adar E, Gev I, Lip J, Yakir D, Gat J (1995) Utilization of oxygen-18 and deuterium in stream flow for the identification of transpiration sources: soil water versus groundwater in sand dune terrain. In: Adar E, Leibundgut C (eds), Application of tracers in arid zone hydrology, Int. Assoc, pp 329–338.

Allison GB, Barnes CJ, Hughes MW (1983) The distribution of deuterium and ^{18}O in dry soils 2. Experimental. J Hydrol 64:377–397.

Bertrand G, Masini J, Goldscheider N, Meeks J, Lavastre V, Celle-Jeanton H, Gobat J-M, Hunkeler D (2014) Determination of spatiotemporal variability of tree water uptake using stable isotopes (δ^{18}O, δ^2H) in an alluvial system supplied by a high-altitude watershed, Pfyn forest, Switzerland. Ecohydrology 7:319–333.

Brandes E, Wenninger J, Koeniger P, Schindler D, Rennenberg H, Leibundgut C, Mayer H, Gessler A (2007) Assessing environmental and physiological controls over water relations in a Scots pine (*Pinus sylvestris* L.) stand through analyses of stable isotope composition of water and organic matter. Plant Cell Environ 30:113–127.

Catinon M, Ayrault S, Boudouma O, Asta J, Tissut M, Ravanel P (2012) Atmospheric element deposit on tree barks: The opposite effects of rain and transpiration. Ecol Indic 14:170–177.

Cernusak LA, Barbour MM, Arndt SK, Cheesman AW, English NB, Feild TS, Helliker BR, Holloway-Phillips MM, Holtum JAM, Kahmen A, McInerney FA, Munksgaard NC, Simonin KA, Song X, Stuart-Williams H, West JB, Farquhar GD (2016) Stable isotopes in leaf water of terrestrial plants. Plant Cell Environ 39:1087–1102.

Cernusak LA, Farquhar GD, Pate JS (2005) Environmental and physiological controls over oxygen and carbon isotope composition of Tasmanian blue gum, *Eucalyptus globulus*. Tree Physiol 25:129–146.

Comas C, del Castillo J, Voltas J, Ferrio JP (2015) Point processes statistics of stable isotopes: Analysing water uptake patterns in a mixed stand of Aleppo pine and Holm oak. For Syst 24(1):e009, doi:http://dx.doi.org/10.5424/fs/2015241-05846.

Craig H, Gordon LI (1965) Deuterium and oxygen-18 variations in the ocean and the marine atmosphere. In: Tongiorgi E (eds), Proceedings of a conference on stable isotopes in oceanographic studies and paleotemperatures. Laboratory of Geology and Nuclear Science, Pisa, pp 9–130.

Dawson TE (1993) Water sources of plants as determined from xylem-water isotopic composition: perspectives on plant competition, distribution, and water relations. In: Ehleringer JR, Hall AE, Farquhar GD (eds), Stable isotopes and plant carbon-water relations. Academic Press, Inc., New York, pp 465–496

Dawson TE, Ehleringer JR (1993) Isotopic enrichment of water in the ‘woody’ tissues of plants: Implications for plant water source, water uptake, and other studies which use the stable isotopic composition of cellulose. Geochim Cosmochim Acta 57:3487–3492.

del Castillo J, Comas C, Voltas J, Ferrio J.P (2016) Dynamics of competition over water in a mixed oak-pine Mediterranean forest: Spatio-temporal and physiological components. Forest Ecology and Management 382:214-224.

Do F, Rocheteau A (2002) Influence of natural temperature gradients on measurements of xylem sap flow with thermal dissipation probes. 2. Advantages and calibration of a noncontinuous heating system. Tree Physiol 22:649–654.

Dongmann G, Nürnberg HW, Förstel H, Wagener K (1974) On the enrichment of $H_2^{18}O$ in the leaves of transpiring plants. Radiat Environ Biophys 11:41–52.

Dubbert M, Cuntz M, Piayda A, Máguas C, Werner C (2013) Partitioning evapotranspiration – Testing the Craig and Gordon model with field measurements of oxygen isotope ratios of evaporative fluxes. J Hydrol 496:142–153.

Ehleringer JR, Dawson TE (1992) Water uptake by plants: perspectives from stable isotope composition. Plant Cell Environ 15:1073–1082.

Ellsworth PZ, Sternberg LSL (2014) Seasonal water use by deciduous and evergreen woody species in a scrub community is based on water availability and root distribution. Ecohydrology 8:538–551.

Ellsworth PZ, Williams DG (2007) Hydrogen isotope fractionation during water uptake by woody xerophytes. Plant Soil 291:93–107.

Farquhar GD, Lloyd J (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In: Ehleringer JR, Hall AE, Farquhar GD (eds), Stable isotopes and plant carbon–water relations. Academic Press, San Diego, pp 47–70

Filella I, Peñuelas J (1999) Altitudinal differences in UV absorbance, UV reflectance and related morphological traits of *Quercus ilex* and *Rhododendron ferrugineum* in the Mediterranean region. Plant Ecol 145:157–165.

Filella I, Peñuelas J (2004) Indications of hydraulic lift by *Pinus halepensis* and its effects on the water relations of neighbour shrubs. Biol Plant 47:209–214.

Gat J (1996) Oxygen and hydrogen isotopes in the hydrologic cycle. Annu Rev Earth Planet Sci 24:225–262.

Gibson JJ, Birks SJ, Edwards TWD (2008) Global prediction of δ_A and δ^2H- δ^{18}O evaporation slopes for lakes and soil water accounting for seasonality. Global Biogeochem Cycles 22:1–12.

Landais A, Barkan E, Yakir D, Luz B (2006) The triple isotopic composition of oxygen in leaf water. Geochim Cosmochim Acta 70:4105–4115.

Lin GH, Sternberg LDL, Ehleringer JR, Hall AE, Farquhar GD (1993) Hydrogen isotopic fractionation by plant roots during water uptake in coastal wetland plants. In: J.R. Ehleringer, A.E. Hall and G.D. Farquhar (eds), Stable isotopes and plant carbon–water relations. Academic Press, San Diego, pp 497–510

Máguas C, Rascher KG, Martins-Loução A, Carvalho P, Pinho P, Ramos M, Correia O, Werner C (2011) Responses of woody species to spatial and temporal ground water changes in coastal sand dune systems. Biogeosciences 8:3823–3832.

Martín-Gómez P, Barbeta A, Voltas J, Peñuelas J, Dennis K, Palacio S, Dawson TE, Ferrio JP (2015) Isotope-ratio infrared spectroscopy: a reliable tool for the investigation of plant-water sources? New Phytol 207:914–927.

Nardini A, Salleo S, Jansen S (2011) More than just a vulnerable pipeline: xylem physiology in the light of ion-mediated regulation of plant water transport. J Exp Bot 62:4701–4718.

Phillips SL, Ehleringer JR (1995) Limited uptake of summer precipitation by bigtooth maple (*Acer grandidentatum* Nutt) and Gambel's oak (*Quercus gambelii* Nutt). Trees Struct Funct 9:214–219.

Orlowski N, Breuer L, McDonnell JJ (2016) Critical issues with cryogenic extraction of soil water for stable isotope analysis. Ecohydrology 9:3-10.

Palacio S, Azorín J, Montserrat-Martí G, Ferrio JP (2014) The crystallization water of gypsum rocks is a relevant water source for plants. Nature Communications 5: 4660.

Picarro (2012) Micro-Combustion Module^{TM} (MCM): Elimination of organics datasheet.

Prieto I, Armas C, Pugnaire FI (2012) Water release through plant roots: New insights into its consequences at the plant and ecosystem level. New Phytol 193:830–841.

Resco de Dios V, Díaz-Sierra R, Goulden ML, Barton CVM, Boer MM, Gessler A, Ferrio JP, Pfautsch S, Tissue DT (2013) Woody clockworks: Circadian regulation of night-time water use in *Eucalyptus globulus*. New Phytol 200:743–752.

Schönherr J (1982) Resistance of plant surfaces to water loss: transport properties of cutin, suberin and associated lipids. In: Lange O, Nobel P, Osmond C, Ziegler H (eds), Physiological plant ecology II. Springer-Verlag: Berlin, pp 153–179.

Skrzypek G, Mydłowski A, Dogramaci S, Hedley P, Gibson JJ, Grierson PF (2015) Estimation of evaporative loss based on the stable isotope composition of water using Hydrocalculator. J Hydrol 523:781–789.

Sperry JS, Alder NN, Eastlack SE (1993) The effect of reduced hyraulic conductance on stomatal conductance and xylem cavitation. J Exp Bot 44:1075–1082.

Sperry JS, Hacke UG, Oren R, Comstock JP (2002) Water deficits and hydraulic limits to leaf water supply. Plant, Cell Environ 25:251–263.

Sternberg LDSL, Swart PK (1987) Utilization of freshwater and ocean water by coastal plants of southern Florida. Ecology 68:1898–1905.

Stöhr A, Lösch R (2004) Xylem sap flow and drought stress of *Fraxinus excelsior* saplings. Tree Physiol 24:169–180.

Tang K, Feng X (2001) The effect of soil hydrology on the oxygen and hydrogen isotopic compositions of plants' source water. Earth Planet Sci Lett 185:355–367.

Treydte K, Boda S, Graf Pannatier E, Fonti P, Frank D, Ullrich B, Saurer M, Siegwolf R, Battipaglia G, Werner W, Gessler A (2014) Seasonal transfer of oxygen isotopes from precipitation and soil to the tree ring: Source water versus needle water enrichment. New Phytol 202:772–783.

Voltas J, Lucabaugh D, Chambel MR, Ferrio JP (2015) Intraspecific variation in the use of water sources by the circum-Mediterranean conifer *Pinus halepensis*. New Phytol 208:1031–41.

Wang XF, Yakir D (2000) Using stable isotopes of water in evapotranspiration studies. Hydrol Process 14:1407–1421.

TABLES AND FIGURES

Table 1. Summary statistics of the mixed models to test for time and treatment effects on xylem isotopic composition and water content during the excision experiments. Models include only samples after treatment, *i.e.* excluding initial, reference values. $\delta^{18}O$, $\delta^{2}H$, oxygen and hydrogen isotopic composition, respectively; D-ex, Deuterium excess; % Weight loss, relative weight loss of twigs between excision and xylem sampling; Xylem WC, water content (%) in the xylem, determined gravimetrically from pre- and post-distillation weights. *P*-values are presented only for significant factors ($P<0.05$), otherwise denoted as non-significant (n.s.). For xylem WC in the medium-term excision experiment, $N=24$ (see methods for details).

Factor	$\delta^{18}O$	$\delta^{2}H$	D-ex	% Weight loss	Xylem WC
Medium-term excision experiment (N=30)					
Xylem diameter	n.s.	n.s.	n.s.	n.s.	n.s.
Species (oak, pine)	0.0105	n.s.	0.0033	0.001	<0.0001
Treatment	n.s.	n.s.	n.s.	0.0011	n.s.
Time	0.0062	0.0131	0.0052	0.0357	0.0042
Species × treatment	n.s.	n.s.	n.s.	n.s.	n.s.
Species × time	0.0182	n.s.	0.0046	n.s.	n.s.
Treatment × time	n.s.	n.s.	n.s.	n.s.	n.s.
Short-term excision experiment (N=74)					
Xylem diameter	n.s.	n.s.	n.s.	0.0049	0.0005
Species (oak, pine)	0.0053	n.s.	<0.0001	n.s.	<0.0001
Treatment	n.s.	n.s.	n.s.	<0.0001	n.s.
Time	<0.0001	n.s.	<0.0001	<0.0001	n.s.
Species × treatment	n.s.	n.s.	n.s.	n.s.	n.s.
Species × time	n.s.	n.s.	0.0207	n.s.	n.s.
Treatment × time	n.s.	n.s.	n.s.	0.0254	n.s.
Species x treatment × time	n.s.	n.s.	n.s.	0.0197	n.s.

Table 2. Summary statistics of the mixed models relating changes in water content during the excision experiments with xylem isotopic composition ($\delta^{18}O$, $\delta^{2}H$, oxygen and hydrogen isotopic composition, respectively; D-ex, Deuterium excess). Models combine all treatments and include initial, reference vaules. % Weight loss, relative weight loss of twigs between excision and xylem sampling; Xylem WC, water content (%) in the xylem, determined gravimetrically from pre- and post-distillation weights. *P*-values are presented only for significant factors ($P<0.05$), otherwise denoted as non-significant (n.s.). For xylem WC in the medium-term excision experiment, $N=34$ (see methods for details).

Factor	$\delta^{18}O$	$\delta^{2}H$	D-ex	% Weight loss
Medium-term excision experiment (N=40)				
% Weight Loss	n.s.	n.s.	n.s.	
Species	0.0036	n.s.	0.0001	
Time	<0.0001	<0.0001	<0.0001	
Species × time	0.0001	0.0483	<0.0001	
Xylem WC	n.s.	n.s.	n.s.	0.0423
Species	n.s.	n.s.	n.s.	n.s.
Time	<0.0001	<0.0001	<0.0001	0.0017
Species × time	0.0142	n.s.	0.0018	n.s.
Short-term excision experiment (N=114)				
% Weight Loss	n.s.	n.s.	0.0362	
Species	0.0242	<0.0001	<0.0001	
Time	<0.0001	n.s.	<0.0001	
Species × time	<0.0001	0.0116	0.0002	
Xylem WC	n.s.	n.s.	0.0003	n.s.
Species	n.s.	n.s.	n.s.	n.s.
Time	<0.0001	0.0063	<0.0001	<0.0001
Species × time	<0.0001	0.0095	0.0009	n.s.

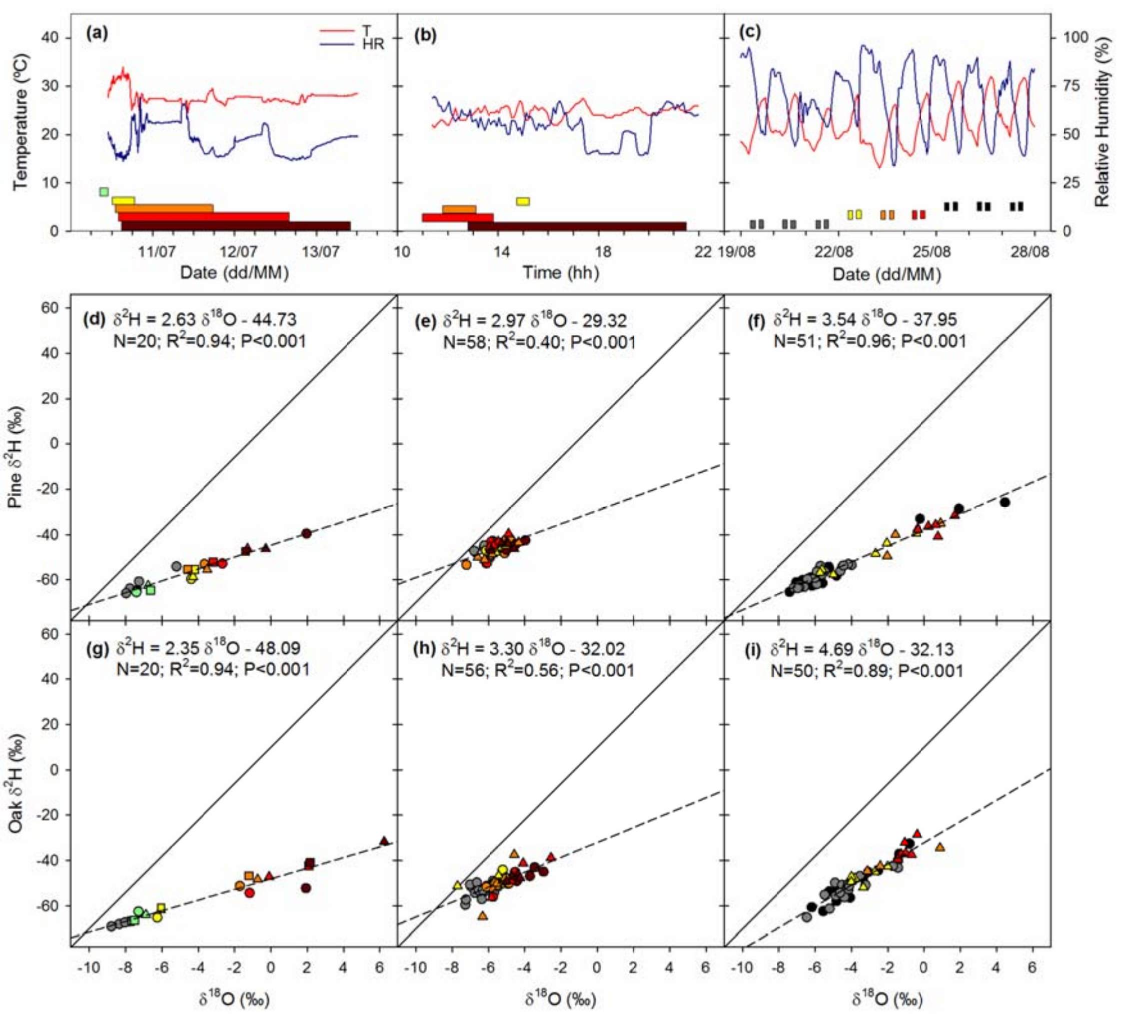

Figure 1. Time-frame and isotopic composition ($\delta^{18}O$ vs. δ^2H) of pine and oak xylem water in the medium-term and short-term excision experiments (left and middle panels, respectively), and in the pot experiment (right panels). Upper panels (a, b and c) display the evolution of Temperature (in red) and Relative Humidity (in blue) and the sampling schedule. Coloured boxes in the excision experiments (a, b) indicate the drying time of twigs between excision and xylem sampling. For the pot experiment (c), boxes indicate the sampling times. In excision experiments (panels d, g, e, h) grey symbols represent samples collected immediately after excision, while green, yellow, orange, red and brown symbols indicate different times after excision. Circles represent non-covered twigs, triangles twigs covered with aluminium foil and squares defoliated twigs. In the pot trial (f, i), yellow, orange and red triangles stand for the three consecutive days of aluminium foil covering; grey and black circles show the values of uncovered samples, before and after the treatment, respectively. Global Meteoric Water Line (GMWL) is shown with a black continuous line and the linear regression between $\delta^{18}O$ and δ^2H for twig xylem water (evaporation line) with a dashed line. Linear regression equations, number of samples (*N*), determination coefficient (R^2) and *P*-values (P) are also presented.

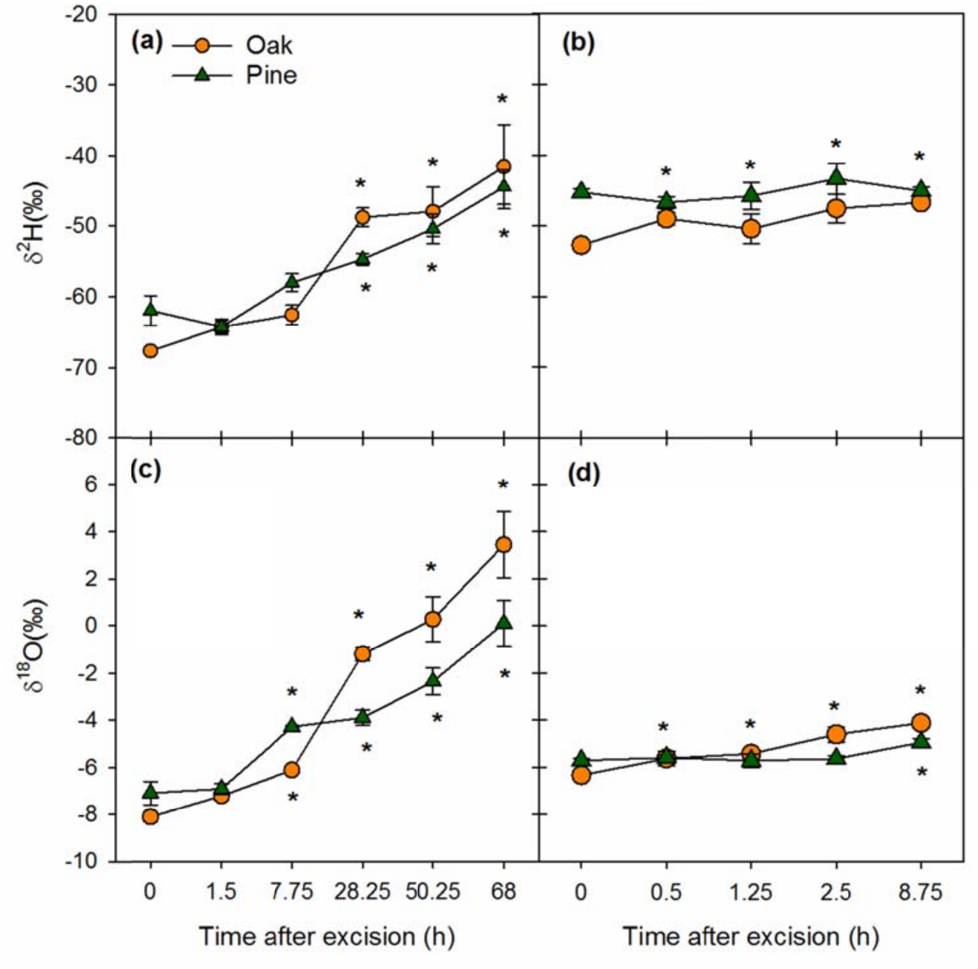

Figure 2. Temporal evolution of hydrogen (δ^2H; a,b) and oxygen (δ^{18}O; c,d) isotope composition in the long-term (a,c) and short-term (b,d) excision experiments. Triangles and circles are used for pines and oaks, respectively. Error bars, ± SE. Differences were tested by Least Square Mean Contrast (α=0.05) on the model including species, time and xylem water content as co-variable (see Table 2). Asterisks (*) indicate significant differences respect to initial time for each species; up, oak; down, pine. Note that the time axis is categorical.

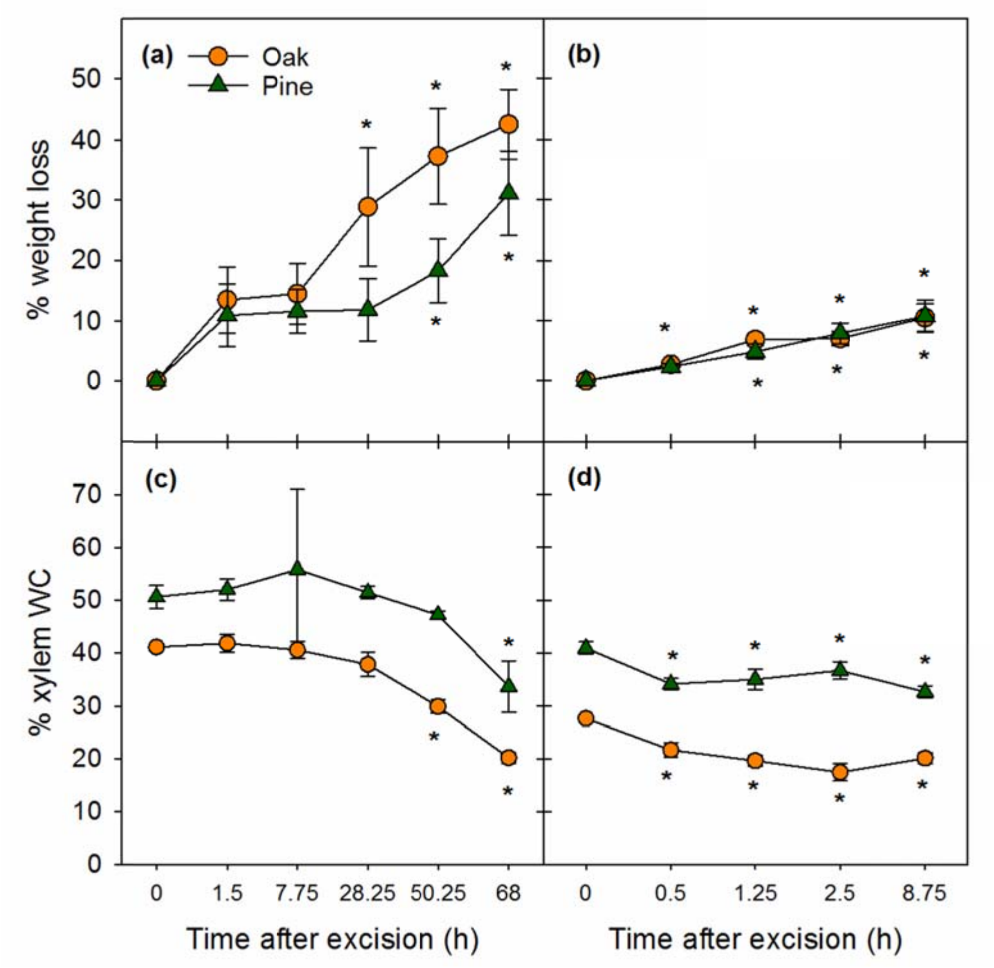

Figure 3. Temporal evolution of water content during the excision experiments: a,b) relative weight loss of twigs (%) between excision and xylem sampling (% Weight loss); c,d) water content (%) in the xylem, determined gravimetrically from pre- and post-distillation weights (Xylem WC). Left panels (a,c), medium-term experiment; right panels (b,d), short-term experiment. Triangles and circles are used for pines and oaks, respectively. Error bars, ± SE. Asterisks denote significant differences by Least Square Mean (α=0.05), including species and time as crossed factors, and xylem diameter as a co-variable. For xylem WC in the medium-term experiment (c), the model excluded the values at 7.75 h. When overlapping, up, oak; down, pine. Note that the time axis is categorical.

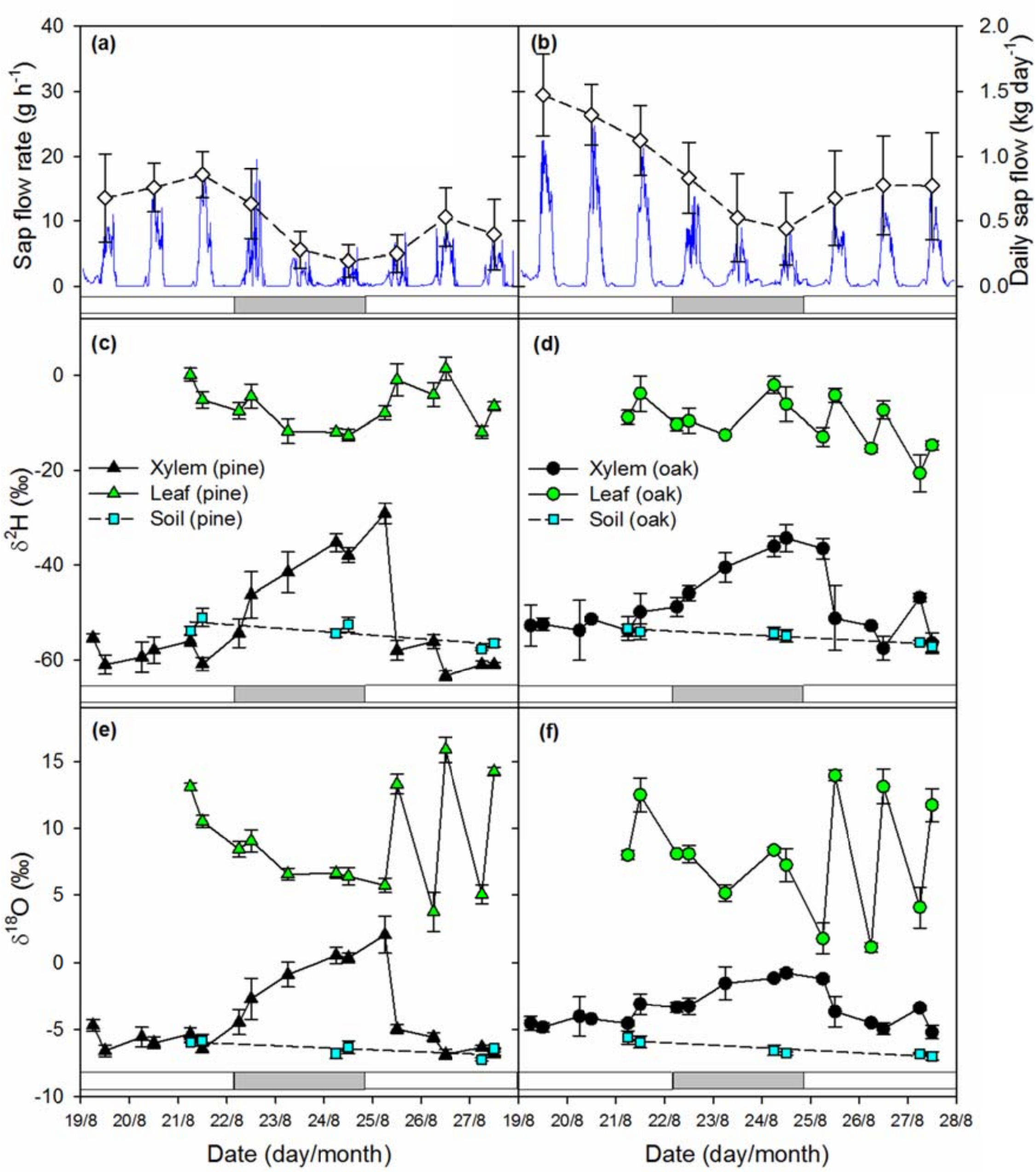

Figure 4. Temporal evolution of sap flow, and isotopic composition (δ^{18}O, δ^2H in ‰) in soil, xylem and leaf water during the pot trial. White and grey boxes denote uncovered and covered periods, respectively. In sap flow panels (a,b) the blue line show mean sap flow rate (g h^{-1}, N=3), and white diamonds show accumulated daily values (kg day^{-1}). In isotope panels (c,d,e,f) triangles and circles are used for pines and oaks, respectively: green symbols, leaf water; black symbols, xylem water. Blue squares stand for soil values. Error bars, ± SE.

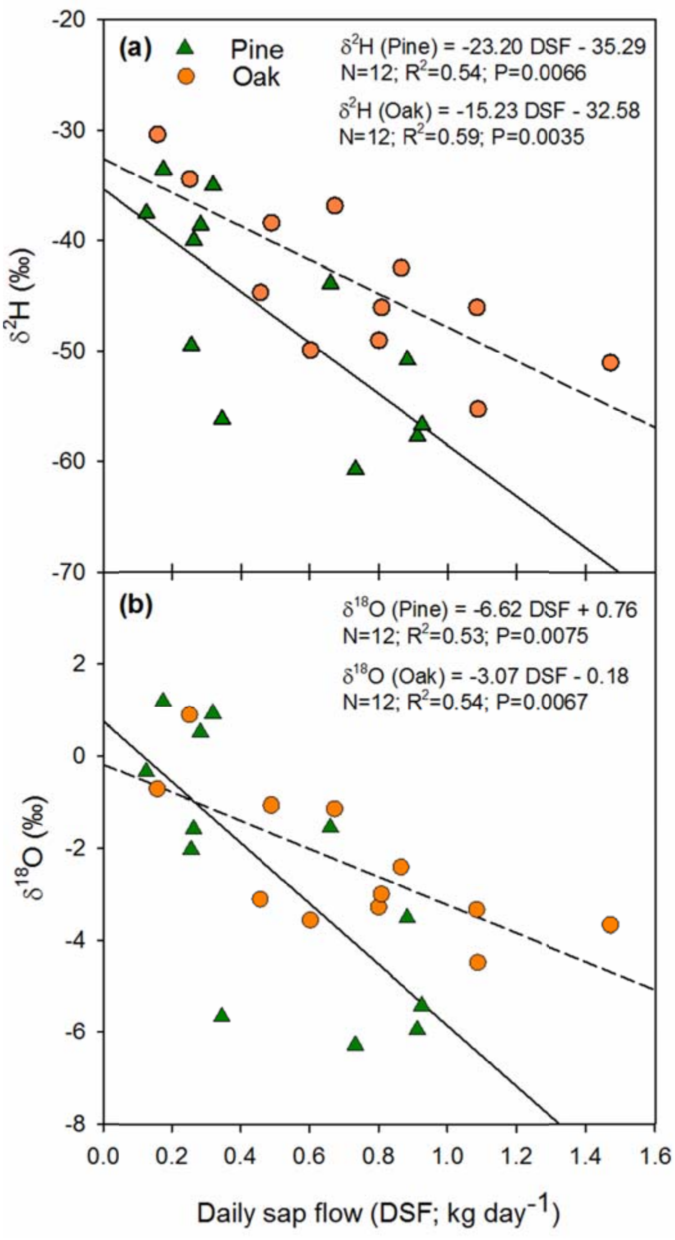

Figure 5. Linear regression between accumulated daily sap flow (kg day^{-1}) and daily means of hydrogen (a) and oxygen (b) isotopic composition (δ^{2}H and δ^{18}O, respectively). Values for each individual, from the last day before aluminium foil covering to the end of the covered period. Triangles and circles are used for pines and oaks, respectively. Regressions are plotted with continuous lines for pines, and dashed lines for oaks.

CHAPTER III.

Contrasting ecophysiological strategies related to drought: the case of a mixed stand of Scots pine (*Pinus sylvestris*) and a submediterranean oak (*Quercus subpyrenaica*)

Paula Martín-Gómez[1], Mònica Aguilera[1], Jesús Pemán[1], Eustaquio Gil-Pelegrín[2], Juan Pedro Ferrio[1,3]

[1] Dept. Crop and Forest Sciences-AGROTECNIO, Universitat de Lleida, Lleida, Spain, [2] Unidad de Recursos Forestales, CITA de Aragón, Zaragoza, Spain, [3] Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile.

SUMMARY

Submediterranean forests are considered an ecotone between Mediterranean and Eurosiberian ecosystems. These transition zones are very sensitive to global change, so models on future climatic scenarios predict dramatic changes in their species composition. A decline of *Pinus sylvestris* and a related expansion of *Quercus* species have been reported in the Spanish Pre-Pyrenees. Although this has been associated to increasing drought stress, the mechanisms behind are not fully understood, and suitable monitoring protocols are lacking. The aim of this study is to bring insight into the physiological mechanisms anticipating selective decline of the pines. For this purpose, we performed a sampling campaign covering two growing seasons in a mixed stand of *P. sylvestris* and *Quercus subpyrenaica*. The studied individuals of the two species were co-dominant and with no visible symptoms of decline. We sampled seasonally twig xylem and soil for water isotope composition ($\delta^{18}O$ and $\delta^{2}H$), leaves for carbon isotope composition ($\delta^{13}C$) and stems to quantify non-structural carbohydrates (NSC) concentration. In addition, we measured twig water potential, leaf gas exchange and sap flow. Isotopic and physiological data reflected the tighter stomatal control and more isohydric strategy for *P. sylvestris*; which resulted in larger limitations on carbon assimilation and carbon storage during drought periods. On the contrary, we did not find this limitation for *Q. subpyrenaica* and we observed a faster recovery of pre-dawn water potential and soil isotopic signature for this species after summer drought, probably associated to a deeper and more reliable water supply. Associated to carbon supply limitation, we found a more pronounced effect of climate on long term growth in pines compared to oaks. All these observations confer evidence of early warning *P. sylvestris* decline and indicate the advantage on survivorship and adaptation of *Q. subpyrenaica* in the area.

Keywords: Decline, drought, isohydric, non-structural carbohydrates, submediterranean forest, water isotopes.

INTRODUCTION

In Europe, the transition zone between the Mediterranean and Eurosiberian region is generally called submediterranean ecotone (Abadía et al. 1996). The change between both regions is mostly gradual and has intermediate climatic characteristics between those (e.g. Sánchez de Dios et al. 2009). The main community in this region is composed by marcescent oaks (*Q. faginea*, *Q. humilis*, *Q. pyrenaica* and their hybrid form, *Q. subpyrenaica*) and conifers, either from natural regeneration or reforested (*P. sylvestris*, *P. nigra* subsp. *salzmannii* and *P. halepensis*). Both groups may appear in the form of pure or mixed stands.

In particular, in the medium-high mountains of the Spanish Pyrenees, one typical association is formed by the marcescent oak *Quercus subpyrenaica* E.H del Villar. and Scots pine (*Pinus sylvestris* L.). These co-ocurring species show different functional traits associated to contrasting water-use strategies. Scots pine as an evergreen conifer has a tracheid-bearing xylem with low hydraulic conductivity and isohydric behavior; based on a tight stomatal control, limiting transpiration when soil moisture deficit reaches a threshold (e.g. Irvine et al. 1998, Poyatos et al. 2008, Martínez-Vilalta et al. 2009). Meanwhile, *Q. subpyrenaica* as its parental species (*Q. faginea* x *Q. humilis*) is a marcescent oak with ring-porous xylem with high maximum hydraulic conductivity and a typical anisohydric behavior, *i.e.* maintaining higher transpiration rates despite soil drought incidence (Nardini and Pitt 2002, Corcuera et al. 2004, Himrane et al. 2004, Tognetti et al. 2007). Additionally, the different physiological behavior between these species is partly associated to their root system distribution (e.g. Filella and Peñuelas 2003, Moreno-Gutiérrez et al. 2012b, West et al. 2012, Barbeta et al. 2015); thus, oaks are able to maintain their continuous physiological activity by relying on stable pools like groundwater or deep soil layers while pines develop an opportunistic strategy of fast use of precipitation water based in a high root density on top soil layers (e.g. Poyatos et al. 2008, Klein et al. 2013, del Castillo et al. 2016, Grossiord et al. 2016). Also, because of the ability of oaks to reach lower water potentials than pines, during dry periods they can have access to more tightly-bound water pools in the soil (Tang and Feng 2001). On the other hand, considering its transitional nature, submediterranean ecotones are thought to be very sensitive to global change (Gosz 1992). Climate-change predictions include increasing temperatures and a reduction of spring and summer precipitation for the next decades (Christensen et al. 2007). In the Mediterranean basin, water is largely the main limiting factor for plant and tree development; thus, the predicted changes in water availability, even subtle, may affect the ecosystem functioning modifying the species composition and dynamic of current forests systems (e.g. Martínez-Vilalta et al. 2002, Poyatos et al. 2008, Allen et al. 2010, Granda et al. 2014). In this regard, there is increasing evidence that extreme drought events are causing extensive tree decline in Scots pine populations, particularly in its southern limit of distribution (e.g. Martínez-Vilalta and Piñol 2002, Gómez-Aparicio et al. 2011, Poyatos et al. 2013, Sánchez-Salguero et al. 2015). Conversely, coexisting deciduous or marcescent oaks generally remain unaffected or even expanded towards higher or fresher locations (e.g. Poyatos et al. 2008, Benito-Garzón et al. 2008, Galiano et al. 2010). There are also some prediction models that suggest an important reduction in the distribution area of marcescent oaks and a displacement towards higher altitudes in favor of Mediterranean evergreen oaks; e.g the holm oak, *Q. ilex* (Sánchez de Dios et al. 2009, Carnicer et al. 2013a, Carnicer 2014). In this context, it remains unclear how the different physiological and adaptive responses of pines and oaks could determine the composition and structure of submediterranean forests in a climate-change scenario. Thus, further research is needed on the species-specific strategies to cope with drought at the submediterranean ecotone, particularly in those areas lacking a clear dominance of one functional group over the other.

In this study, we analyzed the seasonal evolution of water uptake patterns and physiological response in terms of water relations and carbon dynamics, in a mixed forest of *P. sylvestris* and *Q. subpyrenaica* in the submediterranean area of the Spanish Pyrenees. The study spanned over two growing seasons with contrasting water availability. The main objectives were to: (i) understand the physiological advantages (if any) of marcescent oaks in comparison to Scots pine and (ii) anticipate the mechanisms behind Scots pine decline in the area.

MATERIALS AND METHODS

Site description

This study was conducted in an adult mixed stand located in the north-west part of the Spanish Pre-Pyrenees (Boalar Forest - Jaca - Huesca, 30T 693606 4714041, 800m high). The main species in the stand are Scots pine (*Pinus sylvestris* L.) and a Mediterranean marcescent oak (*Quercus subpyrenaica* E.H del Villar) with a 1:1 stand density of 1200 trees/ha aproximately (local source data). Besides, in the area there is a dense (80% in soil cover) understory composed mainly by evergreen *Buxus sempervirens*. Mixed stands of Scots pine and oak in the area mainly originated from natural regeneration, and forest age structure reflects their history. In the past, Iberian forests experienced strong land-use pressures (logging, grazing) until the 1950s, when massive migration of rural population led to an extensive abandonment of cultivated land and in consequence to an enhancement of forest encroachment and increase of canopy and understory cover (García-Ruiz et al. 1996, Ameztegui et al. 2016).

The climate in the region is described as transitional Mediterranean or Submediterranean (Sánchez de Dios et al. 2009). The mean annual precipitation and temperature are 806 mm and 11.7°C, respectively. The summer is mild and relatively dry (mean summer precipitation of 143.2 mm, mean average temperature of 20.1 °C), registering drought periods mainly in July. The highest precipitation events occur during autumn and spring season; in winter it generally snows 5-6 times a year. According to National Geologic Institute (IGME, Geologic map 1:50.000) the dominant geological substrate in the area is a sedimentary combination of sandstone and grey marlstone. In situ soil analyses resulted in a clayey-loam texture with 32% of clay particles and non-limiting calcium carbonate content (<0.3% in most of the cases), electric conductivity (<0.1 dS/m) and organic matter content (1.9%). Average pH was 7.3 and soil water content at field capacity (0.33 MPa) and wilting point (1.5 MPa) was 21.4% and 13.1% respectively.

Remote sensing and meteorological data

Biweekly spectral variation of the Normalized Difference Vegetation Index (NDVI) for the study period were obtained from the MODIS Land Subsetted Products at the MODIS web service (2014). We selected the pixel containing the study stand (250×250 m^2). Seasonal changes in soil water content (SWC) were derived from data acquired from the ESA´s Soil Moisture and Ocean Salinity (SMOS) mission, available at http://www.smos-bec.icm.csic.es; the data is a high resolution (1 x 1 km) soil moisture delayed product, which contains two layers per day, corresponding to SMOS ascending (6 A.M.) and descending (6 P.M.) passes. We selected the four closest pixels containing the study site. These maps are obtained using a downscaling algorithm, which combines the brightness temperature measurements from ESA-SMOS with Land Surface Temperature and NDVI data from Terra/Aqua MODIS. Meteorological data was obtained from the nearest meteorological station (Instituto de Formación Agroambiental de Jaca, < 500m).

Field sampling and measuring campaigns

We selected ten pairs of apparently healthy and codominant neighbor pine – oak trees. The studied individuals in the stand showed an even age and size structure, with diameter at breast height (dbh, mean ± SD) of 30.0 ± 4.4 cm for pines and 19.4 ± 3.4 cm for oaks, and tree height of 12.2 ± 1.5 m and 12.5 ± 1.0 m, for pine and oak respectively, with average age around 55-60 years for both species. Along 2012 and 2013, we performed five intensive field campaigns: on July 31st, October 9th (2012), March 21st, June 27th and August 9th (2013) (Fig. 1). For each sampling time, we collected xylem samples from sun-exposed twigs of the upper third of the crown at two aspects (north and east). In the middle of each pair of trees, soil was sampled at three different depths (0-10 cm, 10-30 cm and 30-50 cm). For xylem sampling, bark and phloem were removed and the peeled xylem was immediately placed in air-tight glass tubes (Duran GL-18). Soil samples were also rapidly placed in air-tight glass tubes after collection. All samples were placed on dry ice directly after sampling, and kept frozen until processing. Twig xylem sampling was complemented with pre-dawn and midday measurements of xylem water potential with a pressure chamber (Scholander et al. 1965). In adjacent twigs, gas exchange measurements were determined with an infra-red gas analyzer (Walz GFS-3000, Heinz Walz GmbH, Effeltrich, Germany). The system was equipped with a LED-Array/PAM-Fluorometer 3055-FL and a cuvette for conifers 3010-V80. Chamber conditions were set to mimic ambient conditions in the upper canopy (CO_2 concentration: 380 ppm; photosynthetic photon flux density: 1000-1500 μmol m^{-2} s^{-1}; cuvette temperature and relative humidity: 18-28ºC, 40-70%). All parameters were recalculated to actual leaf area using the equations from Von Caemmerer and Farquhar (1981). Leaf area was determined by calculating the projected area of an average oak leaf as an ellipse (π × length × width) or pine needle as a thin rectangle (length × width) and multiplying by the total number of leaves inside the chamber.

Water collection, distillation and isotope analyses

Precipitation was collected at the closest meteorological station (Instituto de Formación Agroambiental de Jaca, < 500m), between August 2012 and October 2013. We used a hand-crafted rain collector with paraffin oil to prevent evaporation; samples were collected biweekly and immediately stored in the fridge until analysis (following IAEA protocol for precipitation collection; http://www-naweb.iaea.org/napc/ih/documents/other/gnip_manual_v2.02_en_hq.pdf). After the sampling campaigns, groundwater samples were collected from a nearby fountain (Atarés, <500m from the study site) and its isotopic value was in accordance to other fountains along the medium-high mountains in the Spanish Pyrenees (-8.49 ± 0.10‰ for $\delta^{18}O$ and -57.57 ±0.70‰ for $\delta^{2}H$; Oliván et al. 2011). The extraction of water from the soil and xylem samples was performed by cryogenic vacuum distillation (Dawson and Ehleringer 1993) at the Dept. of Crop and Forest Sciences, Universitat de Lleida (Spain). The extraction system consisted of 10 sample tubes connected with Ultra-Torr™ fittings (Swagelok Company, Solon, Ohio, USA) to 10 U-shaped collection tubes specifically designed for this system. The sample tubes were submerged in mineral oil at a constant temperature (110-120ºC) to evaporate water and the U-tubes were cooled with liquid nitrogen to condense the water vapour. The system was connected to a vacuum pump (model RV3; Edwards, Bolton, UK) to ensure the flow of water vapour from the sample tubes to the collection tubes, and to prevent contamination with atmospheric water vapour. The entire system maintained constant vacuum pressures of *ca.* 10^{-2} mbar. Distillation time was 1.5 hour for xylem samples and 2 hours for soil samples.

We analysed the isotope composition of water samples by Cavity Ring-Down Spectroscopy (CRDS) in a Picarro L2120-*i* isotopic water analyser (Picarro Inc., Sunnyvale, CA, USA) at the Serveis Científico-Tècnics of the Universitat de Lleida (Lleida, Spain). The analyser was coupled to a high-precision vaporiser (A0211) through a Micro-Combustion Module™ (MCM), integrated in-line between the vaporiser and the analyser. The MCM removes the contaminants through oxidation, in a way that only pure water arrives to the analyser (Picarro 2012). After calibration with three internal standards, isotope composition was expressed in per mil notation (δ18O and δ2H, for oxygen and hydrogen, respectively), relative to VSMOW (Vienna Standard Mean Ocean Water). The estimated precision for the L2120-*i*, based on the repeated analysis of 4 reference water samples was 0.10‰ and 0.40‰, for $\delta^{18}O$ and $\delta^{2}H$, respectively.

As described in Martín-Gómez et al. (2015), residual organic compounds in the distilled water can interfere with the analysis of plant and soil samples using CRDS. As a quality assessment of the level of contamination among our samples, we compared the results with and without post-processing correction. We found a very strong correlation between corrected and uncorrected values (R^2=0.998 for $\delta^{18}O$; R^2=0.989 for $\delta^{2}H$, N=620) agreeing with low levels of contamination. For consistency, and taking into consideration that some samples were analysed before the MCM installation, we used software-corrected results for all samples.

Assessment of evaporative processes in soil and xylem water

Potential evaporation processes in xylem and soil water were visually assessed by plotting δ^2H against $\delta^{18}O$. In previous work (Martín-Gómez et al. 2016) we stated that evaporation of soil and xylem water causes a progressive enrichment in the heavy isotopes of the remaining water pools, but this effect is larger in $\delta^{18}O$ than in δ^2H. Consequently, isotopic composition increases along an evaporation line, with a flatter slope than the meteoric water in the $\delta^{18}O$ - δ^2H scatterplot (Craig 1961). Under a particular set of environmental conditions, water samples from the same meteoric origin should move along the same evaporation line, whereas distinct evaporation lines would indicate different origins. As a reference, we used the Global Meteoric Water Line (GMWL), which represents the relationship between the two isotopes in global precipitation ($\delta^2H = 10 + 8\times\delta^{18}O$; Craig 1961), and a Local Meteoric Water Line (LMWL) built from precipitation records (August 2012 - October 2013)

Leaf $\delta^{13}C$ isotopic analysis

For each field campaign, the leaves belonging to every xylem sample collected were immediately frozen in dry ice and stored at -24ºC until processing. The samples were freeze-dried for 24 hours in order to stop cellular activity and subsequently oven-dried for 48 hours at 50ºC. For carbon isotope analysis, 0.9–1.1 mg of dry matter was weighted into tin capsules and analyzed by mass spectrometry at the Stable Isotope Facility of the University of California, Davis (USA) using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). $^{13}C/^{12}C$ ratios were calibrated against internal standards and expressed relative to the international standard Vienna PeeDee Belemnite (VPDB). The accuracy of the analyses (Standard deviation of working standards) was 0.06‰.

Total non-structural carbohydrate concentrations in twig xylem

Once the water was extracted from the twigs, xylem was also analysed for soluble sugars (SS) and starch (St) concentrations. For this purpose the dry xylem samples were grounded to fine powder and analysed twice. Soluble sugars were extracted from 50 mg samples with 80% (v/v) ethanol. The extraction was done in a shaking water bath at 60°C. After centrifugation, the concentration of SS was determined colorimetrically at 490 nm using the phenol– sulphuric method of Dubois et al. (1956), as modified by Buysse and Merckx (1993). For starch concentration, the pellet remaining after ethanol extraction was digested with an enzyme mixture containing amyloglucosidase to convert starch into glucose, as described in Palacio et al. 2007. Concentration of starch-derived glucose was then determined colorimetrically using the same method as for SS.

Concentration of total nonstructural carbohydrates (NSC, % dry matter) was calculated as the sum of SS and starch concentrations.

Tree-ring width

After the last sampling campaign we extracted cores of the studied trees in order to assess their long-term growth patterns. We extracted 10 trees per specie, but 1 pine and 1 oak were discarded as outliers for the chronology. Tree rings were visually cross-dated using a binocular microscope coupled to a PC using the software WinDendro^{TM} (Regent Instruments. Canada Inc., 2009). The COFECHA^{TM} program (Holmes 1983) was used to evaluate the visual cross-dating. Individual tree-ring width was then converted into Basal Area Increment (BAI, in mm^2/year), in order to remove variations in radial growth attributable to size and age effects (e.g. Piovesan et al. 2008, Moreno-Gutiérrez et al. 2012a). BAI was calculated from radial increments assuming a cylindrical shape with the formula: $\mathrm{BAI} = \pi \times (r^2_{\mathrm{t}} - r^2_{\mathrm{t-1}})$, where r is the radius and t is the year of the tree-ring formation.

Statistical analyses

We tested significant differences in time and between species for all physiological variables. In all cases, generalized linear mixed models were based on Restricted Maximum Likelihood - REML ($\alpha = 0.05$). Differences among sampling times for each species were tested with the Tukey test ($\alpha = 0.05$). The association between isotope composition in xylem and soil water at different depths was assessed by simple *Pearson* correlations. The relationship between the water potential and stomatal conductance and NSC was described by fitting exponential and linear regressions, respectively. All the statistical analyses were performed with JMP Pro 11 (SAS Inc., Cary, NC, USA) with the exception of the relation between water potential, g_s and NSC that was adjusted with Sigma Plot version 12.5 (Systat Software, Inc., San Jose California USA). For water potential and gas exchange variables, March sampling-time was excluded from analysis as only data for pine was available. Unless otherwise stated; means are shown together with their associated standard error of the mean.

RESULTS

Evolution of climatic and biophysical site conditions

The year 2012 was a dry year throughout the Iberian Peninsula (Peguero-Pina et al. 2015). In Jaca, the annual total precipitation in 2012 was only 5% under average (806 mm, calculated from 1983 to 2011); however, summer precipitation (June-August) was 43% lower than the average (143 mm). Besides, the preceding winter of 2012 growing season was relatively dry, with very scarce snow events and spring rains. Thus, the scarce precipitation in 2012, along with high summer temperatures and plant water consumption, resulted in a very dry and long drought period. In this regard, we observed a clear reduction in SWC and a sharp decline in NDVI starting from June until late September. At this point, autumn rains increased, soil water reserves were filled again and vegetation recovered physiological activity (Figure 1). In contrast, 2013 was a very wet year (1189 mm annual). In particular, summer precipitation was twice (330 mm) the historical average, and previous winter and spring precipitation events were also abundant. Consequently, SWC in summer was higher than in 2012, and only a short and slight decline in NDVI was found in July-August (Figure 1).

Seasonal changes in the isotopic composition of precipitation, soil and xylem water

Isotopic composition of precipitation events was very variable in time, ranging from -13.90‰ for $\delta^{18}O$ and -99.26‰ for $\delta^{2}H$ in the end of winter period to +5.17‰ for $\delta^{18}O$ and +13.22‰ for $\delta^{2}H$ in summer, whereas the soil remained fairly stable throughout the sampling campaigns (-9.06 ± 0.10‰ for $\delta^{18}O$ and -64.41±0.69‰ for $\delta^{2}H$; Fig. S1). Usually, soil water composition did not correspond strictly to the preceding precipitation events and was only in autumn and after the abundant winter precipitation when the soil approached isotopic composition of previous rains (Fig. S1). Groundwater values (-8.41‰ for $\delta^{18}O$ and -56.36‰ for $\delta^{2}H$) were similar to soil recharge precipitation in autumn (October and November average values: -8.77±0.30‰ for $\delta^{18}O$ and -58.13±2.48‰ for $\delta^{2}H$; Fig. S1). Despite in most cases the top soil was more enriched than deeper soil layers, the evaporative gradient along the soil vertical profile was not very pronounced (average values for surface soil enrichment: +1.03 ± 0.14‰ for $\delta^{18}O$ and +4.51 ± 0.87‰ for $\delta^{2}H$; Fig. 2), and even it was inverse in the wettest period with more depleted values in surface (Fig. 2c). Additionally, xylem water showed in most cases a clear deviation towards more enriched values with respect to soil isotopic values (+2.01±0.21‰ and +2.47±0.29‰ in $\delta^{18}O$; +7.31±0.96‰ and 8.91±1.43‰ in $\delta^{2}H$ for pines and oaks respectively; Fig. 2), particularly during the driest period for pines (+4.08±0.29‰ in $\delta^{18}O$ and +14.82±1.38‰ in $\delta^{2}H$; Fig. 2a), and during leafless stage in oaks (+5.91±0.25‰ in $\delta^{18}O$ and +26.34±1.30‰ in $\delta^{2}H$; Fig. 2c). This isotopic enrichment in the xylem followed a typical evaporative line (Fig. 2a-e), generally overlapping with the one formed by soil water composition with depth. Only in September 2013 (Fig. 2e), xylem and soil isotopic signature were clearly coupled, coinciding with moderate precipitation events occurring several days before the sampling campaign. As a result of the broadly-found enrichment of xylem in respect to soil isotopic composition, we could not make a direct assignation of species to water source at different soil depths by mixing models (Parnell et al. 2010). However, comparing inter-tree variability for a given species and time, we would expect better correlations between xylem and soil water for the soil depth constituting the predominant source of water. As shown in Table 1, the isotope composition of xylem water in pines was best correlated with intermediate depths (0.64 for δ^2H at 30 cm), whereas the oaks were better correlated with the deepest soil layer (0.64 for δ^2H at 50cm). It should be noted that, we found better correlations for δ^2H than $\delta^{18}O$ (Table 1) because evaporative enrichment in stems was the main cause for the observed soil-xylem uncoupling and δ^2H is less affected by evaporative processes than $\delta^{18}O$ (Martín-Gómez et al. 2016).

Pre-dawn and midday leaf water potentials

Overall, throughout the study period, oaks reached lower water potential than pines at midday (ψ_{md}), but recovered to higher water potential at pre-dawn (ψ_{pd}), indicating a preferential access to wet soil layers, together with more active transpiration during the day (Figure 3). During the dry summer in 2012, both species reached very low water potentials for midday (ψ_{md}) and pre-dawn (ψ_{pd}), in accordance with generally dry soil conditions (ψ_{md} = -2.39±0.12 MPa; ψ_{pd} = -2.14±0.11 MPa). In October 2012, despite the milder temperatures and some recent rains, the drought developed during summer was still reflected in the physiological status, particularly for the pines (Figure 3). On the one hand, the higher ψ_{pd} in the oaks suggest than the pines had more limited access to the wetter soil (Figure 3a); on the other hand, the oaks showed much lower values of ψ_{md}, presumably associated to higher transpiration rates (Figure 3b). Contrarily, oaks were reacting faster to the more favorable autumn conditions, showing a clear recovery in pre-dawn water potential (ψ_{pd} = -0.80±0.07 MPa) and more negative midday water potential, indicative of higher transpiration rates (ψ_{md} = -2.27±0.07 MPa). Although midday water potentials in summer 2013 were as low as in summer 2012, pre-dawn values were significantly higher, indicating higher soil water availability and transpiration rates for both species.

Gas exchange and water use efficiency

Pines showed consistently higher $\delta^{13}C$ values than oaks, associated to a greater intrinsic water use efficiency (WUE_i) and tighter stomatal regulation (Fig. 4a,c). In the dry summer of 2012, oak $\delta^{13}C$ values were high and close to those of pines, indicating similar levels of stress in both species. In 2013, the new oak leaves maintained low $\delta^{13}C$ values from early spring to summer. Conversely, the pines showed a sharp decline of $\delta^{13}C$ in early spring, rising in June-August to similar levels to those found in 2012. Although the pine needles sampled in 2013 were not current-year needles, their higher $\delta^{13}C$ cannot be only attributed to a memory-effect of previous-year conditions, considering the highly dynamic changes observed. In summer 2012, both species showed minimal g_s (Fig. 4c), and near-zero or even negative A_n (Fig. 4b) indicating that respiration was in the same order of magnitude as photosynthesis. In October, after the first rain events, both species recovered physiological activity, showing a moderate increase in g_s, and clearly positive A_n. Pine photosynthesis during the following early spring (before budburst in oaks) indicated only moderate physiological activity. Despite the wet conditions of previous winter-spring 2013, neither oak nor pine showed high physiological activity in late spring (June), probably due to relatively low temperatures, and it was not until August that both species reached a maximum in g_s and A_n. At this point, oaks showed significantly higher g_s and A_n than pines.

Total non-structural carbohydrates xylem concentration

Throughout the studied period, oaks showed larger accumulation of starch (Fig. 5b) and soluble sugars (Fig. 5a) in the xylem, as compared to pines. For both groups of carbohydrates, seasonal evolution was rather stable with time in pines, finding only a clear peak of starch accumulation in October and a slightly higher starch concentration in March. Conversely, the seasonal pattern of carbohydrate accumulation in oaks was highly variable, in particular for starch. In this sense, in the severe drought event in July 2012, we found the lowest starch concentrations of the study period, whereas the concentration of soluble sugars was higher than usual. In October, oak starch concentration was recovered, and remained constant until the next season, just before the budburst. In June 2013, starch concentration in oaks increased substantially (maximum values of the study period), recovering to normal levels in August.

Drought limitation of photosynthesis

We found a significant exponential relation between ψ_{pd} and gas exchange parameters (Fig. 6a – A_n, Fig. 6b – g_s) for pines, showing higher g_s and A_n for higher ψ_{pd}, *i.e.* associated to higher soil water content. Contrarily, this relationship was not found for oaks, for which gas exchange parameters were largely independent from water potential. In this regard, we also found a significant correspondence between ψ_{pd} and Total NSC for pines, and not for oaks, which could be linked to stomatal limitation and thus reduced assimilation in pines.

Historical evolution of Basal Area and growth patterns

The long-term evolution of Basal Area Increment (BAI, Fig. 7) indicated differential patterns in growth for both species, although they had similar age (around 55-60 years old). Growth rates in both species were higher and more variable during the first years of establishment. However, pine oscillation was higher and the slope of the decreasing trend with age was higher in comparison to oaks (-12.64 mm^2 $year^{-1}$ in pines, -1.27 in oaks). Conversely, BAI in oaks remained nearly constant (also slower) throughout the study period, and seemed less affected by the accumulation of non-favorable climatic conditions. Furthermore, after the extreme dry years around mid-nineties, pine decrease in BAI was exacerbated, in contrast with the lack of response in the oaks.

DISCUSSION

As summarized in Figure 8, we found species-specific seasonal patterns on physiological variables and isotope composition, reflecting the contrasting climatic conditions during the two measured growing seasons, and the distinct physiological strategies and ability to cope with drought. 2012 was a very dry year with limited autumn precipitation and scarce snow events, whereas 2013 vas a very wet year with important rain and snow events in autumn and winter. On the other hand, the marcescent oak (*Q. subpyrenaica*), in comparison to Scots pine (*P. sylvestris*), was able to reach lower midday water potential throughout the studied period, thus being able to maintain transpiration and physiological activity under drier soil conditions (Picon et al. 1996, Poyatos et al. 2008, Klein et al. 2013, Forner et al. 2014). Despite the lower values in midday water potential, the oaks showed higher water potential overnight, indicating that they could also have access to deeper (and wetter) soil layers than the pines (e.g. Poyatos et al. 2008, Klein et al. 2013, del Castillo et al. 2016, Grossiord et al. 2016). Indeed, transpiration was only restricted in oaks during the extremely dry conditions in summer 2012 (Figure 8a), but rapidly recovered after the first rains in early autumn (Figure 8b). In this sense, marcescence is a well-known phenomenon of extending physiological activity during early and even late autumn when climatic conditions still allow sufficient gas exchange (e.g. Abadía et al. 1996; Peguero-Pina et al. 2015). Although a partial recovery was also noticeable for Scots pine in early autumn, full recovery was only evidenced in early spring 2013 (Figure 8c). During the wet summer in 2013, near-optimal conditions allowed for a complete recovery of stomatal conductance, leading to higher photosynthetic rates and starch accumulation, and a tight coupling between soil and xylem isotope values (Figure 8d).

The ability of oaks to reach low water potentials at midday and recover hydric status overnight is partly associated to root system distribution and water uptake patterns (e.g. Filella and Peñuelas 2003, Moreno-Gutiérrez et al. 2012b, West et al. 2012, Barbeta et al. 2015). Generally, oaks are able to maintain their conservative strategy and continuous physiological activity by relying on stable water pools like groundwater, deep soil layers or more tightly-bound water in the soil, while the pines with greater Water Use Efficiency (in this study $\delta^{13}C$ as proxy of WUE) develop an opportunistic strategy of fast use of precipitation water based in a high root density on top soil layers (e.g. Poyatos et al. 2008, Klein et al. 2013, del Castillo et al. 2016, Grossiord et al. 2016). In this sense, despite the offset between soil and xylem isotopic composition in our data (Brandes et al. 2007, Ellsworth and Sternberg 2014, Bertrand et al. 2014), the analysis of correlation for all the studied period showed maximum $\delta^{2}H$ correlations for soil water at 50 cm depth for oaks and at 30 cm for pines, reinforcing the general knowledge of oak reliance on deeper soil water pools in comparison to pines (e.g. David et al. 2007, Barbeta et al. 2015). In support of the more opportunistic strategy of Scots pine, a study in the Eastern Pyrenees (Poyatos et al. 2008) reported larger sap flow restrictions during summer drought in a Scots pine stand than in a plot with pubescent oak (*Quercus humilis*), but a faster increase in pine transpiration after the first rain events. Besides the differences in water uptake patterns, pines are generally regarded as more isohydric species, with quicker and tighter stomatal control (e.g. Irvine et al. 1998, Poyatos et al. 2008, Martínez-Vilalta et al. 2009) . However, the more dynamic stomatal control of pines does not always imply a faster recovery of physiological activity after drought, since it is linked to greater xylem vulnerability (Martínez-Vilalta et al. 2004). Therefore, after long and severe drought periods, the greater vulnerability to xylem embolism may lead to more limited recovery of transpiration flow in pines, as compared to oaks (e.g. Poyatos et al. 2008, Salmon et al. 2015). Furthermore, in mixed stands, as in the present study, the risk of xylem embolism in Scots pine might be enhanced due to the larger ability of oak species to exhaust soil water (Willaarts 2012, Klein et al. 2013). In agreement with our findings, del Castillo et al. (2016) found a faster recovery after a long summer drought in the evergreen oak *Quercus ilex*, with respect to the Mediterranean pine *Pinus halepensis*. Similar to our study, the larger water uptake capacity of the evergreen oaks also resulted in a tighter association between soil and xylem water isotope composition during drought and subsequent recovery. This can be explained by the enhanced effect of evaporative enrichment of xylem water under conditions of limited sap flow, as has been recently shown for the same studied species (Martín-Gómez et al. 2016). In consequence, pines in summer 2012 and leafless oaks showed maximum separation; oaks recovered physiological activity and soil isotopic signature faster during the first rains after summer and was only from 2013 spring when the soil is completely hydrated when pine and oak practically coupled with soil.

The limited ability of pines to access deep water reservoirs was also evidenced by the response of gas exchange parameters to ψ_{pd}. In accordance to previous studies (Gallego et al. 1994, Irvine et al. 1998, Perks et al. 2002, Poyatos et al. 2008, Klein et al. 2013, Salmon et al. 2015, Nardini et al. 2016) we found a significant relationship between soil water status (as reflected in ψ_{pd}) and g_s and A_n in pines, showing that photosynthesis was largely constrained by drought due to stomatal closure. Hence, when pines reached levels of ψ_{pd} below -1.5 MPa, stomatal conductance was lower than 30 mmol m^{-2} s^{-1}, and consequently carbon assimilation was heavily affected. Similarly, Salmon et al. (2015) showed a decrease in g_s from about 120 mmol m^{-2} s^{-1} at ψ_{pd} =-1.3 MPa to values below 20 mmol m^{-2} at ψ_{pd} =-1.8 MPa, whereas Poyatos et al. (2008) and Perks et al. (2002) showed that midday g_s was already reduced to 10-20% of its maximum value with ψ_{pd} below -0.8 MPa. Conversely, although oaks also showed a tendency towards lower ψ_{pd} associated to lower g_s and A_n, this was not significant, suggesting a lesser restriction of water availability for this species; and hence, continued physiological activity despite the harsh climatic conditions. Poyatos et al. (2008) also found a rather scattered response of g_s against ψ_{pd} in pubescent oaks, which maintained g_s above 30% of their maximum over the entire range of ψ_{pd} recorded (from -0.2 to -0.8 MPa). On the other hand, Gallego et al. (1994) did not find substantial changes in g_s and ψ_{pd} of *Q. pyrenaica* as soil water decreased, concluding that this was a consequence of the ability of this species to access deep water reservoirs. Indeed, in the two sites included in their study, soil water potential at 20 cm declined up to -4.0 MPa during summer drought, whereas ψ_{pd} was generally above -1.0 MPa, coinciding with that found in the soil at 40-60 cm depth. Notably, this range of soil depth agrees with our observation of a tight association between isotope composition in oak xylem water and soil water at 50 cm.

We found lower concentrations of both SS and starch for pines in comparison to oaks throughout the studied period. This is in accordance to general knowledge that stem in conifers has very low content of NSC relative to angiosperm species (Martínez-Vilalta et al. 2016) and it is associated to the lower amount of parenchyma in conifer sapwood (Morris et al. 2016, Plavcová et al. 2016). Our results for *P. sylvestris* (average NSC content: 3.99 ± 1.34%) are in the range with those presented for defoliated individuals in Poyatos et al. (2013), but higher than other studies under water-limited conditions, such as Voltas et al. (2013) (<1%) and Sangüesa-Barreda et al. (2012) (1% of starch, 1.5% SS) in the submediterranean ecotone, or Gruber et al. (2012) (1-2%) in a xeric Alpine valley. Overall, even considering the additional uncertainty associated to different methods of carbohydrate quantification (Quentin et al. 2015), the values obtained do not suggest a strong NSC depletion in the studied pines. Nevertheless, given the strong drought-induced limitation of carbon uptake (A_n) in the pines, we still found a positive association between ψ_{pd} and NSC for pines, suggesting that drought periods actually imposed some limitations in carbon storage. Conversely, the oaks did not show any correlation between carbon storage and water potential, and NSC values (7.76 ± 2.39%) were generally high, e.g. as compared to those presented in Nardini et al. 2016 for *Q. humilis* (1.5% of starch, 3.5% SS), although lower than those in Hoch et al. (2003), Körner (2003) or Li et al. (2013) (around 15%). Unlike in our study, Nardini et al. (2016) found decreasing water potential and NSC associated to hydraulic failure and drought-induced decline. Together with the evidences on water uptake and gas exchange, carbon storage patterns in the studied oaks suggest that the particular site conditions allow them to keep sufficient storage pools, minimizing drought effects and allowing a rapid recovery after a severe drought. In this regard, the close link between carbon pools and xylem repair indicate that large storage pools may contribute to a drought-tolerance strategy (Tyree et al. 1999, Salleo et al. 2009, Secchi and Zwieniecki 2011, Johnson et al. 2012, Brodersen and McElrone 2013). Along with this, the independence between ψ_{pd} and NSC in oaks, highlights that carbon storage in deciduous oaks is less sensitive to source limitations (e.g. under drought stress) and reflects a more complex dynamics of carbohydrate accumulation and probably starch-SS conversion (Salleo et al. 2009, Johnson et al. 2012).

Overall, our study on the seasonal patterns of physiological variables suggest that, despite the lack of visible symptoms of tree decline, the pines in our study site are largely limited by drought conditions, and cannot recover easily from severe drought episodes. In line with this, in both species radial growth patterns were affected by contrasting climatic years, but *Pinus sylvestris* showed higher variations and far more pronounced decreasing trend with time. In this sense, it is well known that carbon storage has priority over growth because ultimately survival depends more on carbon demands for metabolism than for growth (McDowell 2011, Sala et al. 2012, Palacio et al. 2013). Therefore, since we observed an effect of drought on NSC patterns, we would expect an even larger effect on growth. In this regard, *P.sylvestris*, which showed a long-lasting accumulated effect of drought limitation on NSC patterns, was also the most affected in terms of growth, particularly after the extreme dry years around mid-nineties. In fact, Galiano et al. (2012) probed in *P. sylvestris* that extreme drought events can induce long-term physiological disorders that would cause a slower recovery of the survival trees, and point to carbohydrate reserves as a key factor determining tree survivorship.

CONCLUSIONS

In conclusion, as it can be seen in our results, an important number of physiological traits confer the advantage for survivorship and adaptation to *Q.subpyrenaica* in comparison to *P. sylvestris*, which is not only reflected in the short-term, but has already clear consequences on long-term trends in secondary growth. Despite the repeated drought events reported in the area, *Q. subpyrenaica* seems to stay within its 'confort zone' of physiological performance, being able to survive and grow steadily; anyhow, *P. sylvestris* seems to be largely drought-limited in the driest conditions. The isohydric strategy of *P. sylvestris* confers a very sensitive behavior, where hydraulic and carbon limitations are difficult to separate; soil dryness affects stomatal closure, which in turn influences carbon assimilation and in consequence carbon storage. Also, these lower NSC concentrations in conifer sapwood may result in lower capacity to refill embolized xylem conduits. Along with this, even though the studied pine trees did not exhibit clear symptoms of decline, the physiological and isotopic data reported here constitute an early warning evidence, confirming our initial hypothesis that these variables could be used to anticipate potential tree decline. Our results encourage the implementation of long-term studies to monitor the evolution of mixed stands in the area, in order to validate whether forest decline of *P. sylvestris* is consistently associated to the climatic and physiological conditions reported here.

ACKNOWLEDGEMENTS

This research was supported by the Spanish Government through projects AGL 2012-40039-C02 and AGL 2012-40151-C03, the PhD fellowship to P.M.G. (FPU12/00648) and *Ramón y Cajal* contract to J.P.F. (RYC-2008-02050). We thank Pilar Sopeña, Maria Josep Pau and Mireia Oromí for laboratory assistance on isotope and carbohydrate analyses; *Instituto de Formación Agroambiental de Jaca*, *Unidad de Salud de los Bosques de Aragón*, Miguel Ángel Lázaro and Jorge del Castillo for their support in field campaigns.

Author contributions

P.M.G., M.A., J.P., E.G.P. and J.P.F. designed the research and collected the samples. P.M.G and J.P.F analysed the data and wrote the manuscript.

REFERENCES

Abadía A, Gil E, Morales F, Montañés L, Montserrat G, Abadía J (1996) Marcescence and senescence in a submediterranean oak (*Quercus subpyrenaica* E.H. del Villar): photosynthetic characteristics and nutrient composition. Plant Cell Environ 19: 685–694.

Allen CD, Macalady AK, Chenchouni H, Bachelet D, McDowell N, Vennetier M, Kizberger T, Rigling A, Breshears DD, Hogg EH, Gonzalez P, Fensham R, Zhang Z, Castro J, Demidova N, Lim JH, Allard G, Running SW, Semerci A, Cobb N (2010) A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. Forest Ecology and Management. 259: 660-684.

Ameztegui A, Coll L, Brotons L, Ninot JM (2016) Land-use legacies rather than climate change are driving the recent upward shift of the mountain tree line in the Pyrenees. Glob Ecol Biogeogr 25:263–273.

Barbeta A, Mejía-Chang M, Ogaya R, Voltas J, Dawson TE, Peñuelas J (2015) The combined effects of a long-term experimental drought and an extreme drought on the use of plant-water sources in a Mediterranean forest. Glob Chang Biol 21: 1213–1225.

Benito-Garzón M, Sánchez de Dios R, Sainz Ollero H (2008) Effects of climate change on the distribution of Iberian tree species. Appl Veg Sci 11: 169–178.

Bertrand G, Masini J, Goldscheider N, Meeks J, Lavastre V, Celle-Jeanton H, Gobat J-M, Hunkeler D (2014) Determination of spatiotemporal variability of tree water uptake using stable isotopes ($\delta^{18}O$, δ^2H) in an alluvial system supplied by a high-altitude watershed, Pfyn forest, Switzerland. Ecohydrology 7: 319–333.

Brandes E, Wenninger J, Koeniger P, Schindler D, Rennenberg H, Leibundgut C, Mayer H, Gessler A (2007) Assessing environmental and physiological controls over water relations in a Scots pine (*Pinus sylvestris* L.) stand through analyses of stable isotope composition of water and organic matter. Plant Cell Environ 30: 113–127.

Brodersen CR, McElrone AJ (2013) Maintenance of xylem network transport capacity: a review of embolism repair in vascular plants. Front Plant Sci 4:108.

Buysse J, Merckx R (1993) An improved colorimetric method to quantify sugar content of plant tissue. J Exp Bot 44: 1627-1629

del Castillo J, Comas C, Voltas J, Ferrio JP (2016) Dynamics of competition over water in a mixed oak-pine Mediterranean forest: Spatio-temporal and physiological components. For Ecol Manage 382: 214–224.

Carnicer J, Barbeta A, Sperlich D, Coll M, Peñuelas J (2013a) Contrasting trait syndromes in angiosperms and conifers are associated with different responses of tree growth to temperature on a large scale. Frontiers in Plant Science, 4: 409

Carnicer J, Coll M, Ninyerola M, Pons X, Vayreda J, Peñuelas J (2014) Large-scale recruitment limitation in Mediterranean pines: the role of *Quercus ilex* and forest successional advance as key regional drivers. Glob. Ecol. Biogeogr 23: 371–384

Christensen JH, Hewitson B, Busuioc A, Chen A, Gao X, Held I, Jones R, Kolli RK, Kwon W-T, Laprise R, Rueda VM, Mearns L, Menéndez CG, Räisänen J, Rinke A, Sarr A, Whetton P (2007) Regional climate projections. In: Climate Change 2007: The physical science basis. contribution of working Group I to the fourth assessment report of the intergovernmental panel on climate change (eds), pp 847–940.

Corcuera L, Camarero JJ, Gil-Pelegrín E (2004) effects of a severe drought on growth and wood anatomical properties of *Quercus faginea*. 25: 185–204.

Craig H (1961) Isotopic variations in meteoric waters. Science 133: 1702–1703.

David TS, Henriques MO, Kurz-Besson C, Nunes J, Valente F, Vaz M, Pereira JS, Siegwolf R, Chaves MM, Gazarini LC, David JS (2007) Water-use strategies in two co-occurring Mediterranean evergreen oaks: surviving the summer drought. Tree Physiol 27: 793–803.

Dawson T, Ehleringer JR (1993) Isotopic enrichment of water in the 'woody' tissues of plants: Implications for plant water source, water uptake, and other studies which use the stable isotopic composition of cellulose. Geochim Cosmochim Acta 57: 3487–3492.

DuBois M, Gilles K, Hamilton J, Rebers P, Smith F (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28: 350–356

Ellsworth PZ, Sternberg LSL (2014) Seasonal water use by deciduous and evergreen woody species in a scrub community is based on water availability and root distribution. Ecohydrology 8: 538–551.

Filella I, Peñuelas J (2003) Partitioning of water and nitrogen in co-occurring Mediterranean woody shrub species of different evolutionary history. Oecologia 137: 51–61.

Forner A, Aranda I, Granier A, Valladares F (2014) Differential impact of the most extreme drought event over the last half century on growth and sap flow in two coexisting Mediterranean trees. Plant Ecol 215: 703–719.

Galiano L, Martínez-Vilalta J, Lloret F (2010) Drought-induced multifactor decline of Scots Pine in the Pyrenees and potential vegetation change by the expansion of co-occurring oak species. Ecosystems 13: 978–991.

Galiano L, Martínez-Vilalta J, Sabaté S, Lloret F (2012) Determinants of drought effects on crown condition and their relationship with depletion of carbon reserves in a Mediterranean holm oak forest. Tree Physiol 32: 478–489.

Gallego HA, Rico M, Moreno G, Santa-Regina I (1994) Leaf water potential and stomatal conductance in *Quercus-Pyrenaica* Willd Forest - Vertical gradients and response to environmental factors. Tree Physiol 14: 1039–1047.

García-Ruiz JM, Lasanta T, RuizFlano P, Ortigosa L, White S, Gonzalez C, Marti C (1996) Land-use changes and sustainable development in mountain areas: A case study in the Spanish Pyrenees. Landsc Ecol 11: 267–277.

Gómez-Aparicio L, García-Valdés R, Ruíz-Benito P, Zavala M.A (2011). Disentangling the relative importance of climate, size and competition on tree growth in Iberian forests: implications for forest management under global change. Glob. Change Biol. 17: 2400–2414.

Gosz JR (1992) Ecological functions in a biome transition zone: translating local responses to broad-scale dynamics. In: Hansen AJ, di Castri F (eds), Landscape boundaries: consequences for biotic diversity and ecological flows. Springer New York, New York, NEW YORK,pp 55–75.

Granda E, Rossatto DR, Camarero JJ, Voltas J, Valladares F (2014) Growth and carbon isotopes of Mediterranean trees reveal contrasting responses to increased carbon dioxide and drought. Oecologia, 174: 307–317.

Grossiord C, Sevanto S, Dawson TE, Adams HD, Collins AD, Dickman LT, Newman BD, Stockton EA, McDowell NG (2016) Warming combined with more extreme precipitation regimes modifies the water sources used by trees. New Phytol 213: 584-596

Gruber A, Pirkebner D, Florian C, Oberhuber W (2012) No evidence for depletion of carbohydrate pools in Scots pine (*Pinus sylvestris* L.) under drought stress. 14: 142–148.

Himrane H, Camarero J, Gil-Pelegrín E (2004) Morphological and ecophysiological variation of the hybrid oak *Quercus subpyrenaica* (*Q. faginea* x *Q. pubescens*). Trees 18: 566–575.

Hoch G, Richter A, Körner C (2003) Non-structural carbon compounds in temperate forest trees. Plant Cell Environ 26: 1067–1081.

Holmes RL (1983) Computer-assisted quality control in tree-ring dating and measurement. Tree -Ring Bulletin 43: 69-78

Irvine J, Perks MP, Magnani F, Grace J (1998) The response of *Pinus sylvestris* to drought: stomatal control of transpiration and hydraulic conductance. Tree Physiol 18: 393–402.

Johnson DM, McCulloh K a, Woodruff DR, Meinzer FC (2012) Hydraulic safety margins and embolism reversal in stems and leaves: why are conifers and angiosperms so different? Plant Sci 195: 48–53.

Klein T, Shpringer I, Fikler B, Elbaz G, Cohen S, Yakir D (2013) Relationships between stomatal regulation, water-use, and water-use efficiency of two coexisting key Mediterranean tree species. For Ecol Manage 302: 34–42.

Körner C (2003) Carbon limitation in trees. J Ecol 91: 4–17.

Li MH, Cherubini P, Dobbertin M, Arend M, Xiao WF, Rigling A (2013) Responses of leaf nitrogen and mobile carbohydrates in different *Quercus* species/provenances to moderate climate changes. Plant Biol 15: 177–184.

Martínez-Vilalta J, Cochard H, Mencuccini M, Sterck F, Herrero A, Korhonen JFJ, Llorens P, Nikinmaa E, Nolè A, Poyatos R, Ripullone F, Sass-Klaassen U, Zweifel R (2009) Hydraulic adjustment of Scots pine across Europe. New Phytol 184: 353–64.

Martínez-Vilalta J, Piñol J (2002) Drought-induced mortality and hydraulic architecture in pine populations of the NE Iberian Peninsula. For Ecol Manage 161: 247–256.

Martínez-Vilalta J, Piñol J, Beven K (2002) A hydraulic model to predict drought-induced mortality in woody plants: an application to climate change in the Mediterranean. Ecol Model 155: 127–147

Martínez-Vilalta J, Sala A, Asensio D, Galiano L, Hoch G, Palacio S, Piper FI, Lloret F (2016) Dynamics of non-structural carbohydrates in terrestrial plants: a global synthesis. Ecol Monogr 86: 495-516

Martínez-Vilalta J, Sala A, Piñol J (2004) The hydraulic architecture of Pinaceae–a review. Plant Ecol 171: 3–13.

Martín-Gómez P, Barbeta A, Voltas J, Peñuelas J, Dennis K, Palacio S, Dawson TE, Ferrio JP (2015) Isotope-ratio infrared spectroscopy: a reliable tool for the investigation of plant-water sources? New Phytol 207: 914–927.

Martín-Gómez P, Serrano L, Ferrio JP (2016) Short-term dynamics of evaporative enrichment of xylem water in woody stems: implications for ecohydrology. Tree Physiol doi: 10.1093/treephys/tpw115

McDowell NG (2011) Mechanisms linking drought, hydraulics, carbon metabolism, and vegetation mortality. Plant Physiol 155: 1051–1059.

Moreno-Gutiérrez C, Battipaglia G, Cherubini P, Saurer M, Nicolás E, Contreras S, Querejeta JI (2012a) Stand structure modulates the long-term vulnerability of *Pinus halepensis* to climatic drought in a semiarid Mediterranean ecosystem. Plant, Cell Environ 35: 1026–1039.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI (2012b) Isotopes reveal contrasting water use strategies among coexisting plant species in a mediterranean ecosystem. New Phytol 196: 489–496.

Morris H, Plavcová L, Cvecko P, Fichtler E, Gillingham MAF, Martínez-Cabrera HI, Mcglinn DJ, Wheeler E, Zheng J, Ziemińska K, Jansen S (2016) A global analysis of parenchyma tissue fractions in secondary xylem of seed plants. New Phytol 209: 1553–1565.

Nardini A, Casolo V, Dal Borgo A, Savi T, Stenni B, Bertoncin P, Zini L, Mcdowell NG (2016) Rooting depth, water relations and non-structural carbohydrate dynamics in three woody angiosperms differentially affected by an extreme summer drought. Plant, Cell Environ 39: 618–627.

Nardini A, Pitt F (2002) Drought resistance of *Quercus pubescens* as a function of root hydraulic conductance, xylem embolism and hydraulic architecture. New Phytol 143: 485–493.

Oliván C, Lambán L, Cuchí J, Villaroel J (2011) Caracterización hidrogeoquímica e isotópica del acuífero carbonatado-kárstico drenado por el manantial de Fuenmayor (Huesca). Asociación Internacional de Hidrogeólogos (Grupo Español), Zaragoza.

Palacio S, Hoch G, Sala A, Körner C, Millard P (2013) Does carbon storage limit tree growth? New Phytol 201: 1096-1100

Palacio S, Millard P, Maestro M, Montserrat-Martí G (2007) Non-structural carbohydrates and nitrogen dynamics in mediterranean sub-shrubs: an analysis of the functional role of overwintering leaves. Plant Biol. 9: 49–58

Parnell AC, Inger R, Bearhop S, Jackson AL (2010) Source partitioning using stable isotopes: Coping with too much variation. PLoS One 5: 1–5.

Peguero-Pina JJ, Sancho-Knapik D, Martín P, Saz MÁ, Gea-Izquierdo G, Cañellas I, Gil-Pelegrín E (2015) Evidence of vulnerability segmentation in a deciduous Mediterranean oak (*Quercus subpyrenaica* E. H. del Villar). Trees - Struct Funct 29: 1917–1927.

Perks MP, Irvine J, Grace J (2002) Canopy stomatal conductance and xylem sap abscisic acid (ABA) in mature Scots pine during a gradually imposed drought. Tree Physiol 22: 877–883.

Picarro (2012) Micro-Combustion Module^{TM} (MCM): elimination oforganics datasheet. http://www.picarro.com/sites/default/files/Micro-Combustion Module Datasheet.pdf (24 December 2016, date last accessed).

Picon C, Guehl JM, Ferhi A (1996) Leaf gas exchange and carbon isotope composition responses to drought in a drought-avoiding (*Pinus pinaster*) and a drought-tolerant (*Quercus petraea*) species under present and elevated atmospheric CO_2 concentrations. Plant Cell and Environment 19: 182 - 190

Piovesan G, Biondi F, Di Filippo A, Alessandrini A, Maugeri M (2008) Drought-driven growth reduction in old beech (*Fagus sylvatica* L.) forests of the central Apennines, Italy. Glob Chang Biol 14: 1265–1281.

Plavcová L, Hoch G, Morris H, Ghiasi S, Jansen S (2016) The amount of parenchyma and living fibers affects storage of nonstructural carbohydrates in young stems and roots of temperate trees. Am J Bot 103: 603–612.

Poyatos R, Aguadé D, Galiano L, Mencuccini M, Martínez-Vilalta J (2013) Drought-induced defoliation and long periods of near-zero gas exchange play a key role in accentuating metabolic decline of Scots pine. New Phytol 200: 388–401.

Poyatos R, Llorens P, Piñol J, Rubio C (2008) Response of Scots pine (*Pinus sylvestris* L.) and pubescent oak (*Quercus pubescens* Willd.) to soil and atmospheric water deficits under Mediterranean mountain climate. Ann For Sci 65: 306.

Quentin AG, Pinkard EA, Ryan MG, Tissue DT, Baggett LS, Adams HD, Maillard P, Marchand J, Landhäusser SM, Lacointe A, Gibon Y, Anderegg WRL, Asao S, Atkin OK, Bonhomme M, Claye C, Chow PS, Clément-Vidal A, Davies NW, Dickman LT, Dumbur R, Ellsworth DS, Falk K, Galiano L, Grünzweig JM, Hartmann H, Hoch G, Hood S, Jones JE, Koike T, Kuhlmann I, Lloret F, Maestro M, Mansfield SD, Martínez-Vilalta J, Maucourt M, McDowell NG, Moing A, Muller B, Nebauer SG, Niinemets Ü, Palacio S, Piper F, Raveh E, Richter A, Rolland G, Rosas T, Joanis B Saint, Sala A, Smith RA, Sterck F, Stinziano JR, Tobias M, Unda F, Watanabe M, Way DA, Weerasinghe LK, Wild B, Wiley E, Woodruff DR (2015) Non-structural carbohydrates in woody plants compared among laboratories. Tree Physiol 35: 1146–1165.

Sala A, Woodruff DR, Meinzer FC (2012) Carbon dynamics in trees: Feast or famine? Tree Physiol 32:

764–775.

Salleo S, Trifiló P, Esposito S, Nardini A, Lo Gullo MA (2009) Starch-to-sugar conversion in wood parenchyma of field-growing *Laurus nobilis* plants: A component of the signal pathway for embolism repair? Funct Plant Biol 36: 815–825.

Salmon Y, Torres-Ruiz JM, Poyatos R, Martinez-Vilalta J, Meir P, Cochard H, Mencuccini M (2015) Balancing the risks of hydraulic failure and carbon starvation: a twig scale analysis in declining Scots pine. Plant Cell Environ 38: 2575-88

Sánchez de Dios R, Benito-Garzón M, Sainz-Ollero H (2009) Present and future extension of the Iberian submediterranean territories as determined from the distribution of marcescent oaks. Plant Ecol 204: 189–205.

Sánchez-Salguero R, Camarero JJ, Hevia A, Madrigal-González J, Linares JC, Ballesteros-Cánovas JA, Sánchez-Miranda A, Alfaro-Sánchez R, Sangüesa-Barreda S, Galván JD, Gutiérrez E, Génova M, Riglin, A (2015) What drives growth of Scots pine in continental Mediterranean climates: drought, low temperatures or both? Agric. For. Meteorol. 206: 151–162

Sangüesa-Barreda G, Linares JC, Camarero JJ (2012) Mistletoe effects on Scots pine decline following drought events: Insights from within-tree spatial patterns, growth and carbohydrates. Tree Physiol 32: 585–598.

Scholander PF, Hammel HT, Bradstreet ED, Hemmingsen EA.1965. Sap pressure in vascular plants. Science 148: 339–346.

Secchi F, Zwieniecki MA (2011) Sensing embolism in xylem vessels: The role of sucrose as a trigger for refilling. Plant, Cell Environ 34: 514–524.

Tang K, Feng X (2001) The effect of soil hydrology on the oxygen and hydrogen isotopic compositions of plants' source water. 185: 355–367

Tognetti R, Cherubini P, Marchi S, Raschi A (2007) Leaf traits and tree rings suggest different water-use and carbon assimilation strategies by two co-occurring *Quercus* species in a Mediterranean mixed-forest stand in Tuscany, Italy. Tree Physiol 27: 1741–1751.

Tyree MT, Salleo S, Nardini A, Gullo MA Lo, Mosca R, Lo Gullo MA, Mosca R, Thomas Tyree M, Salleo S, Nardini A, Lo Gullo MA, Mosca R (1999) Refilling of embolized vessels in young stems of laurel. Do We need a new paradigm? Plant Physiol 120: 11–21.

Voltas J, Camarero JJ, Carulla D, Aguilera M, Ortiz A, Ferrio JP (2013) A retrospective, dual-isotope approach reveals individual predispositions to winter-drought induced tree dieback in the southernmost distribution limit of Scots pine. Plant, Cell Environ 36: 1435–1448.

Von Caemmerer S, Farquhar GD (1981) Some relationships between the photochemistry and the gas exchange of leaves. Planta 153: 376-387

West AG, Dawson TE, February EC, Midgley GF, Bond WJ, Aston TL (2012) Diverse functional responses to drought in a Mediterranean-type shrubland in South Africa. New Phytol 195: 396–407.

Willaarts B (2012) Linking land management to water planning: estimating the water consumption of Spanish forests. In: Water, agriculture and the environment in Spain: Can we square the circle?. Lucia De Stefano and M. Ramon Llamas (eds), Water Observatory of the Botin Foundation; Complutense University of Madrid, Spain, pp 139–151

TABLES AND FIGURES

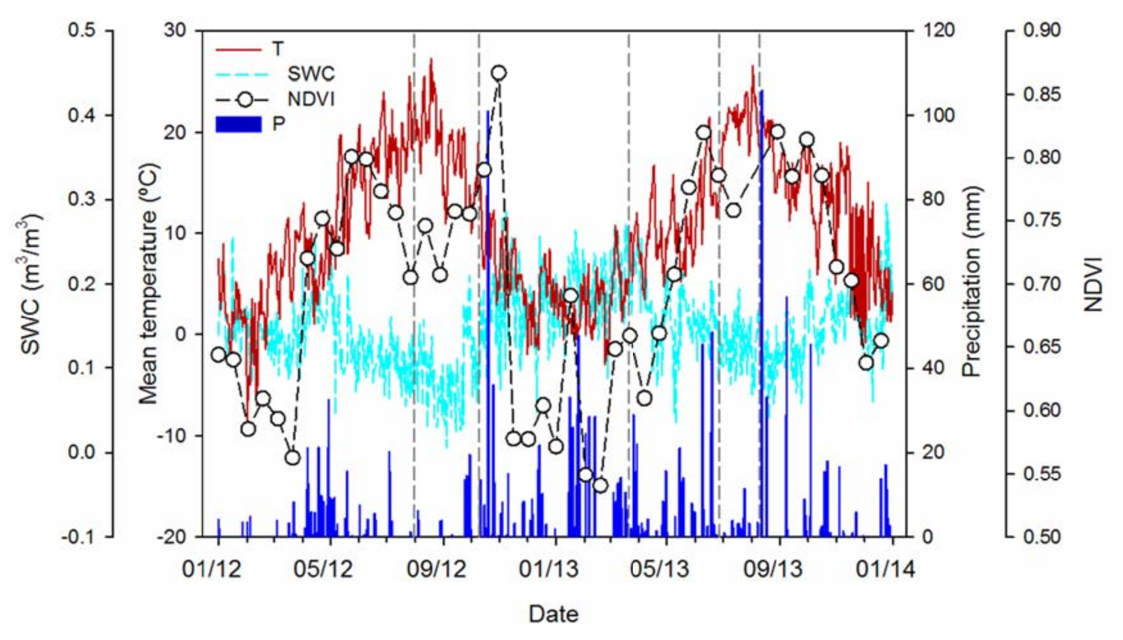

Figure 1. Physiological and environmental variables of the study site during the 2012-2013 seasonal sampling: soil water content (SWC) derived from ESA-SMOS remote sensing data for the four closest pixels (50×50 m^2) to the study site, daily mean temperature (T) and precipitation (P), and vegetation index (NDVI) from MODIS data of the pixel containing the study site (250×250 m^2). Dotted vertical lines indicate the five sampling campaigns.

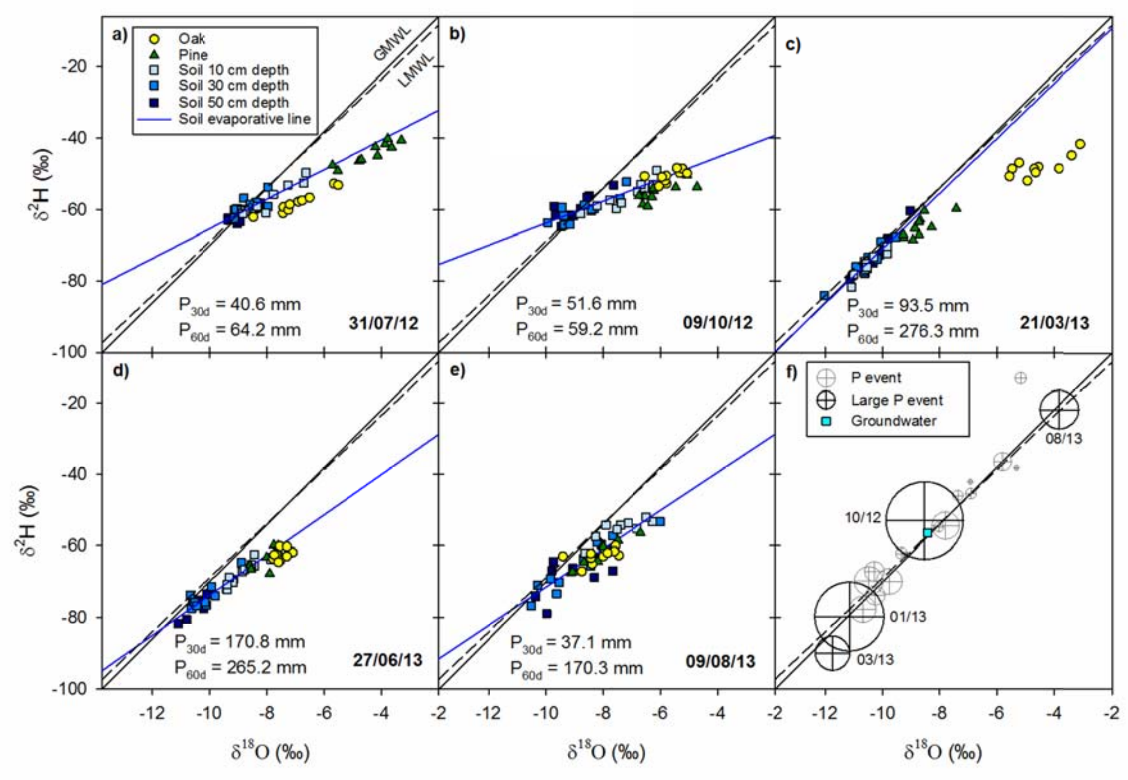

Figure 2. Bi-plots representing the seasonal evolution of the isotope composition of oxygen ($\delta^{18}O$) and hydrogen (δ^2H) of precipitation (f), groundwater (f), and water extracted from twig xylem of pines and oaks and from the 10, 30 and 50 cm deep soil layers (a, b, c, d, e) during the five sampling campaings. Crossed circles in f panel, $\delta^{18}O$ and δ^2H of precipitation during the study period collected in the closest climatic station to the forest; size proportional to precipitation quantity; the largest precipitation events are highlighted with thicker and black lines and date (month/year) is indicated. The precipitation of the previous 30 and 60 days to the sampling date is displayed. Blue square in f; δ-values for groundwater collected in a fountain within the forest. In a-e panels, green triangles, pine; yellow circles oak; light blue, blue and dark blue squares, 10, 30 and 50 cm depth soil, respectively. Black solid and dashed lines indicate, respectively, the global (GMWL, solid) and local (LMWL, dashed) meteoric water lines. Blue solid line represents soil water evaporative line.

Table 1. Correlation between isotope composition in xylem water and in soil water collected at different depths (p = 0.05). *Ps* and *Qs*, xylem water of *Pinus sylvestris* and *Quercus subpyrenaica*, respectively; S10, S30 and S50, soil water at 10, 30 and 50 cm, respectively. Lower and upper triangle show correlations for oxygen (δ^{18}O) and hydrogen (δ^{2}H) isotope composition, respectively.

δ^{2}H **δ^{18}O**	***Ps***	***Qs***	**S10**	**S30**	**S50**
Ps (*n*=50)	-	0.41	*0.48*	**0.64**	**0.54**
Qs (*n*=40)	**0.55**	-	0.35	*0.56*	**0.64**
S10 (*n*=50)	0.43	*n.s.*	-	**0.77**	**0.56**
S30 (*n*=50)	*0.50*	*n.s.*	**0.71**	-	**0.77**
S50 (*n*=48)	0.32	*n.s.*	**0.55**	**0.64**	-

n.s., non significant ($p \geq 0.05$); $p < 0.001$, italics; $p < 0.0001$, bold

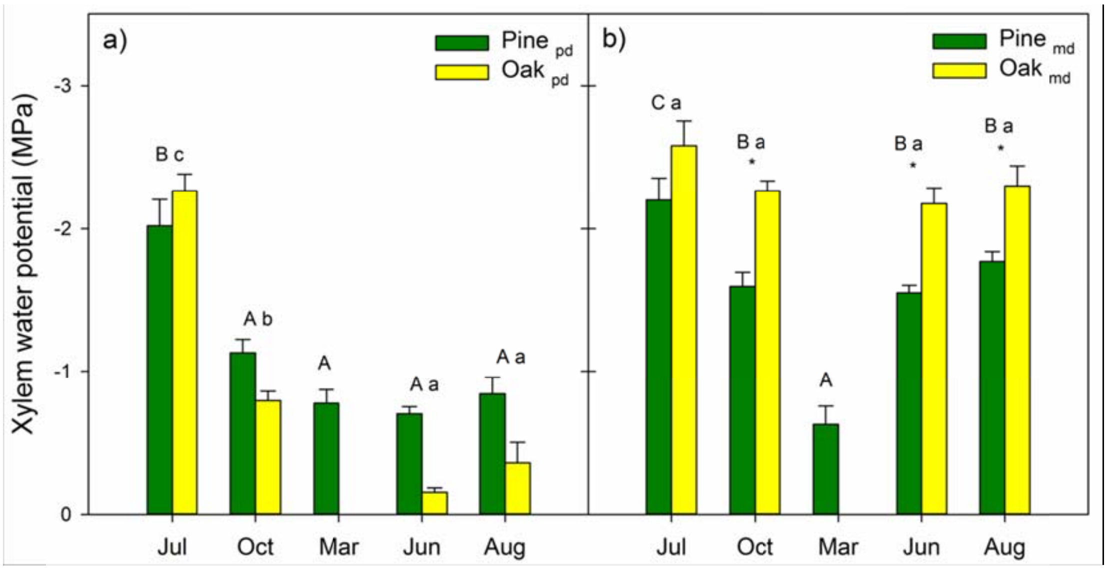

Figure 3. Seasonal evolution of pre-dawn (a) and midday (b) xylem water potential (MPa) of the two species, pine (green) and oak (yellow). Error bars represent standard errors. Differences were calculated based on Restricted Maximum Likelihood - REML (α = 0.05). Asterisks denote significant differences between species for each time. Letters show significant differences among sampling times for each species (Tukey test, $p < 0.05$); capital letters, pine; lower case, oak.

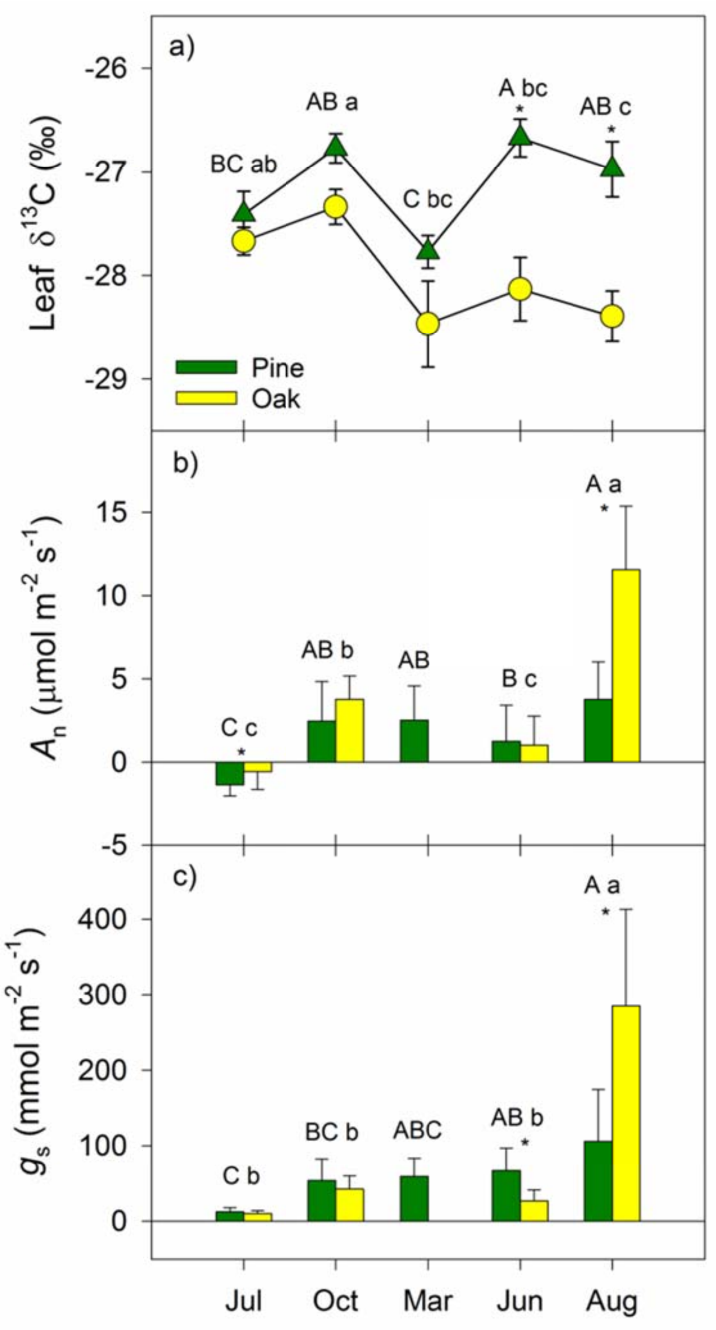

Figure 4. Seasonal evolution of leaf-level carbon and water balance for the two studied species, pine (green) and oak (yellow): (a) carbon isotope composition ($\delta^{13}C$) of bulk leaves, (b) photosynthetic rate (A_n), and (c) stomatal conductance (g_s). Error bars represent standard errors. Differences were calculated based on Restricted Maximum Likelihood - REML ($\alpha = 0.05$). Asterisks denote significant differences between species for each time. Letters show significant differences among sampling times for each species (Tukey test; $p < 0.05$): capital letters, pine; lower case, oak.

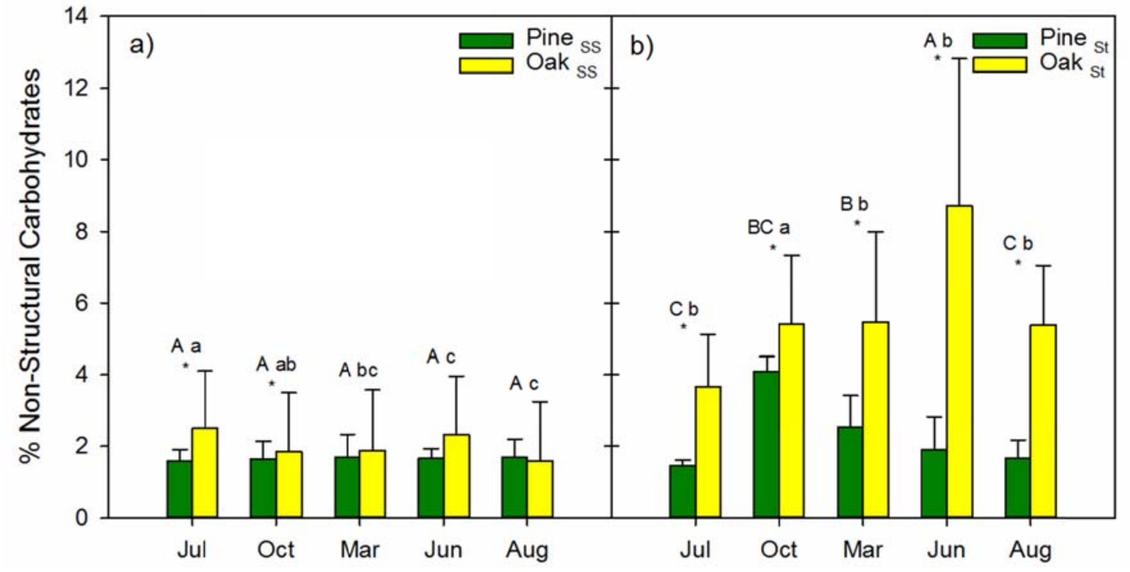

Figure 5. Seasonal evolution of soluble sugars (a) and starch (b) concentration in xylem for the two studied species, pine (green) and oak (yellow). Error bars represent standard errors. Differences were calculated based on Restricted Maximum Likelihood - REML ($\alpha = 0.05$). Asterisks denote significant differences between species for each time. Letters show significant differences among sampling times for each species (Tukey test; $p < 0.05$): capital letters, pine; lower case, oak.

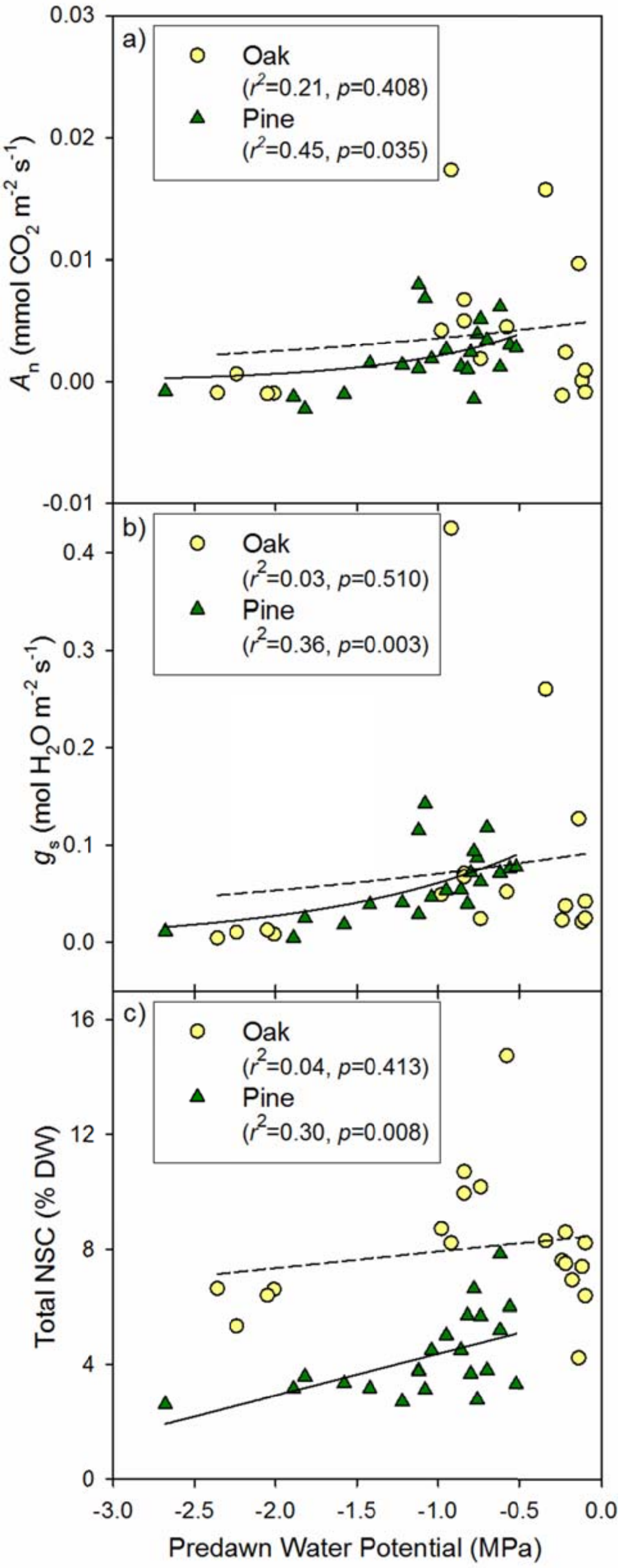

Figure 6. Relationship between pre-dawn water potential and (a) photosynthesis - A_n. (b) stomatal conductance - g_s and (c) total carbohydrates content - Total NSC, for all the sampling times and both species. Yellow circles and dashed line, oaks; green triangles and solid line, pines. The regression coefficient (r^2) and significance (p) is represented in each panel.

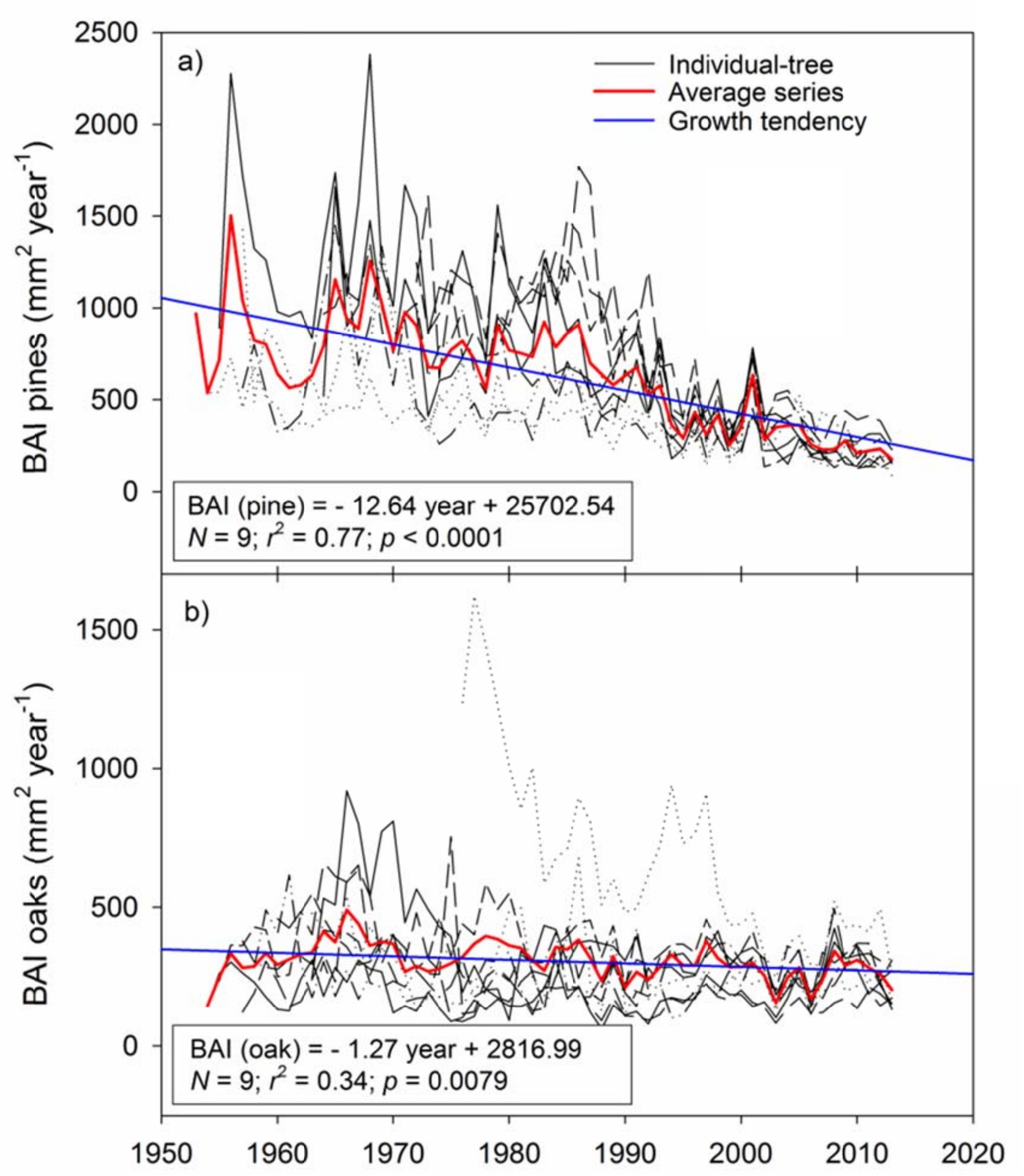

Figure 7. Historical evolution of Basal Area Index (BAI in mm/year) for the same individuals included in the seasonal sampling: (a) pines (*N*=9) and (b) oaks (*N*=9). Grey thin lines, individual-tree values; thick red line, average values for the 9 trees; linear regression in a thick blue line represents the decreasing trend for BAI (named growth tendency). The regression equation, number of chronologies (*N*), regression coefficient (r^2) and significance (p) is represented in each panel.

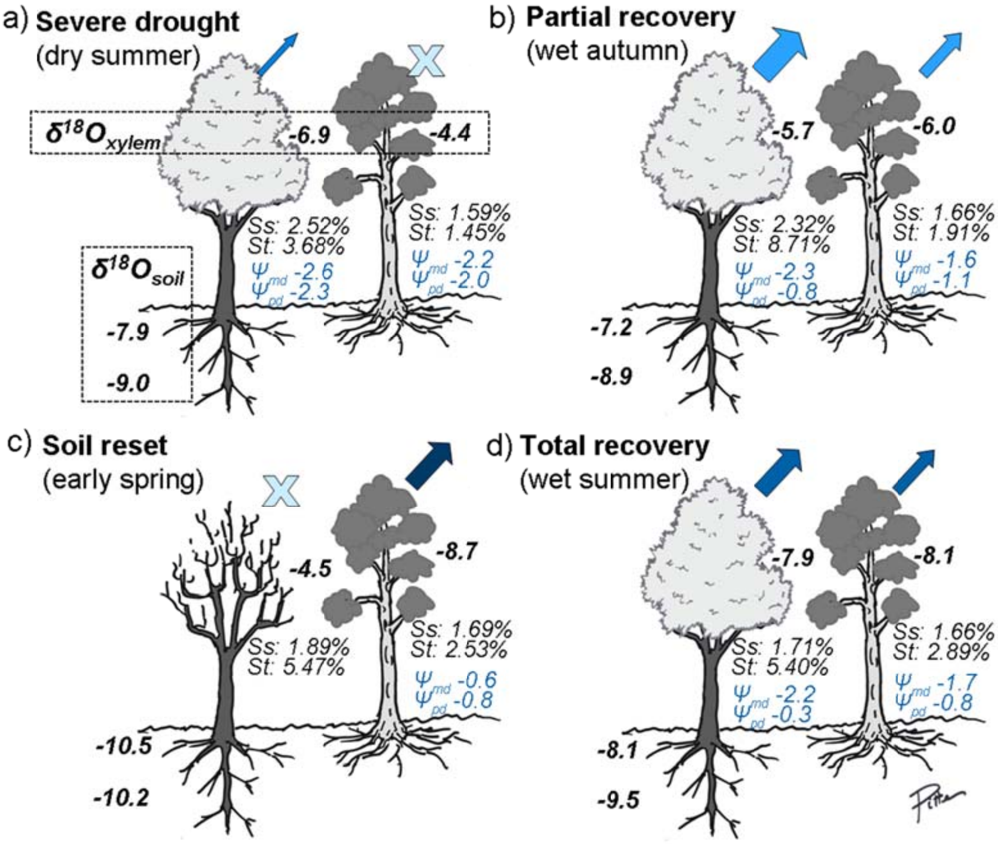

Figure 8. Graphical summary of seasonal changes in soil and xylem water isotope composition ($\delta^{18}O$ in ‰), midday and pre-dawn water potential (Ψ_{md}, Ψ_{pd} in MPa) and carbohydrate concentration in xylem (SS: soluble sugars; St: starch in %) for oaks and pines during the studied period. The size of the arrows on top of the trees denotes the estimated range of transpiration rates for each species; a cross denotes stomatal closure. Light to dark blue in arrows indicate enriched to depleted isotopic values in xylem water. In the dry summer of 2012 (a) the soil profile shows the strongest evaporative gradient, with more enriched values in the upper layers. In both species the transpiration is minimal and there is not recovery of soil water potential overnight. In early autumn after the first precipitation events (b) there is a recovery of transpiration rates reflected in a lower water potential at pre-dawn and in less enriched xylem water, although still uncoupled. Oak recovery is much more evident than pine. After the soil water recharge phase in winter (c), soil isotopic signature changes to very negative values due to the snow and there is no evaporative gradient. Oaks remained leafless with highly enriched xylem. Pines showed very low water potentials in relation to higher transpiration rates and xylem moving in the direction of new soil isotope values. Finally, in the second summer, were climatic conditions were very wet (d), transpiration is fully recovered and xylem and soil couple.

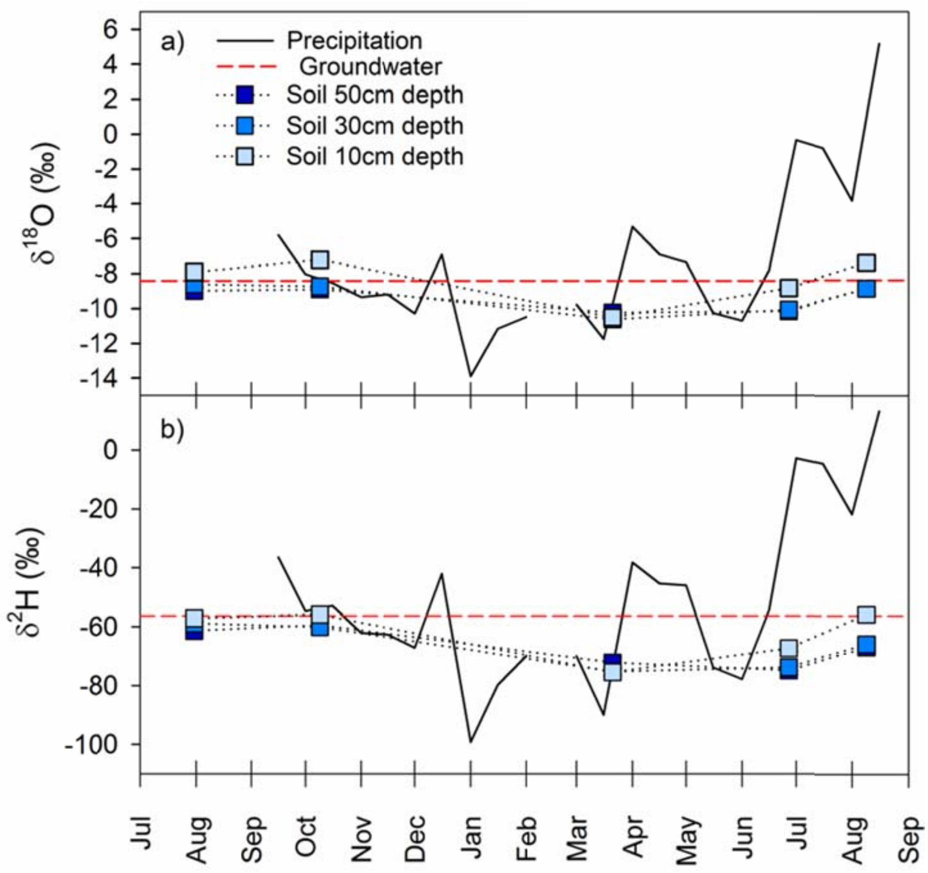

Figure S1. Seasonal evolution the isotope composition of oxygen (a - $\delta^{18}O$) and hydrogen (b - $\delta^{2}H$) in precipitation, groundwater, and soil water extracted at 10, 30 and 50 cm depth (in this order, light to dark blue squares). Black solid and red dashed lines indicate, respectively, precipitation and groundwater isotopic values.

CHAPTER IV.

Soil- or plant-driven fractionation cause isotopic uncoupling between soil and xylem water during drought and recovery

Paula Martín-Gómez[1], Ulises Rodríguez-Robles[2], Juan Pedro Ferrio[3]

[1]Dept. Crop and Forest Sciences-AGROTECNIO, Universitat de Lleida, Lleida, Spain, [2]División de Ciencias Ambientales, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa de San José No. 2055 Lomas 4ta, C.P. 78216, San Luís Potosí, México, [3]Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile.

SUMMARY

Currently, the analysis of the isotopic composition of xylem water to determine the source of water used by plants is being challenged by increasing evidence of isotopic uncoupling between precipitation, soil and xylem. This phenomenon has been ascribed to fractionation mechanisms taking place in roots at water uptake in some xerophytic and halophytic species, to fractionation processes during water storage associated to evaporation from the stem surface, as well as to the existence of non-well mixed pools of water in soil and the species-specific use of them. In this context, we conducted a laboratory experiment with pot trees of the contrasting strategy species, Scots pine (*P. sylvestris*) and marcescent oak (*Q. faginea).* We designed two cycles of watering-drought irrigating with isotopically different water and continuously measuring physiological status and sampled soil and xylem for water isotopes. We confirmed that isohydric strategy in pines limited their water uptake during the first stages of drought while oaks, with a more conservative strategy, were maintaining transpiration and physiological activity under drier soil conditions. These different strategies were linked to contrasting water use patterns and resulting soil drying profile, showing certain evidence for species-specific use of different soil water pools. In any case, we could not confirm the "two water world hypothesis" as the occurring evaporative process in xylem was displacing isotopic composition in the same direction as the tightly-attached previous soil labelling. We conclude that further assessment is needed to understand and separate the effects of isotopic fractionation occurring in soil and xylem, before straightly assume that xylem water represents source water.

Keywords: drought, evaporative enrichment, isotopic fractionation, soil water pools, uncoupling, water strategy

INTRODUCTION

The isotopic composition of precipitation, groundwater, soil and plant xylem presents a great potential for characterizing water movement along the soil-plant-atmosphere continuum (see e.g. Meinzer et al. 1999, Moreira et al. 2000, Ferrio et al. 2005, Li et al. 2007, Querejeta et al. 2007, Moreno-Gutiérrez et al. 2012, Bertrand et al. 2014, del Castillo et al. 2016). The analysis of the isotopic composition of xylem water has been applied to determine the source of water used by plants (Ehleringer and Dawson 1992), under the assumption that there is no fractionation during the uptake process by roots and water transportation (see e.g. Zimmermann et al. 1967, Dawson 1991, Dawson and Ehleringer 1993, Thorburn et al. 1993). The basis of this approach is that the potential water sources available to plants show contrasting isotopic signatures (see e.g. Gat 1996, Tang and Feng 2001, Máguas et al. 2011) and that the preferential loss of light isotopes during evaporation creates strong isotopic gradients along the soil profile during dry periods (Allison et al. 1983). Taking advantage of this variability, isotopic tracing has revealed the use of contrasting water sources among adjacent plants, related to differences in physiological strategies (e.g. Sternberg and Swart 1987, Filella and Peñuelas 2004, Máguas et al. 2011, del Castillo et al. 2016). In this sense, in a previous study (Martín-Gómez et al. 2016b) we associated the isohydric strategy of Scots Pine (*Pinus sylvestris*) to the use of water of top soil layers; and also, the more conservative strategy of a marcescent oak (*Quercus subpyrenaica*) to the access to deeper or more tightly-bound water in the soil.

Nevertheless, despite the high amount and variety of studies based on these approaches there is increasing evidence of isotopic uncoupling between xylem water and soil water (Brooks et al. 2009, Máguas et al. 2011, McDonnell 2014, Bertrand et al. 2014, Martín-Gómez et al. 2016). This phenomenon has been ascribed to fractionation mechanisms taking place in roots at water uptake in some xerophytic and halophytic species (Lin et al. 1993, Ellsworth and Williams 2007), as well as to fractionation processes during water storage in the stem, either due to potential isotopic exchange with organic compounds (Zhao et al. 2016), or more often linked to isotopic enrichment associated to evaporation from the stem surface (Ellsworth and Sternberg 2014, Martín-Gómez et al. 2016, Bögelein et al. 2017). Along with fractionation processes occurring within the plant, several studies show evidence for the existence of non-well mixed pools of water in soil; the mobile water linked to infiltrating precipitation, and the tightly-bound water that reflects the unsaturated soil water and plant transpiration (Tang and Feng 2001, Brooks et al. 2009, Goldsmith et al. 2012, McDonnell 2014, Evaristo et al. 2015). 2015). The proportion of these two water pools, and its isotopic signature would depend mainly on soil physical properties, and plants would show a differential ability to use the different soil water fractions, according to their physiological status and strategy.

Certainly, these new findings on soil and stem processes, have important implications for the study of plant water uptake, as well as for ecosystem- and global-scale hydrological models by means of adding new sources of variation between precipitation, water source and water transpired by plants. In this sense, in order to assess the water dynamics of plant water use under different ecological and environmental circumstances, it is necessary to clarify the magnitude of the fractionation processes occurring in the soil and the xylem, particularly during drought.

For this purpose, we conducted a laboratory experiment with pot trees of the previously studied Scots pine (*P. sylvestris*) and a closely-related marcescent oak (*Q. faginea).* We designed two cycles of watering-drought irrigated with isotopically different water and we continuously avoided soil evaporation and controlled tree sap flow. During the experiment, we sampled soil and xylem water and measured water potential and gas exchange parameters. By precisely controlling the water entering the system and understanding plant physiological status, we expected to whether confirm the existence of xylem and soil driven fractionation and separate their effects in order to, finally, advance in the knowledge of water uptake patterns and recovery after drought in the studied species.

MATERIALS AND METHODS

Description of the experiment

In a pot experiment, we studied the dynamics of water use of pine and oak saplings during drought and recovery, and their effect on xylem and soil isotopic signatures. Commercial saplings of Scots pine and a marcescent oak (*Quercus faginea* L.) located at the Experimental Fields of the Universitat de Lleida (Lleida, 31T 0.596293 41629816, 170m high) were used during summer 2015. Ten saplings per species (4 and 6 years old, for oaks and pines, respectively) were originally cultivated in nursery containers with standard substrate. One year and a half before the experiment, they were transplanted to 20-liter pots filled with a forest loamy soil, collected in a pine-oak mixed stand located in the Spanish Pre-Pyrenees (Boalar Forest - Jaca - Huesca, 30T 693606 4714041, 800m high). To minimize isotopic fractionation due to soil evaporative enrichment, soil pot surface was directly covered with aluminium foil and a cone-shaped roof was fixed around the tree stem to prevent water inputs from eventual precipitation events. Two plants per species were kept aside from the main experimental procedure, and used to quantify stem evaporation, soil evaporation, and the potential effect of thermal gradients on sap flow measures. The remaining eight plants per species were divided into two groups, in which two different water labelling treatments were applied during two consecutive soil irrigation-drought cycles (Fig. 1). All the trees were initially irrigated, dried for 8 days until reaching soil wilting point (-1.5 MPa, 13% of soil water content); then irrigated with water with different isotopic composition and dried for 8 days again. The first group was initially irrigated with tap water (-9.79 ± 0.24‰ for $\delta^{18}O$ and -68.25 ± 3.42‰ for $\delta^{2}H$) and afterwards with depleted water (-23.25‰ for $\delta^{18}O$ and -166.102‰ for $\delta^{2}H$). The second group was first irrigated with deuterated water (-9.16 ± 0.04‰ for $\delta^{18}O$ and +343.02 ± 0.96 for $\delta^{2}H$), followed by tap water. Twigs were sampled at the time of maximum vegetative activity (around 10 a.m.) on the day before watering, the day after and on the 3rd, 5th and 7th-8th day of drought. Soil cores (15 mm diameter × *ca.* 150-200 mm height) were sampled on the same days as twigs and the soil profile was divided in 3 samples: 0-5cm, 5-10 and 10-15cm depth. Twig xylem and soil sampling was complemented with measurements, in the same sampled twigs, of xylem water potential (ψ_{xylem}) with a pressure chamber (Scholander and Hammel 1965) and gas exchange with an infra-red gas analyzer (Walz GFS-3000, Heinz Walz GmbH, Effeltrich, Germany). The IRGA was equipped with a LED-Array/PAM-Fluorometer 3055-FL and a cuvette for conifers 3010-V80. Chamber conditions were set to mimic ambient conditions (CO_2 concentration: 380 ppm; photosynthetic photon flux density: 1200 µmol m^{-2} s^{-1}; cuvette temperature and relative humidity: 25-33ºC, 40-70%). All parameters were recalculated to actual leaf area following Von Caemmerer and Farquhar (1981). Leaf area was determined by calculating the projected area of an average oak leaf as an ellipse (π × length × width) or pine needle as a thin rectangle (length × width) and multiplying by the total number of leaves inside the chamber. Throughout the experiment, pot weight was measured and sap flow was monitored with "baby gauges" SF62, coupled to the Sap flow meter T4.2 (EMS Brno, Brno, Czech Republic). These data were downloaded and analyzed with Mini32 software ver.403.34 (EMS Brno, Brno, Czech Republic).

Assessment of soil and twig evaporation influence

Firstly, in order to assess whether aluminium foil covering on pot soil and the pot shelter were effective in preventing soil evaporation, we did a test in one tree per species. We irrigated the pots until field capacity, defoliated the trees to avoid plant water consumption and left them without watering throughout the experiment. We weighted the pots several times along the experiment to prove that soil water loss was minimal.

Secondly, in order to assess evaporative enrichment in twigs, we followed the procedure described by Martín-Gómez et al. (2016) in one tree per species. Following the main experiment, we did two watering-drought cycles; for the first one we used tap water, and for the second deuterated water (same as above). After watering, plants remained uncovered until the third night, when all the leaves were covered with aluminium foil. The leaves remained covered for three nights, when the aluminium foil was removed. After three days of recovery, plants were watered again and the cycle was repeated. We sampled twig xylem and soil water on the last day of covering and on the first once uncovered.

Sample collection and water extraction for isotopic analyses

For xylem sampling, bark and phloem were removed and the peeled xylem was immediately placed in air-tight glass tubes (Duran GL-18). The tubes were frozen on liquid nitrogen directly after sampling, and kept frozen until processing. Xylem and soil water was extracted by cryogenic vacuum distillation (Ehleringer and Dawson 1992) at the Dept. of Crop and Forest Sciences of the Universitat de Lleida. Sample tubes were placed in a heated silicone oil bath (120°C), and connected with Ultra-Torr^{TM} unions (Swagelok Company, Solon, Ohio, USA) to a vacuum system (*ca.* 10^{-2} mbar), in series with U-shaped collector tubes, cooled with liquid N_2. After an extraction time of 2 h (soil) and 1.5 h (xylem), trapped water was transferred into 2 ml vials, and stored at 4°C until analysis. Preliminary recovery tests showed that these were the most suitable conditions to ensure complete distillation (Martín-Gómez et al. 2014, 2016; Palacio et al. 2014). All xylem and soil samples were weighted before and after distillation, in order to calculate xylem and soil water content (XWC, SWC, in %).

Estimation of soil water potential: water retention curves and Rosetta Model

In order to estimate soil water potential (ψ_s in MPa) from soil water content (SWC, in %) we used the software Rosette V1.0 (company, city, country) to calculate unsaturated hydraulic properties (e.g. water retention parameters or hydraulic conductivity) from surrogate soil data such as soil texture and bulk density. From previous soil analysis, we had texture data (29.14% sand, 38.49% silt, 32.37% clay) and SWC at field capacity (0.33MPa, 21% SWC) and wilting point (1.5MPa, 13% SWC). We calculated dry bulk density using wet and dry distillation weight, field capacity pot weight and pot volume, obtaining an average value of 0.64±0.06 g/cm^3. Using these data as model inputs, and resolving the Van Genuchten (1980) equations with the calculated parameters (saturated and residual water content, and curve shape parameters), we obtained ψ_s values for a defined SWC.

Isotopic analyses

We analysed the isotope composition of water samples by Cavity Ring-Down Spectroscopy (CRDS) in a Picarro L2120-*i* isotopic water analyser (Picarro Inc., Sunnyvale, CA, USA) at the Serveis Científico-Tècnics of the Universitat de Lleida (Lleida, Spain) and a Picarro L2130-*i* in the Stable Isotopes Facility of the Institute for Landscape Biogeochemistry at ZALF (Müncheberg, Germany). Generally, 6 replicates of 1 µl were injected into the vaporizer, keeping the last three injections for calculation; but when analysing deuterium-enriched samples, in order to minimize memory effect, 9 replicates were injected and only the last three injections were used for calculation. With this method, we observed negligible memory effects, and rather homogeneous values. Average within-sample standard deviation was 0.16‰ for $\delta^{18}O$ and 0.67‰ for δ^2H, in the range of estimated analytical precision (0.10‰ and 0.40‰, for $\delta^{18}O$ and δ^2H, respectively). After calibration with three internal standards (the same in both laboratories), isotope composition was expressed in per mil notation ($\delta^{18}O$ and δ^2H, for oxygen and hydrogen, respectively), relative to VSMOW (Vienna Standard Mean Ocean Water).

As described in Martín-Gómez et al. (2015), residual organic compounds in the distilled water can interfere with the analysis of plant and soil samples using CRDS, but it is possible to overcome this with a post-processing correction. As a quality assessment of the level of contamination among our samples, we compared the results with and without post-processing correction (δ_{corr} and δ_{raw}, respectively). We found a very strong correlation between corrected and uncorrected values ($\delta^{18}O_{raw}=-0.33+0.9997\times\delta^{18}O_{corr}$, $R^2=0.998$; $\delta^2H_{raw}=-2.60+1.0035\times\delta^2H_{corr}$, $R^2=0.989$ for δ^2H; $N=369$). The offset between raw and corrected values was significant, but small, and the slope of the relationship between raw and corrected values did not differ significantly from unity. Due to the low level of contamination, and for consistency, we used raw results for all samples.

Statistical analyses

We tested significant differences in time and between species for all physiological variables. In all cases, generalized linear mixed models were based on Restricted Maximum Likelihood - REML ($\alpha = 0.05$). Differences among sampling times for each species were tested with the Tukey test ($\alpha = 0.05$). The association between isotope composition in xylem and soil water at different depths was assessed by simple *Pearson* correlations. All the statistical analyses were performed with JMP Pro 11 (SAS Inc., Cary, NC, USA), with the exception of the relationship between the water potential and gas exchange variables, that was described by fitting exponential regressions using Sigma Plot version 12.5 (Systat Software, Inc., San Jose California USA). Unless otherwise stated; means are shown together with their associated standard error of the mean.

RESULTS

Evolution of sap flow rates and water potential under drought limitation

The two irrigation events increased instant and total daily sap flow rates in the same day the water was added, followed by a progressive reduction in sap flow, associated to the decrease in soil water content (Fig. 2a,b). It should be noted that, due to the progressive reduction in total leaf area with time (as a consequence of twig sampling) and the changing environmental conditions, we did not observe a complete recovery of initial daily sap flow values after the second irrigation. The decrease in sap flow rates was slightly larger in pines than in oaks (33.6±12.0% and 30.4±19.9% of maximum decrease in midday sap flow for pines −Fig. 2a − and oaks −Fig. 2b −respectively) and they showed different temporal patterns in relation to the species-specific effects of drought and recovery on their physiological activity. As explained in methods section, the covering of soil in pots was very effective and the drought conditions in the soil were generated by plant water consumption, not by direct soil evaporation; thus, the patterns of soil drying and hence the consequent drought status relied on species-specific water use. In line with sap flow changes, soil and xylem water potential increased with irrigation and started to decline concurrently as long as the trees were consuming the water in the pots (Fig. 2c,d). During the first day of irrigation, we found in both species similar values of ψ_{soil} (-0.23±0.03 and -0.28±0.02 MPa, for pines and oaks, respectively) and ψ_{xylem} (-1.16±0.34 MPa for pines, -0.73±0.01 MPa for oaks). However, we found significant differences between species during the experimental drought. Pines reached minimum values for ψ_{soil} and ψ_{xylem} at the 4th day after irrigation, and remained constant for the two following days (e.g. 4th-6th day average for the first drying period: ψ_{soil}=-1.22±0.12 and ψ_{xylem}=-1.53±0.04 MPa), suggesting a limited physiological activity and water consumption during the last days of drought. In contrast, oaks decreased continuously the ψ_{soil} and ψ_{xylem} until the last day of the drought treatment, reaching minimum values during the first drying period of ψ_{soil} =-1.86±0.44 MPa and ψ_{xylem}=-2.74±0.13 MPa in soil (significantly lower than the values of the previous measurement; ψ_{soil} =-1.05±0.15 MPa and ψ_{xylem}=-1.95±0.27 MPa). On the other hand, when we irrigated after the first drought period, the recovery of xylem water potential was faster in pines than in oaks, reaching their maximum ψ_{xylem} on the next day after watering. Conversely, despite the ψ_{soil} indicated that soil was completely wet the next day of irrigation; the oaks did not reach maximum ψ_{xylem} until two days after the watering. Irrigation was always until field capacity, meant around 21% of soil water content for this clayey-loam soil. However, the conditions under which the plants stopped their water uptake under drought (*i.e.* the effective wilting point) were different for both species. The pines dried the soil until 13.7±0.60% of SWC (-1.5MPa). In contrast, the oaks dried up the soil until 12.3±0.70% of SWC (-2.7 MPa), and even under these conditions they maintained a significant transpiration.

Isotopic changes: labelling and drought effects

Soil water isotopes followed the isotopic trend of irrigation water, but with a significant offset between irrigation water and soil, depending on previous soil water signature (Fig. 3). This was particularly large for the pots irrigated with deuterated (average values of +2.86.25±18.02‰ in δ^2H) or depleted water (-9.65±0.03‰ in δ^{18}O), after tap water, but it was also visible in the pots irrigated only with tap water (-2.28±0.04‰ in δ^{18}O). As well, xylem water tracked changes in soil isotopic composition, but showed a significant enrichment relative to the soil, which was relatively higher in pines (+6.86±1.64‰ in δ^{18}O) than in oaks (+2.27±0.35‰ in δ^{18}O). On the first irrigation (13th of July), we found consistent soil and xylem isotopic values for the 8 pots irrigated with tap water (Fig. 3a,b), as they had been irrigated with the same water since transplanting; conversely, it took at least 2 days for the 8 pots irrigated with enriched water to reach the maximum values of the "labelling" (Fig. 3c,d). Anyway, drainage water collected the first day of watering was generally close to irrigation water in terms of isotopic composition (Fig. 3), thus we considered that enough water was added in order to fill or replace all the exchangeable pore water. Just after the watering, we found isotopic differences in soil within depth, due to the progressive infiltration of new water (particularly for deuterium enriched irrigation), but with the progression of water consumption and drought conditions, these isotopic differences become minimal. Anyhow, homogeneity in soil depth profile for water isotopes was not associated to homogeneous soil water content in depth, as some differences were seen in these variables (Fig. 2c,d). At the last day of drought, pine pots showed slightly lower SWC in surface – 5cm – (12.6±0.39%) than in deeper layers (10cm, 13.7±0.16%; 15cm, 13.0±0.57%). However, oaks showed more homogeneous SWC profile, with the lowest values at 10cm depth (5cm, 12.5±0.70%; 10cm, 11.9±0.81%; 15cm, 12.4±0.73%). After drought, the second watering generated an inverted soil profile, with more depleted isotopic values in surface. This was particularly apparent for the pots irrigated first with tap water and later with depleted water (Fig. 3c,d), which could be associated to progressive infiltration (magnified by the low SWC in surface for pines). Moreover, after the second watering, we found species-specific differences of xylem water isotopes, showing contrasting water uptake patterns: oaks reached maximum labelling signature the next day after irrigation (Fig. 3b,d), whereas the pines did not show maximum labelling signature until 3 days after irrigation (Fig. 3a,c). Additionally, at the last day of drought treatment, we noticed more enriched isotopic values of xylem water than the preceding days in the pots watered with tap water (+1.65±1.65‰ and +1.65±1.19‰ in δ^{18}O for pines and oaks respectively) and slightly more depleted for the pine pots irrigated with deuterated water (-12.21±4.53‰ in δ^2H; oaks slightly positive difference +0.39±7.01‰ in δ^2H), agreeing with possible evaporative processes occurring on the stem (Fig. 3). In the biplots relating δ^{18}O with δ^2H (Fig. 4), soil and xylem evaporative lines are represented for pots irrigated with tap water, showing higher differences in terms of slope between those lines in oak trees (significant in the first irrigation cycle, p=0.0183; Fig. 4c,d) than in pines (non-significant, *p*=0.5843; Fig. 4a,b). Generally, the xylem water in pines followed soil evaporative line, although with more enriched values, whereas the slope in the oaks indicated different evaporative line, closer to the new added water than to the average soil water.

Soil and twig evaporation influence

The results for the performance of soil covering in both species showed negligible water losses after 12 consecutive days (less than 0.8% weight loss); indicating that our experimental set-up was largely preventing soil evaporation.

Furthermore, the experiment to assess evaporative enrichment in the twigs evidenced xylem separation from soil isotopic composition towards more positive values for both species and irrigation cycles after three days of leaf-covering (differences of xylem and soil for pines and oaks with tap water; 9.78 and 5.98‰ in δ^2H and for pines and oaks with deuterium enriched water; 34.48 and 10.56‰ in δ^2H respectively). The next day, once uncovered, all the trees coupled their xylem isotopic composition with soil (average differences in δ^2H lower than 0.12‰).

Evolution of gas exchange parameters and hydraulic conductivity

Despite the differences in SWC and ψ_{xylem} with irrigation and drought cycles, oaks showed very regular stomatal conductance g_s (83.31±12.34 mmol m^{-2} s^{-1}) and photosynthesis rate A_n (3.57±0.50 μmol m^{-2} s^{-1}) (Fig. 5b,c) over time. On the contrary, pines presented large reductions in stomatal conductance under drought (Fig. 5b,c): the last days of the drying process in both irrigation cycles pines exhibited minimal g_s (39.06±14.58 mmol m^{-2} s^{-1}) and A_n (1.92±1.55 μmol m^{-2} s^{-1}). However, when irrigated (particularly in the second time), the pines rapidly reacted to new water content in soil by increasing both gas exchange parameters (until maximum values of 309.02±67.78 mmol m^{-2} s^{-1} for g_s and 18.69±3.03 μmol m^{-2} s^{-1} for A_n).

Drought limitation of photosynthesis: stomatal regulation and xylem embolism assessment

We found a significant exponential relation between ψ_{soil} and gas exchange parameters (Fig. 6b – A_n, Fig. 6c – g_s) for pines, showing higher g_s and A_n for higher ψ_{soil} *i.e.* positively associated with water availability. Contrarily, this relationship was not found for oaks, for which gas exchange parameters were largely independent from soil water potential.

DISCUSSION

Species-specific differences in physiological response during drought and recovery

Our results showed that we successfully simulated soil water conditions from field capacity to the wilting point and exhibited the typical species-specific physiological response to these changes. When drying, pines reached their physiological wilting point earlier and at higher water potential (ψ_{xylem} around -1.5MPa) than oaks. Once these values were reached, xylem and soil water potential stabilised and sap flow stayed minimal, suggesting a strongly limited physiological activity and water consumption during the last days of drought. Conversely, the progressive reduction of ψ_{soil} in oaks, together with the smaller reduction in sap flow rates, suggested that they continued water uptake until the last day of drought treatment, reaching values around -2.7MPa (ψ_{xylem}). These results are in agreement with the ones we obtained in field campaigns for the same species and soil type (Martín-Gómez et al. under review) and many others (Picon et al. 1996, Poyatos et al. 2008, Forner et al. 2014, Klein 2014), confirming that marcescent oaks are able to maintain transpiration and physiological activity under drier soil conditions than Scots pine.

On the other hand, although soil was completely wet for both species the next day after irrigation, xylem water potential recovery was faster in pines than in oaks: pines reached their maximum ψ_{xylem} on the next day after watering, whereas oaks needed two days. These results are in agreement with previous studies (e.g. Irvine et al. 1998, Poyatos et al. 2008, Martínez-Vilalta et al. 2008) describing the more isohydric and opportunistic strategy of Scots pine in comparison to oaks. For example, Poyatos et al. (2008) reported faster increase transpiration after the first rain events in Scots pine compared to pubescent oak (*Quercus humilis*), despite the larger sap flow restrictions in the preceding summer drought. Contrarily, in our previous work at field conditions (Martín-Gómez et al. under review), together with other studies such as del Castillo et al. (2016), we demonstrated that the recovery after drought was faster in oaks than in pines. In the field, the faster recovery could be associated to oaks' access to deeper (and wetter) soil layers in comparison to pines (e.g. Poyatos et al. 2008, Klein et al. 2013, del Castillo et al. 2016, Grossiord et al. 2016); thus, implying that oaks were able to avoid to some extent the effect of summer drought. In the pot experiment, we cannot associate physiological divergence to clear differences in depth of water uptake, as the root growth is restricted by the size of the containers.

Nevertheless, and despite the homogeneity in soil water isotopic composition profile along all experiment, we found that, at the end of the drought treatment the pines dried mostly superficial soil (5cm), whereas the maximum water use in oaks was located in the intermediate soil layer (10cm). This fact was also reflected after the addition of new water after drought, when the infiltration process, together with the specific differences in soil water uptake, resulted in an inverted profile for water isotopes, which was more marked in pines than oaks. Hence, despite the 'unnatural' development of the root system in pot trials, the two species showed certain intrinsic differences in the water uptake profile.

In contrast to the faster recovery of ψ_{xylem} in pines once irrigated, xylem isotopic composition was considerably distant from irrigation water until 3 days after watering; *i.e.* the isohydric pines apparently restored their transpiration rates rapidly, but they did not replace all their water content by new moving water. Conversely, oaks reached maximum labelling signature on the next day after irrigation, despite their physiological performance was not completely restored until the next two days. This divergence between sap flow and isotopic patterns is intriguing, and might be associated with distinct radial sap flow profile in conifers and ring-porous oaks, being more evenly distributed in the former than in the latter (Granier et al. 1994, Poyatos et al. 2007, Brandes et al. 2007, Kumagai et al. 2009).

Drought limitation of photosynthesis

Gas exchange parameters confirmed the limited physiological activity in pines during the first stages of drought and their stagnation once reached the wilting point. This contrasted with the constant and continuous activity in oaks, despite of declining water availability, as generally observed in the field (Picon et al. 1996, Poyatos et al. 2008, Forner et al. 2014, Klein 2014, Martín-Gómez et al. under review). In accordance with previous studies (Gallego et al. 1994, Irvine et al. 1998, Perks et al. 2002, Poyatos et al. 2008, Klein et al. 2013, Salmon et al. 2015, Nardini et al. 2016), we found a significant relationship between soil water status (as reflected in ψ_{soil}) and g_s and A_n in pines, showing that photosynthesis was largely constrained by drought due to stomatal closure. Hence, in agreement with our results in field campaigns (Martín-Gómez et al. under review), when pines reached levels of ψ_{xylem} around -1.5 MPa, stomatal conductance was lower than 30 mmol m^{-2} s^{-1}, and consequently carbon assimilation was heavily affected. Similarly, Salmon et al. (2015) showed a decrease in g_s from about 120 mmol m^{-2} s^{-1} at ψ_{pd} =-1.3 MPa to values below 20 mmol m^{-2} at ψ_{pd} =-1.8 MPa, whereas Poyatos et al. (2008) and Perks et al. (2002) showed that midday g_s was already reduced to 10-20% of its maximum value with ψ_{pd} below -0.8 MPa. Conversely, although oaks also showed a tendency towards lower ψ_{xylem} associated to lower g_s and A_n, this was not significant, suggesting a limited restriction of water uptake for this species; and hence, extended physiological activity during drought episodes. Poyatos et al. (2008) also found a rather scattered response of g_s against ψ_{pd} in pubescent oaks, which maintained g_s above 30% of their maximum over the entire range of ψ_{pd} recorded (from -0.2 to -0.8 MPa). On the other hand, Gallego et al. (1994) did not find substantial changes in g_s and ψ_{pd} of *Q. pyrenaica* as soil water decreased, concluding that this was a consequence of the ability of this species to access deep water reservoirs.

Therefore, our results suggest that, despite Scots pine was faster-limited by drought treatment than the marcescent oak, the limitation was not long or strong enough to cause severe hydraulic damage in Scots pine due to xylem embolism. Nevertheless, because of its isohydric behaviour, the reaction to drought implied a fast stop of vegetative activity, but was recovered immediately once the SWC was restored. Conversely, the oaks were rather unaffected by drought conditions, and kept their physiological state more or less constant along the irrigation-drought cycles.

Soil-xylem uncoupling evidence stem evaporative enrichment and the complexity of soil water pools

In accordance with several studies (see e.g. Dawson and Ehleringer 1993, Cernusak et al. 2005, Ellsworth and Sternberg 2014, Martín-Gómez et al. 2016), we found isotopic evaporative enrichment in xylem water, associated to limited transpiration rates. In the supplementary sample used to assess xylem evaporative enrichment, we observed that plants irrigated with both tap and enriched water moved towards more enriched or evaporated values after leaf-covering and subsequent sap flow reduction, in agreement with a similar experiment with saplings of the same species (Martín-Gómez et al. 2016). This provides a straightforward mechanistic explanation for our observations during the drought experiment. When sap flow and stomatal conductance were reduced, changes in xylem water were in accordance with the expected isotopic fractionation associated with evaporation. Thus, whereas the trees watered with depleted water showed a significant isotopic enrichment during drought, those labelled with deuterated water became more depleted after the exchange with more depleted atmospheric water.

Nevertheless, evaporative enrichment may not explain completely the observed uncoupling between soil and xylem throughout the experiment, even in well-watered moments (particularly for pines). Besides, neither the negligible soil evaporation nor the plant water consumption could determine the notable offset between irrigation and soil water. In this sense, despite our attempt to test experimentally the “two water worlds” hypothesis (Tang and Feng 2001, Brooks et al. 2009, Goldsmith et al. 2012, McDonnell 2014, Evaristo et al. 2015), by controlling isotopic composition of irrigation water and avoiding soil variability in depth caused by evaporation, we could not associate the changes in xylem to the use of different water pool, because of the fact that evaporative process in xylem were displacing isotopic composition in the same direction as the tightly-attached previous soil labelling. In fact, we can assume that the important evidence of xylem evaporative enrichment in this and the previous study (Martín-Gómez et al. 2016) might certainly mask the complex effect of variability in soil water pools.

In any case, our results evidenced a very homogeneous isotopic soil profile in depth at the driest point, which could be associated to the limited contribution of the free pore water pool, thus prevailing in the more tightly-bound water pool. This water pool would show a more homogeneous isotopic composition, being a mixture of older water, previous to the contrasting irrigation cycles of the experiment. Along with this, we observed that xylem followed soil evaporation line in pines irrigated with tap water (Fig. 4a,b), whereas the slope of the evaporative line for the oaks was closer to the new added water (Fig. 4c,d). This fact exhibits certain evidence for species-specific use of different soil water pools, suggesting that pines are consuming a water which isotopic composition is a mixture of new and old water (that get mixed in soil or maybe in xylem) while oaks have greater capacity to exchange all its xylem water with mobile water. This results for species-specific use of different soil water pools is in consonance with the findings of del Castillo et al. (2016), who found indirect evidence for changes in soil water isotopic composition during drought (not related with evaporation), and that could be followed only by the species with greater ability to retrieve water from dry soils (holm oak). They speculated that changes in the soil could be caused by the existence of non-well mixed water pools that were accessible for the more drought-tolerant species.

CONCLUSIONS

In this experiment we confirmed that isohydric strategy in pines limited their water uptake during the first stages of drought while oaks, with a more conservative strategy, were maintaining transpiration and physiological activity under drier soil conditions. These different strategies were linked to contrasting water use patterns and resulting soil drying profile, showing certain evidence for species-specific use of different soil water pools. Although we could not unequivocally show the specific use of different water pool in relation to drought conditions, confirming the “two water world hypothesis”, our results illustrate that not all the water in the soil is well-mixed. This is shown by the inability to replace completely the original soil isotopic signature with that of the new irrigation water, even when the soil reaches full capacity and allowing enough time for equilibration. This is in line with field studies in which free or stream water has different isotopic composition as bulk soil or plant, or labelling studies in which labelling is not efficient in changing completely isotopic of soils. In this sense, we can assume that in fine textured soils (clayey-loamy), an important portion of the micro-pores do not exchange with irrigation water and thus these soil water pools would keep un-mixed. Finally, we conclude that further assessment is needed to understand and separate the effects of isotopic fractionation occurring in soil and xylem, before straightly assume that xylem water represents source water.

ACKNOWLEDGEMENTS

We thank Pilar Sopeña, Maria Josep Pau and Mireia Oromí for laboratory assistance on water isotope analyses; Luis Serrano, Jesús Pemán and Youness El Karkri for experimental assistance. We also thank Todd Dawson and Kevin Simonin for their guidance in the experimental design during P.M.G stay in U.C Berkeley. This research was supported by the Spanish Government through projects AGL 2012-40039-C02 and AGL 2012-40151-C03, the PhD fellowship (FPU12/00648) to P.M.G., the National Council for Science and Technology of México (CONACyT) scholarship to U.R.R and Ramón y Cajal contract to J.P.F. (RYC-2008-02050).

Author contributions

P.M.G., J.P.F. and U.R.R. planned and designed the research, collected and analysed the data, and wrote the manuscript.

REFERENCES

Allison GB, Barnes CJ, Hughes MW (1983) The distribution of deuterium and ^{18}O in dry soils 2. Experimental. J Hydrol 64:377–397.

Bertrand G, Masini J, Goldscheider N, Meeks J, Lavastre V, Celle-Jeanton H, Gobat J-M, Hunkeler D (2014) Determination of spatiotemporal variability of tree water uptake using stable isotopes (δ^{18}O, δ^{2}H) in an alluvial system supplied by a high-altitude watershed, Pfyn forest, Switzerland. Ecohydrology 7:319–333.

Bögelein R, Thomas FM, Kahmen A (2017) Leaf water ^{18}O and ^{2}H enrichment along vertical canopy profiles in a broadleaved and a conifer forest tree. Plant Cell Environ, doi: 10.1111/pce.12895.

Brandes E, Wenninger J, Koeniger P, Schindler D, Rennenberg H, Leibundgut C, Mayer H, Gessler A (2007) Assessing environmental and physiological controls over water relations in a Scots pine (*Pinus sylvestris* L.) stand through analyses of stable isotope composition of water and organic matter. Plant Cell Environ 30:113–127.

Brooks JR, Barnard HR, Coulombe R, McDonnell JJ (2009) Ecohydrologic separation of water between trees and streams in a Mediterranean climate. Nat Geosci 3:100–104.

Von Caemmerer S, Farquhar GD (1981) Some relationships between the photochemistry and the gas exchange of leaves. Planta 153:383–387.

del Castillo J, Comas C, Voltas J, Ferrio JP (2016) Dynamics of competition over water in a mixed oak-pine Mediterranean forest: Spatio-temporal and physiological components. For Ecol Manage 382:214–224.

Cernusak L a, Farquhar GD, Pate JS (2005) Environmental and physiological controls over oxygen and carbon isotope composition of Tasmanian blue gum, *Eucalyptus globulus*. Tree Physiol 25:129–146.

Dawson TE (1991) Stream side trees that do not use stream water. Nature 350:335-337

Dawson TE, Ehleringer JR (1993) Isotopic enrichment of water in the ‘woody’ tissues of plants: Implications for plant water source, water uptake, and other studies which use the stable isotopic composition of cellulose. Geochim Cosmochim Acta 57:3487–3492.

Ehleringer JR, Dawson TE (1992) Water uptake by plants: perspectives from stable isotope composition. Plant Cell Environ 15:1073–1082.

Ellsworth PZ, Sternberg LSL (2014) Seasonal water use by deciduous and evergreen woody species in a scrub community is based on water availability and root distribution. Ecohydrology 8:538–551.

Ellsworth PZ, Williams DG (2007) Hydrogen isotope fractionation during water uptake by woody xerophytes. Plant Soil 291:93–107.

Evaristo J, Jasechko S, McDonnell JJ (2015) Global separation of plant transpiration from groundwater and streamflow. Nature 525:91–94.

Ferrio JP, Resco V, Williams DG, Serrano L, Voltas J (2005) Stable isotopes in arid and semi-arid forest systems. Invest Agrar Sist Recur For 14:371–382.

Filella I, Peñuelas J (2004) Indications of hydraulic lift by *Pinus halepensis* and its effects on the water relations of neighbour shrubs. Biol Plant 47:209–214.

Forner A, Aranda I, Granier A, Valladares F (2014) Differential impact of the most extreme drought event over the last half century on growth and sap flow in two coexisting Mediterranean trees. Plant Ecol 215:703-719.

Gallego HA, Rico M, Moreno G, Santa-Regina I (1994) Leaf water potential and stomatal conductance in *Quercus-Pyrenaica* Willd Forest - Vertical gradients and response to environmental factors. Tree Physiol 14:1039–1047.

Gat J (1996) Oxygen and hydrogen isotopes in the hydrologic cycle. Annu Rev Earth Planet Sci 24:225–262.

Van Genuchten MT (1980) A closed-form equation for predicting the hydraulic conductivity of unsaturated soils. Soil Sci Soc Am J 44:892–898.

Goldsmith GR, Muñoz-Villers LE, Holwerda F, Mcdonnell JJ, Asbjornsen H, Dawson TE (2012) Stable isotopes reveal linkages among ecohydrological processes in a seasonally dry tropical montane cloud forest. Ecohydrology 5:779–790.

Granier A, Anfodillo T, Sabatti M, Cochard H, Dreyer E, Tomasi M, Valentini R, Bréda N (1994) Axial and radial water flow in the trunks of oak trees: a quantitative and qualitative analysis. Tree Physiol 14:1383–1396.

Grossiord C, Sevanto S, Dawson TE, Adams HD, Collins AD, Dickman LT, Newman BD, Stockton EA, McDowell NG (2016) Warming combined with more extreme precipitation regimes modifies the water sources used by trees. New Phytol 213:584-596

Irvine J, Perks MP, Magnani F, Grace J (1998) The response of *Pinus sylvestris* to drought: stomatal control of transpiration and hydraulic conductance. Tree Physiol 18:393–402.

Klein T (2014) The variability of stomatal sensitivity to leaf water potential across tree species indicates a continuum between isohydric and anisohydric behaviours. Funct Ecol 28:1313–1320.

Klein T, Shpringer I, Fikler B, Elbaz G, Cohen S, Yakir D (2013) Relationships between stomatal regulation, water-use, and water-use efficiency of two coexisting key Mediterranean tree species. For Ecol Manage 302:34–42.

Kumagai T, Aoki S, Otsuki K, Utsumi Y (2009) Impact of stem water storage on diurnal estimates of whole-tree transpiration and canopy conductance from sap flow measurements in Japanese cedar and Japanese cypress trees. Hydrol Process 23:2335–2344.

Li SG, Romero-Saltos H, Tsujimura M, Sugimoto A, Sasaki L, Davaa G, Oyunbaatar D (2007) Plant water sources in the cold semiarid ecosystem of the upper Kherlen River catchment in Mongolia: A stable isotope approach. J Hydrol 333:109–117.

Lin GH, Sternberg LDL, Ehleringer JR, Hall AE, Farquhar GD (1993) Hydrogen isotopic fractionation by plant roots during water uptake in coastal wetland plants. In: Ehleringer, J.R., Hall, A.E., Farquhar, G.D. (eds.), Stable Isotopes and Plant Carbon–Water Relations. Academic Press Inc., San Diego, pp 497–510.

Máguas C, Rascher KG, Martins-Loução A, Carvalho P, Pinho P, Ramos M, Correia O, Werner C (2011) Responses of woody species to spatial and temporal ground water changes in coastal sand dune systems. Biogeosciences 8:3823–3832.

Martín-Gómez P, Barbeta A, Voltas J, Peñuelas J, Dennis K, Palacio S, Dawson TE, Ferrio JP (2015) Isotope-ratio infrared spectroscopy: a reliable tool for the investigation of plant-water sources? New Phytol 207:914–927.

Martín-Gómez P, Serrano L, Ferrio JP (2016) Short-term dynamics of evaporative enrichment of xylem water in woody stems: implications for ecohydrology. Tree Physiol, doi: 10.1093/treephys/tpw115.

Martínez-Vilalta J, López BC, Adell N, Badiella L, Ninyerola M (2008) Twentieth century increase of Scots pine radial growth in NE Spain shows strong climate interactions. Glob Chang Biol 14:2868–2881.

McDonnell JJ (2014) The two water worlds hypothesis: ecohydrological separation of water between streams and trees? WIREs Water 2014, 1: 323-329.

Meinzer FC, Andrade JL, Goldstein G, Holbrook NM, Cavelier J, Wright SJ (1999) Partitioning of soil water among canopy trees in a seasonally dry tropical forest. Oecologia 121:293–301 .

Moreira M, Sternberg L, Nepstad D (2000) Vertical patterns of soil water uptake by plants in a primary forest and an abandoned pasture in the eastern Amazon: an isotopic approach. Plant Soil 222, 95-107.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI (2012) Isotopes reveal contrasting water use strategies among coexisting plant species in a Mediterranean ecosystem. New Phytol 196:489–96.

Nardini A, Casolo V, Dal Borgo A, Savi T, Stenni B, Bertoncin P, Zini L, Mcdowell NG (2016) Rooting depth, water relations and non-structural carbohydrate dynamics in three woody angiosperms differentially affected by an extreme summer drought. Plant, Cell Environ 39:618–627.

Perks MP, Irvine J, Grace J (2002) Canopy stomatal conductance and xylem sap abscisic acid (ABA) in mature Scots pine during a gradually imposed drought. Tree Physiol 22:877–883.

Picon C, Guehl JM, Ferhi A (1996) Leaf gas exchange and carbon isotope composition responses to drought in a drought-avoiding (*Pinus pinaster*) and a drought-tolerant (*Quercus petraea*) species under present and elevated atmospheric CO_2 concentrations. Plant Cell Environ 19:182–190.

Poyatos R, Cermák J, Llorens P (2007) Variation in the radial patterns of sap flux density in pubescent oak (*Quercus pubescens*) and its implications for tree and stand transpiration measurements. Tree Physiol 27:537–548.

Poyatos R, Llorens P, Piñol J, Rubio C (2008) Response of Scots pine (*Pinus sylvestris* L.) and pubescent oak (*Quercus pubescens* Willd.) to soil and atmospheric water deficits under Mediterranean mountain climate. Ann For Sci 65:306.

Querejeta JI, Egerton-Warburton LM, Allen MF (2007) Hydraulic lift may buffer rhizosphere hyphae against the negative effects of severe soil drying in a California Oak savanna. Soil Biol Biochem 39:409–417.

Sack L, Melcher PJ, Zwieniecki MA, Holbrook NM (2002) The hydraulic conductance of the angiosperm leaf lamina: a comparison of three measurement methods. J Exp Bot 53:2177–84.

Salmon Y, Torres-Ruiz JM, Poyatos R, Martinez-Vilalta J, Meir P, Cochard H, Mencuccini M (2015) Balancing the risks of hydraulic failure and carbon starvation: A twig scale analysis in declining Scots pine. Plant, Cell Environ 38:2575–2588.

Scholander PF, Hammel HT (1965) Sap pressure in vascular plants. Science 148:339–346.

Sternberg LDSL, Swart PK (1987) Utilization of freshwater and ocean water by coastal plants of southern Florida. Ecology 68:1898–1905.

Tang K, Feng X (2001) The effect of soil hydrology on the oxygen and hydrogen isotopic compositions of plants' source water. Earth Planet Sci Lett 185:355–367.

Thorburn PJ, Walker GR, Brunel JP (1993) Extraction of water from *Eucalyptus* trees for analysis of deuterium and oxygen18: laboratory and field techniques. Plant Cell Environ 16:269–277.

Zhao L, Wang L, Cernusak LA, Liu X, Xiao H, Zhou M, Zhang S (2016) Significant difference in hydrogen isotope composition between xylem and tissue water in *Populus euphratica*. Plant, Cell Environ. 39: 1848-1857

Zimmermann U, Ehhalt D, Münnich KO (1967) Soil-water movement and evapotranspiration: Changes in the isotopic composition of the water. Proc. Symp. Isot. Hydrol., Vienna, Austria, pp 567–584.

TABLES AND FIGURES

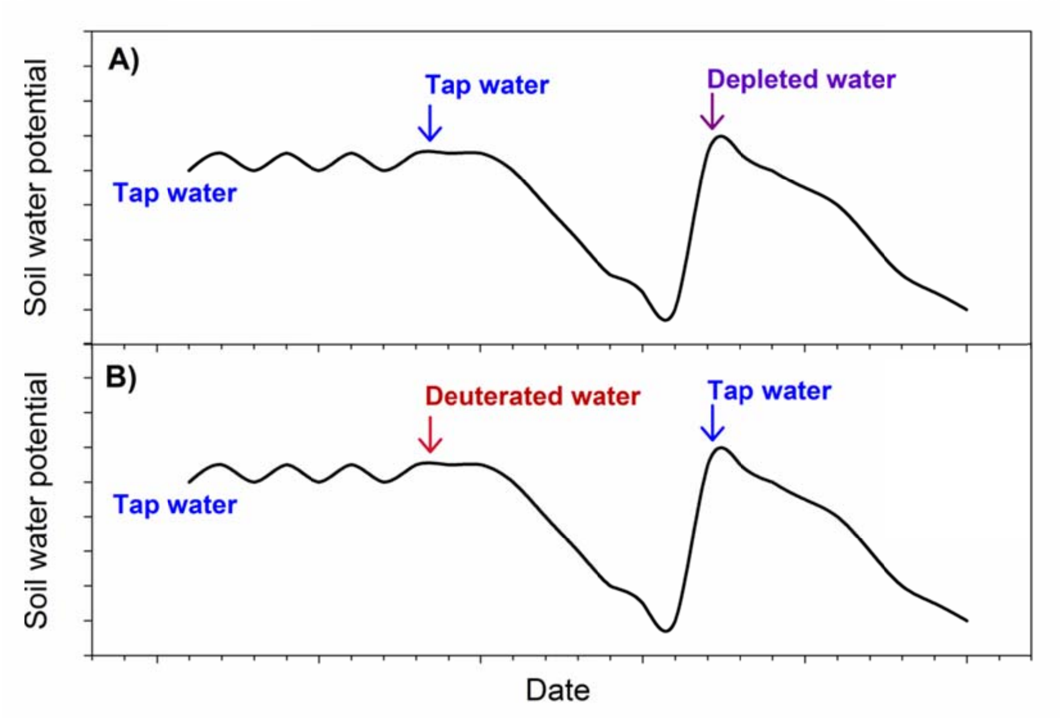

Figure 1. Schematic diagram for the irrigation method procedure used in the experiment. Plants were divided into two groups, 8 plants for “normal labelling” (tap + depleted water, a) and 8 plants for “deuterium-enriched” labelling (deuterium-enriched + tap water, b). All the trees were initially irrigated, dried for 8 days until wilting point; irrigated with different water and dried for 8 days again.

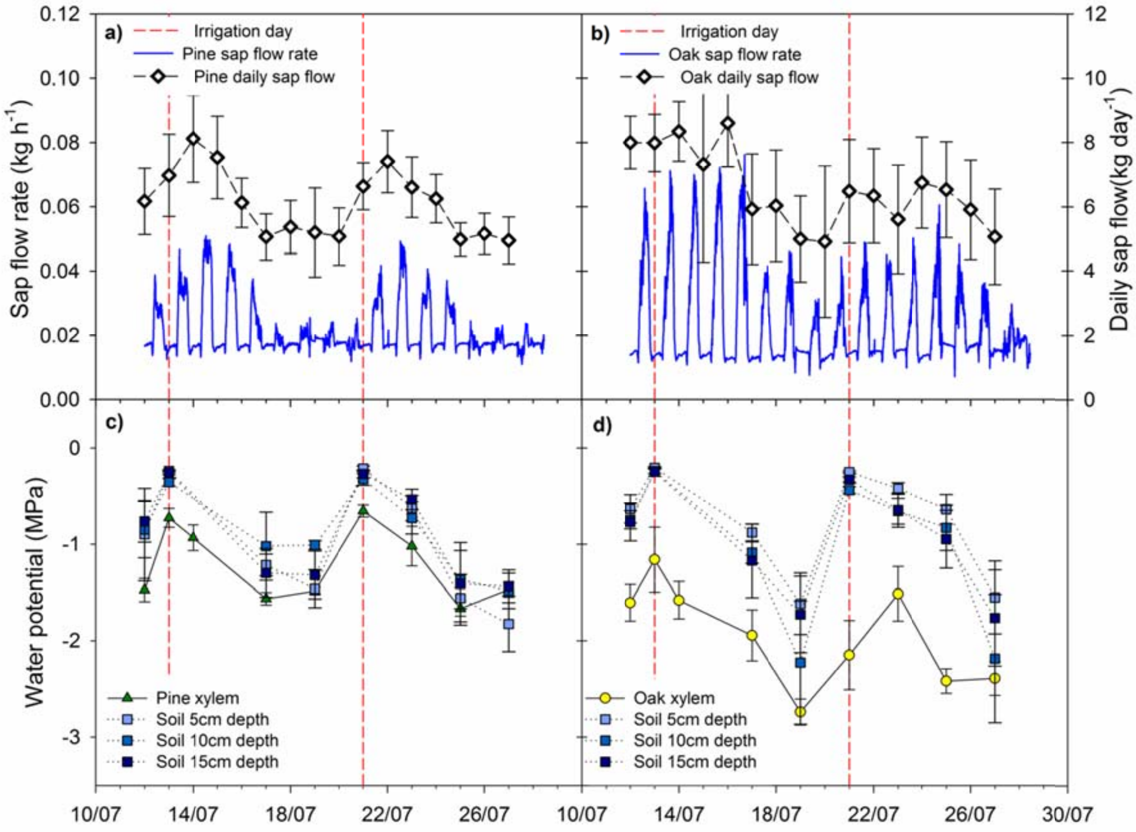

Figure 2. Evolution of sap flow (a, b) and soil and xylem water potential (c, d) with time. Red vertical lines indicate the days we irrigated (13/07/15 and 21/07/15). Blue continuous line represent instantly sap flow rate and open black diamonds the sum of daily sap flow, for pines (a) and oaks (b). Light to dark blue squares represent soil water potential at 0-5, 5-10 and 10-15 cm depth in pine (c) and oaks (d) pots. Xylem potential in pines in displayed with green triangles (c) and with yellow circles in oaks (d). Error bars represent standard errors.

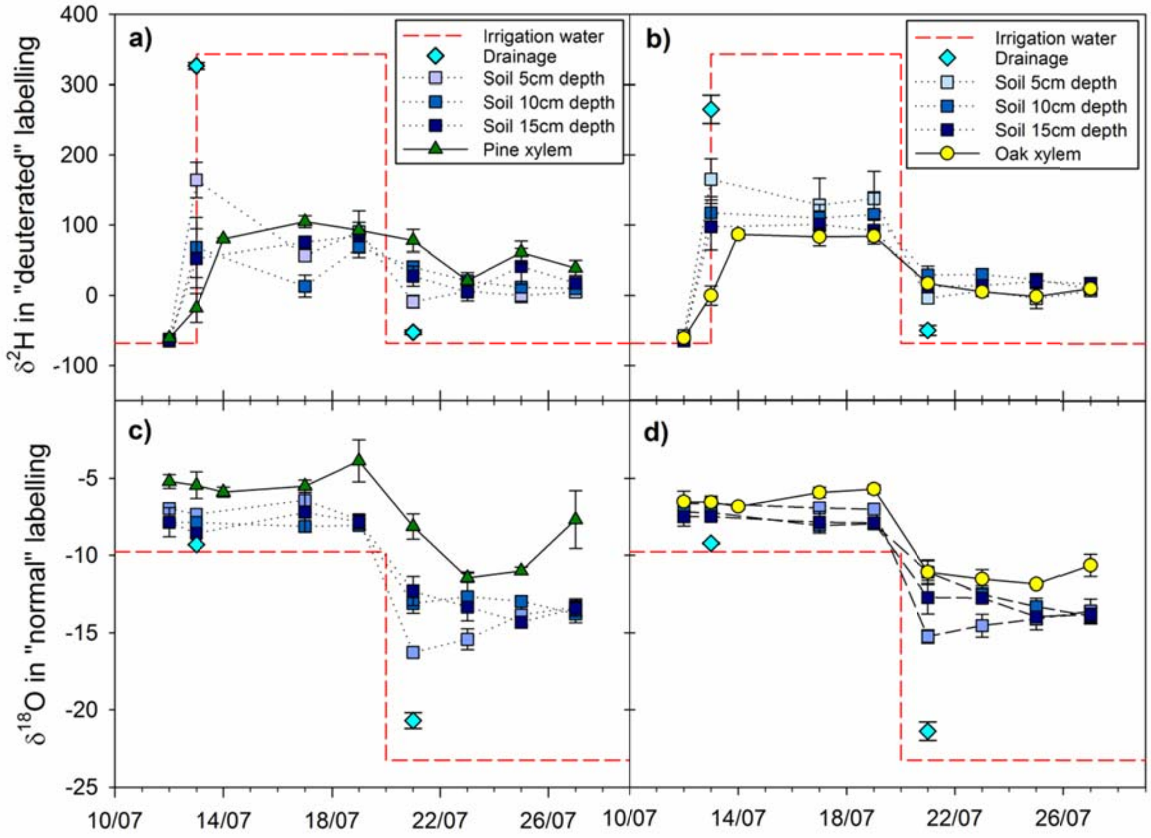

Figure 3. Evolution of soil (light to dark blue squares), xylem (green triangles, pine; yellow circles, oak), irrigation (red-dashed line) and drainage (blue diamond) water isotopic composition with time. Pots irrigated with deuterium-enriched water in the first cycle and tap water in the second in pines (a) and in oaks (b); pots irrigated with tap water in the first cycle and depleted water in the second in pines (c) and in oaks (d). Error bars represent standard errors.

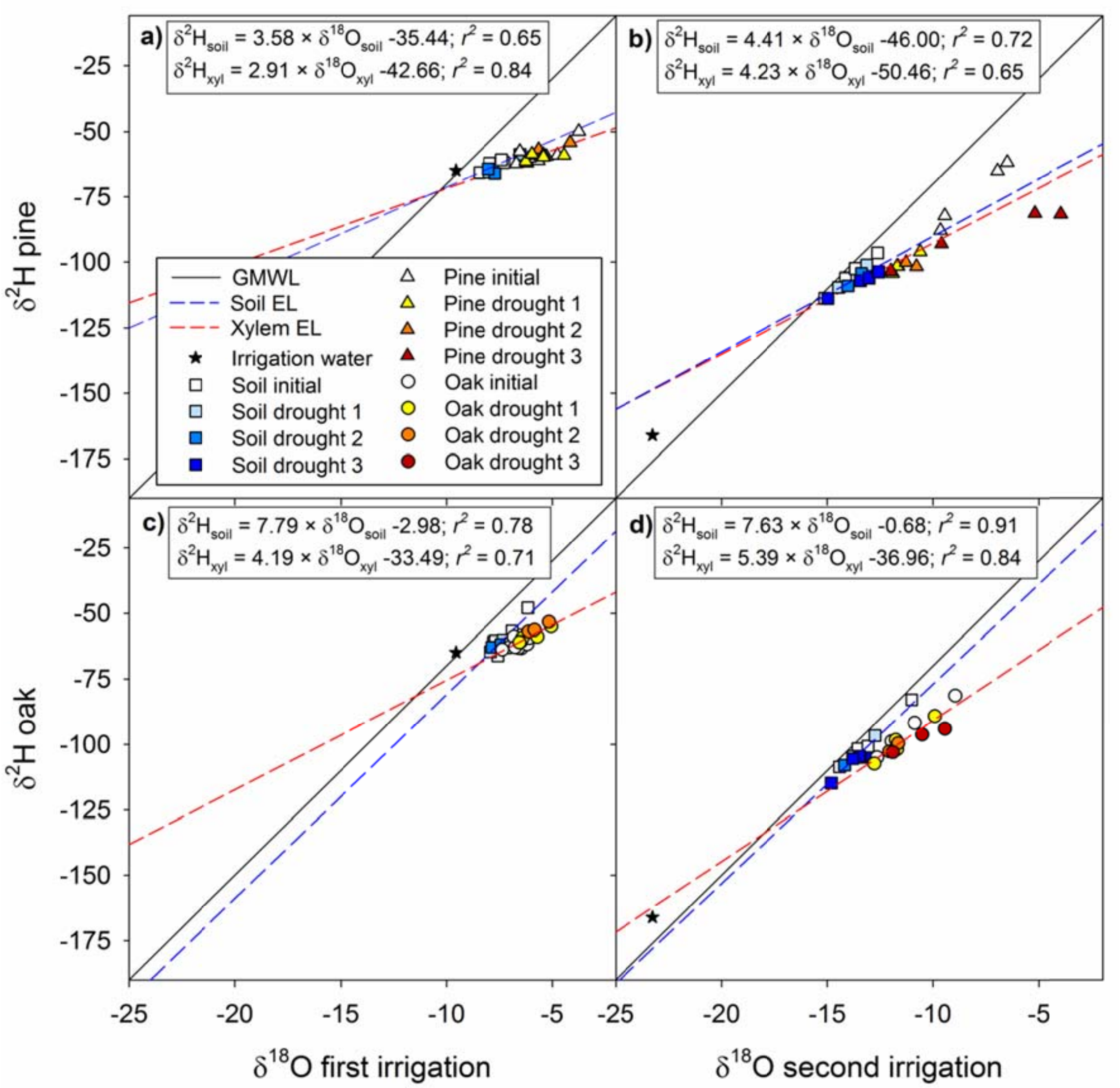

Figure 4. Bi-plots representing the seasonal evolution of the isotope composition of oxygen ($\delta^{18}O$) and hydrogen ($\delta^{2}H$) of irrigation water, water extracted from twig xylem of pines and oaks and from the 10, 30 and 50 cm deep soil layers for the “normal” labeling. The first irrigation cycle, with tap water, is represented in a (pines) and c (oaks) panels; the second irrigation cycle, with depleted water, is represented in b (pines) and d (oaks) panels. Black star, irrigation water; white square, triangle and circle indicate initial values for soil, pine and oak xylem respectively; light to dark blue indicate wetter to dry soil values with time; yellow, orange and red triangles (pine) and circles (oaks) display xylem water corresponding to wetter to dry soil values with time. Solid and dashed lines indicate, respectively, the global meteoric water line (GMWL, solid), soil (blue dashed) and xylem (red dashed) evaporative water lines. The regression equation and coefficient (r^2) of soil and xylem evaporative lines are represented in each panel.

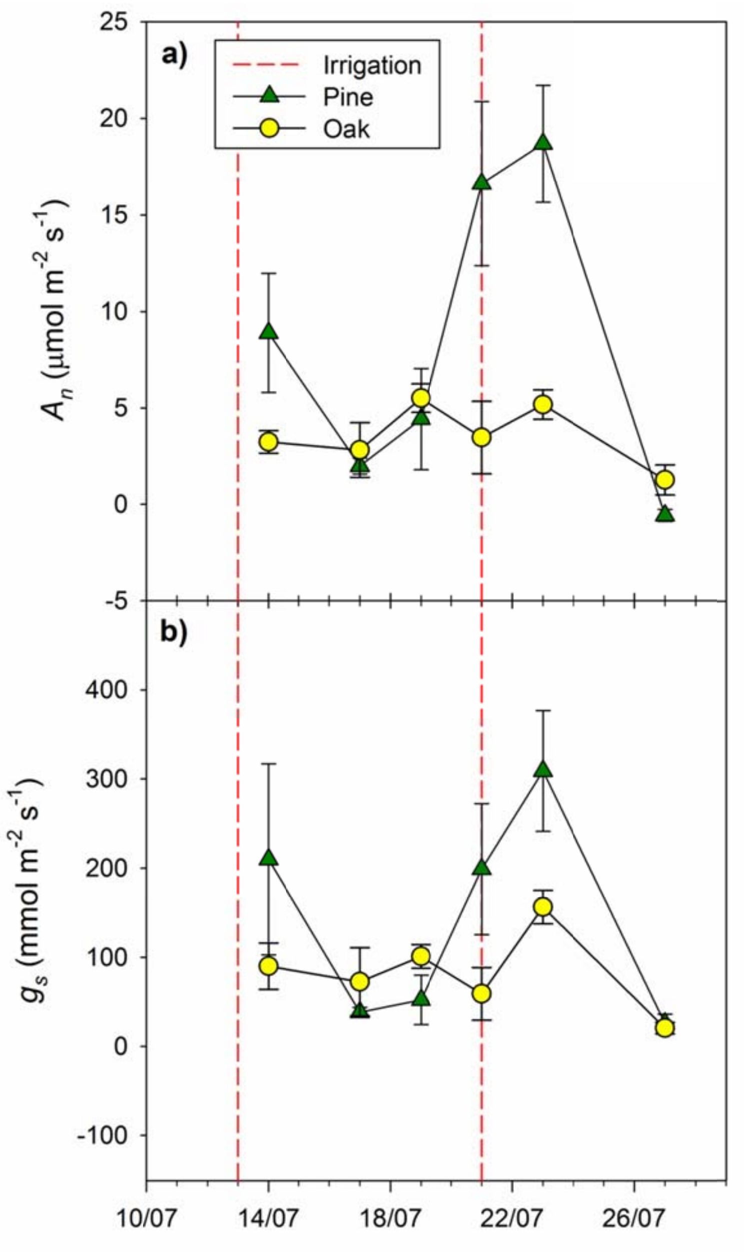

Figure 5. Time evolution of leaf-level gas exchange for the two studied species, pine (green triangles) and oak (yellow circles): (a) photosynthetic rate (A_n), and (b) stomatal conductance (g_s). Red vertical dashed lines indicate the days we irrigated the pots. Error bars represent standard errors.

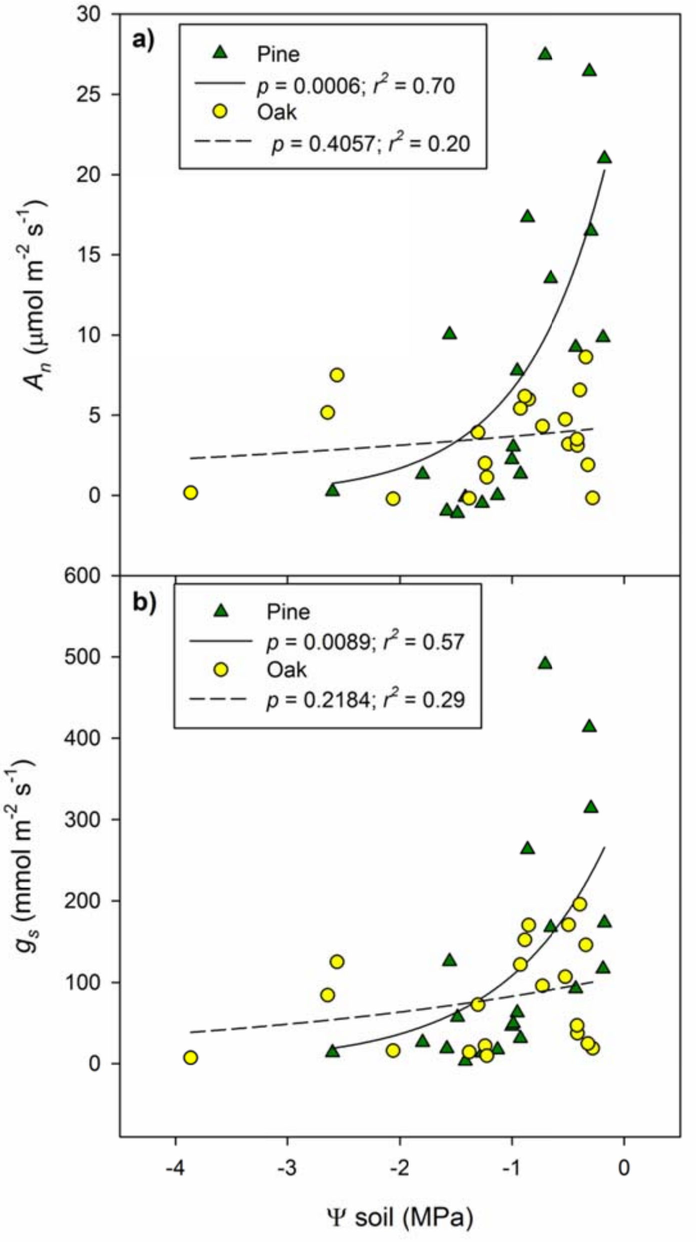

Figure 6. Relationship between soil water potential and (a) photosynthesis - A_n. (b) stomatal conductance - g_s, for all the sampling times and both species. Yellow circles and dashed line, oaks; green triangles and solid line, pines. The regression coefficient (r^2) and significance (p) is represented in each panel.

GENERAL DISCUSSION

1. Uncoupling between soil and xylem samples. Limitations associated to the study of water uptake patterns with stable isotopes.

In Chapter III and IV we found a general deviation of soil water isotopic composition in respect to precipitation or irrigation water, and also from soil to xylem water. Generally, xylem water showed more enriched values with respect to soil and soil was either more enriched or depleted than incoming water according to previous isotopic composition, the magnitude of precipitation or irrigation event, and soil water content. In this sense, there is increasing evidence of non-perfect correspondence between precipitation-soil-xylem water (see references bellow), which certainly have important implications for the study of plant water uptake, as well as for ecosystem- and global-scale hydrological models, by means of adding new sources of variation between precipitation, water source and water transpired by plants not taken into account before. For this reason, in the current thesis, before characterising the ecophysiological functioning of submediterranean forests by means of water stable isotopes, we aimed to clarify the main fractionation causes, and their magnitude and circumstances.

1.1 Spectral interference of organic contaminants in isotope-ratio infrared spectroscopy (IRIS) methods

The first source of error that we studied in Chapter I was the interference of organic contaminants in water isotopic composition analysed with IRIS methods. The presence of organic contaminants (such as methanol, ethanol or more than two carbon alcohols) is broadly found in plant and soil samples. Using the traditional methods of isotope-ratio mass spectrometry (IRMS), the effect of contaminants is relatively small, unless they are very abundant and hence contribute significantly to total amount of oxygen and hydrogen atoms entering the spectrometer. However, in IRIS, the magnitude of the error not only depends on contaminant concentration but also their spectral properties, hence the associated analytical errors may become unacceptable starting at very small concentrations for some compounds (e.g. <0.1% for methanol (Brand et al. 2009)). Despite this fact is commonly recognized, many studies using IRIS did not consider or correct it, due to the lack of substantial information by the IRIS companies on how to operate. Only some previous studies proposed correction curves, e.g. as a function of the degree of contaminant concentration (Brand et al. 2009, Schultz et al. 2011, Leen et al. 2012), but obtaining lower precision and following a rather empirical approach, *i.e.* without considering the spectral features of the sample. In this context, and trying to solve the limited information on this subject, in Chapter II we examined two approaches to cope with contaminated samples in IRIS: post-processing correction and on-line oxidation of organic compounds (MCM). In our study we proved that the post-processing correction proposed by Picarro strongly reduced the effects of contamination, even in cases of heavily contaminated samples. The correction limits for methanol were relatively low due to its strong spectral interference (up to concentrations of 0.4% methanol for $\delta^{18}O$) and, conversely, the deviation for ethanol was minimal even at the highest tested concentration (8%). On the other hand, we observed that MCM outperformed the post-processing correction in removing methanol interference, but was not effective for high concentrations of ethanol, because the MCM causes significant changes in the isotope composition of water with high concentrations of contaminant. Additionally, applying the post-processing correction to samples previously treated by micro-combustion apparently overcorrected the isotopic values, with the exception of highly contaminated samples with residual methanol. In conclusion, for contaminated samples we can generally recommend the post-processing correction in isotopic analyses, and only when the main (and mostly unique) contaminant detected is methanol, the use of MCM (eventually combined with post-processing correction). Also, we suggest the development of an integrated post-processing correction as a way to account for changes in water isotope composition caused by the MCM (e.g. considering spectral information before and after MCM operation) and encourage the IRIS companies to improve recommendations and protocols to develop in the presence of organic contaminants in water samples.

1.2 Root fractionation during water uptake

The general assumption of no fractionation in roots during water uptake has been questioned by Lin et al. (1993) in xerophyitic plants, and by Ellsworth and Williams (2007), in halophytic species. The transport of water through the symplastic pathway in these species has been suggested to cause isotopic fractionation, leading to a depletion of $\delta^{2}H$ in root xylem compared to surrounding soil water (ranging from 3‰ to 9‰, as observed in 12 species by Ellsworth and Williams (2007)). Lin et al. (1993) hypothesized that the fractionation of hydrogen isotopes in water occurred as water moved through the endodermis, as the energy required to disassociate individual water molecules from water aggregates before passing through the plasma membrane is less for $^{1}H^{1}H^{16}O$ than for $^{1}H^{2}H^{16}O$ (Chacko et al. 2001). Substitution of ^{18}O for ^{16}O in water causes only a slight relative difference in vibrational energy compared to the substitution of ^{2}H for ^{1}H; so, fractionation of isotopes in water during uptake by roots is expected to be more apparent for hydrogen than for oxygen. In any case, root isotopic fractionation was never found for $\delta^{18}O$ and has been generally discarded for the apoplastic pathway, which is the main route for soil water to enter the root in the majority of plants. Under conditions where apoplastic movement of water predominates, water can enter the plant without the dissociation into single water molecules, *i.e.* moving in a mass flow, thus, no fractionation is likely to occur.

In this sense, in order to examine whether root fractionation could be a major factor explaining the uncoupling between soil and xylem water described in Chapters III and IV, at the end of the drought treatment in the experiment designed in Chapter IV, we sampled the complete trees, separating the material in three samples: tap roots, trunk and twig xylem, and compared its value with that of soil water. The obtained results showed no significant differences between soil, tap root and trunk. Nevertheless, the tap root and trunk isotopic composition was slightly more depleted with respect to the soil, which would agree with a potential root fractionation. However, this slight difference was equally important for $\delta^{18}O$ than for $\delta^{2}H$ which would challenge the hypothesis by Lin et al. (1993) that establishes only root fractionation for $\delta^{2}H$. In this sense, further investigation is needed and other explanations related to soil properties (developed in the section d) or wood capacitance (section e) could be behind this fractionation.

1.3 Evaporative enrichment in xylem water during limited transpiration

In Chapter II, we studied the enrichment of xylem water as another source of error when we want to relate the isotopic composition of soil and xylem water. Previous studies had only proved the existence of xylem isotopic enrichment for green, unsuberized stems (Dawson and Ehleringer 1993) or in suberized stems of deciduous plants during long leafless periods (Phillips and Ehleringer 1995, Ellsworth and Sternberg 2014, Treydte et al. 2014, Bertrand et al. 2014, del Castillo et al. 2016). However, the short-term (hourly) dynamic of evaporative enrichment in active, leaved branches, was unknown until our work. In this sense, in Chapter II we reported evidence of fast evaporative enrichment in metabolically active stems as a consequence of a temporal decline in sap flow rates, proving that evaporative enrichment in the xylem is a reversible, highly dynamic process in fully active plants, and that xylem water isotopic composition does not only reflect source water, but also stem hydraulic processes. Indeed, when leaf transpiration is limited, the gradient in water potential between the stem and the leaves is smaller, hydraulic flow decreases and water storage may increase to mitigate xylem cavitation. Under such conditions, the limited stem flow increases water turnover time, reducing the input of fresh, unenriched xylem water, and allowing for accumulative evaporative enrichment. Additionally, and in opposition to other studies (Dawson and Ehleringer 1993, Bertrand et al. 2014), we did not find evidence of a feedback effect of leaf water on stem values, suggesting that evaporation through the stem surface is the main driver of xylem isotopic enrichment during periods of limited sap flow (in line with Stöhr and Lösch 2004, Catinon et al. 2012). In conclusion, in plant-source studies, observed seasonal fluctuations, e.g. in response to drought (Bertrand et al. 2014, Voltas et al. 2015), or even daily variations (Filella and Peñuelas 1999) might not always reflect changes in source water, as previously assumed, but may result from a confounding effect of xylem evaporative enrichment under limited sap flow (see e.g. del Castillo et al. 2016). Although stem evaporative enrichment can be seen as a handicap, once evaporative effects are constrained, it could provide a new insight into xylem water dynamics.

1.4 Soil extraction methods give contrasting results: the existence of different non-well mixed pools of water in soil.

As we thoroughly explained in the introduction of this thesis, it has been proved that soil extraction methods have an influence on the isotopic composition of the extracted water (see e.g. Ingraham and Shadel 1992, Leaney et al. 1993, Araguás-Araguás et al. 1995, Kelln et al. 2001). As well, the extraction conditions such as, e.g. for cryogenic distillation, the duration, the temperature and the vacuum level (Walker et al. 1994, Araguás-Araguás et al. 1995, West et al. 2006, Orlowski et al. 2013), and certain physical and biogeochemical soil properties, or the water content in soil and microbial activity, have been associated to deviations in isotope composition from presumed water source (see e.g. Ingraham and Shadel 1992, Araguás-Araguás et al. 1995, Sacchi et al. 2001, Koeniger et al. 2011, Orlowski et al. 2013, Meißner et al. 2013, Oerter et al. 2014, Palacio et al. 2014, Chen et al. 2016)

In this thesis, following the vast amount of plant-source studies (see e.g. Koeniger et al. 2011, Munksgaard et al. 2014), we always used cryogenic distillation at high temperature (110-120ºC) and vacuum (*ca.* 10^{-2} mbar), and long extraction times (2 h for soil samples and 1.5 h for xylem samples), in order to guarantee a complete extraction of water in the samples. However, considering all the variability associated to methods (often linked to incomplete extractions) and inter-dependence of sample characteristics with fractionation processes, in this thesis we included some methodological assessments in order to verify the validity of the chosen method. For example, in a test with 13 soil and 69 xylem samples, we compared the weight just after distillation, and after oven-drying at 60ºC for 24h. The result was that 93% of the samples gained weight in the oven, indicating that they were dry enough to rehydrate under such conditions. Also, for a subset of soil samples, we carried out cryogenic distillation at two different temperatures (35 and 120ºC); at 35ºC we got almost the whole amount of water in the sample, so the temperature did not make a difference between water sources, but the results were more consistent at 120ºC, probably due to some incomplete distillations at 35ºC. Finally, we also tested alternative distillation times, showing no significant differences in water content between the samples distilled at 120°C for 2h and 5h. Hence, we could state that the cryogenic distillation method used ensured complete extraction of water.

Nevertheless, it should be noted that even if the water extraction in cryogenic distillation is complete (and thus is truly representative of total soil water), not all this soil water may be available for plant consumption. It is well known that, particularly in clayey soils, a substantial proportion of the water is tightly bound to the mineral matrix, hence requiring very low water potentials to be extracted (see e.g. Hillel, 1998). Assuming a good mixing of all water pools in the soil, this would not be a problem, since water retained at different water potential should not differ isotopically. However, the *two water world hypothesis* considers that at least two independent unmixed water pools exist in the soil (mobile water-stream vs. tightly bound water-plant water) as a function of soil texture and other soil properties, intensity and quantity of the precipitation event (Tang and Feng 2001, Brooks et al. 2010, McDonnell 2014, Evaristo et al. 2015). However, although there is some indirect evidence of the existence of isotopically distinct water pools in the soil, there is a lack of experimental proof, particularly in relation to water use by plants. Thus, the key point would be to properly define which soil water fraction (mobile, weakly or tightly bounded) can be captured by each extraction method, and which fraction would be used by plants. On the other hand, since the different soil fractions are retained under different water potentials, plant's ability to uptake these water pools depends on the species itself, its physiological status and water-use strategy and environmental conditions.

Considering this, and taking into account that in Chapter III we observed a generalized uncoupling between soil and precipitation events (soil isotopic composition was very constant over the season, despite large variations in precipitation), we made some attempts to separate different water pools in the soil by centrifugation. We chose centrifugation because of its easiness of implementation, discarding other extraction methods in which the involved evaporation-condensation processes could include fractionation that would be mixed up with the differences on water sources. Then, with the interest to prove the centrifugation method, firstly, we dried soil samples totally in the oven and irrigated them with snow and evaporated water (with very disparate isotopic values). In one of the samples we irrigated first with (isotopically depleted) snow water, drained and later irrigated with (isotopically enriched) evaporated water. In the other sample we did the opposite. Thus, we expected to have two different soil profiles, one in which the tightly-bound water will retain the snow isotopic signature, and mobile water has the signal of evaporated water, and the other with the inverse profile. Also we had some “natural” samples for winter, spring and summer for the *Pinus-Quercus* mixed forest studied in Chapter III. We designed medium size tubes and small vials with two zones separated by metallic and paper filter (one for the sample and one for the centrifuged water) and calculated the rotation speed related to different pressure or retention forces, particularly from field capacity (0.33MPa) until wilting point (-1.5 MPa). Even though we could separate two water fractions in soils close to saturation, we could not recover a mobile fraction in drier soils. Anyway, we were always obtaining a "mixing infusion" of all the water in soil and we didn't find clear differences between the water extracted by centrifugation and the one with cryogenic distillation. Centrifugation was also problematic because the soil got stacked and the water could not come out (probably due to the high percentage of clay in the samples and, thus, the high percentage of immobile fraction retained water). We cannot fully discard a good mixing among all water pools in the soil (which would explain the similar values in centrifuged and vaccuum-distilled water). However, we believe that our lack of success might be related to the size of the centrifuge we used (a common lab centrifuge with small quantity of soil), as other researchers have obtained better results with bigger centrifuges (with at least one kilo of sample, Hissler et al. pers. comm). As well, centrifugation could fractionate water isotopic sample because of the differences in mass of different oxygen and hydrogen isotopes. On the other hand, we studied the possibility to extract water using the Richard's chamber, but as wetting of the porous lamina was needed it was almost impossible to separate new water from soil water. We also considered the use of other types of pressure chambers but as high pressures would facilitate water condensation, fractionation would happen. Similarly, if we use freezers to condensate mobile water at very low temperatures we would face similar problems.

Not having solved the controversy of the two water worlds by evaluating the extraction methods, we designed the experiment described in Chapter IV, with a double objective; firstly, to complete our analysis of water-use strategy of Scots pine and marcescent oak in controlled conditions (in line with Chapter III) and secondly, to assess the use of different labelled pools of water during plant drought and recovery. Somehow, we thought to use the plant as soil water "extractor". Therefore, by controlling isotopic composition of irrigation water and avoiding soil variability in depth caused by evaporation, we tried to test the existence of non-mixed water pools in the soil and the differential use of them by plants. In this sense, we would expect that plants would spend first the free-pore water in the soil until it is exhausted, and later they would start to consume progressively more tightly-bound soil water until they approach their physiological wilting point. Contrary to our expectations, the results presented in Chapter IV showed very stable xylem and soil values until the last day of drought treatment. Afterwards, when sap flow and stomatal conductance were reduced until minimum values; we could not clearly associate the changes in xylem to the use of different water pool, because evaporative enrichment was displacing isotopic composition in the same direction as the (tightly-bound) initial soil labelling. According to our results, we can assume that 1) even in clayey soils, there is a substantial mixing between soil water pools and 2) the important evidence of xylem evaporative enrichment in this and the previous study (Chapter II) may have certainly masked any effect of variability among soil water pools. However, the results in Chapter III and IV showed a clear and continuous uncoupling or displacement between soil and xylem water, which cannot be associated to evaporative enrichment, as they are independent from soil drought conditions. In this line, these results evidence that not all the water in the soil can be exchanged with new irrigation or precipitation water, despite reaching full capacity and even having enough time for equilibration. In this regard, it is worth to add that soil labelling was more difficult than expected. Soil texture, properties and water content had a notable effect on the results obtained in Chapter IV and the centrifugation proves. Even when the soil was completely dry and labelled immediately, the total water recovered after distillation had different isotopic composition than the initially added water. This is in agreement with previous studies showing divergences between theoretical and retrieved values after labelling (Oerter et al. 2014; Meißner et al. 2013). Although still not empirically tested for the liquid water-mineral matrix interphase, the most likely explanation is that water adsorbed to the mineral organic and mineral matrices undergoes fractionation, as has been shown for water vapour in contact with organic porous material (Chen et al. 2016). Also, when we wanted to label into two different fractions we were getting always a unique labelling with mixed signature although, as stated above, this may actually indicate that the isotopic isolation between water pools is not a general phenomenon, or at least requires certain conditions that may not be easily reproduced in the laboratory. In this sense, further investigation on soil characteristics and labelling procedures and times is needed if we want to experimentally test the existence of different and separated pools of water in soil.

1.5 When, where, how to sample xylem can make the difference: water transport delay and wood capacitance

The way we sample xylem for water extraction can also be a source of variability. Therefore, in order to answer the questions above; *when, where and how?* we conducted a more extensive sampling accompanying the results presented in Chapters II, III and IV: in our sampling campaigns and experiments we included samples of different parts of the tree, such as roots, trunk, big branches and twigs, together with pre-dawn and midday measurements.

Firstly, in this thesis, unlike several works that do not account for this problematic (e.g. Dawson and Ehleringer 1993, Phillips and Ehleringer 1995, Ellsworth and Williams 2007), we peeled the branches before sampling, in order to avoid direct contamination from phloem water. Also, sometimes it is recommended to sample older or thick branches, because it would reduce the potential effect of xylem-phloem exchange on xylem water and also the effect of stem evaporation due to the lower surface/volume ratio. In this regard, in experiments in Chapter II, twig diameter explained part of the variability in stem water loss, but was not associated with isotopic composition; also, we found only minor differences between small branches and twigs in the field measures (Chapter III). In contrast, δ^2H in the trunk (but not δ^{18}O) was consistently more depleted than soil values, placing trunk values outside the evaporative line of soil, branches and twig samples and suggesting some fractionation process. This was the case not only in heartwood, but also in sapwood samples, pointing to a non-negligible radial water transport within the main stem (see e.g. James et al. 2003) and to a probable fractionation caused by aquaporin-mediated transport in living parenchyma cells (Mamonov et al. 2007). In this regard, in the samples taken in the end of the experiment described in Chapter IV, we observed this depletion for trunk samples in comparison to soil as well, but was equally important for both isotopes and concurred with root isotopic values, suggesting another explanation. Alternatively, the long residence time of trunk water could favour the exchange of hydrogen atoms in water with isotopically-depleted starch (Luo and Sternberg 1992), and/or with other organic compounds resulting from fermentation processes (Gansert et al. 2001). However, exchange rates and metabolic activity in the trunk should be higher than generally assumed to explain the differences reported in this and other studies (Zhao et al. 2016; del Castillo et al. 2016). Thus, the causes for this fractionation remain unclear, and deserve further consideration, but in any case the use of trunk water for xylem sampling is discouraged.

Regarding the sampling time, Dawson and Ehleringer (1993) reported that evaporative enrichment was still present at pre-dawn, but was lower than at midday, so they proposed to collect xylem at pre-dawn to avoid evaporation. Conversely, considering that it takes some hours for water in the soil to reach the stem (Dawson 1993), Filella and Peñuelas (2003) established that morning measurements likely correspond to water absorbed by the roots the previous day, and afternoon measurements would correspond to water obtained by the roots early in the morning. Then, it is crucial to consider potential time lags in the isotopic signature between source water in the soil and water in the xylem of the tree in different heights (Waring et al. 1979, Brandes et al. 2007). In this regard, in our field campaign (Chapter III) we observed comparable results for twigs sampled at pre-dawn and midday. On the other hand, after uncovering the leaves in the pot trial (Chapter II), the xylem water remained unchanged (*i.e.* enriched) at pre-dawn, and did not reflect source water until midday. This suggests that pre-dawn sampling, which in principle implies more limited sap flow rates, may not be appropriate if night-time conditions favour evaporative losses (see e.g. Resco de Dios et al. 2013). Therefore, we recommend to sample medium-size twigs at the time of maximum transpiration, avoiding pre-dawn measurements and afternoon depression of stomatal conductance during drought stress.

1.6 To summarize: the necessity to include other complementary data in water isotopes studies

Even though it has been clearly shown that stable isotopes are a powerful tool to study water tracing on plant, soils and ecosystems, from a physiological to a global scale; our findings also highlight the need to combine this data with other physiological tools if we want a more complete study of plant water status and plant response to drought and subsequent recovery. The measure of water potential, tree sap flow, leaf gas exchange in the moment of sampling water isotopes, together with more integrative measures such as tree-ring width or remote sensing, could help to clarify the meaning of water isotope variations.

2. What we learned about submediterranean forests: specific physiological strategies in front of climate change

As it has been broadly developed in the introduction, the submediterranean forests in the Spanish Pyrenees are being affected by extensive drought-induced decline episodes in the last years. However, this is not affecting all species in the same way. Whereas marcescent oaks (*Q.faginea* and *Q. subpyrenaica*) seem unaffected or even favoured, the Scots pine (*P.sylvestris*) is suffering from important weakening and mortality in the worst circumstances. According to climate change projections, this situation is expected to be aggravated. However, the way how the different physiological and adaptive responses of pines and oaks are going to determine the composition and structure of submediterranean forests still remains unclear. On that account, in this thesis we studied the physiological behaviour and water use patterns in relation to drought in submediterranean pines and oaks under field and laboratory conditions (Chapters III and IV, respectively).

In Chapters III and IV we found species-specific patterns on physiological variables and isotope composition along the season, and during the changing conditions in the pot trial, reflecting the distinct physiological strategies and ability to cope with drought. In both studies, the marcescent oaks were able to reach lower midday water potential in comparison to Scots pine throughout the studied period; thus, showing their ability in maintaining transpiration and physiological activity under drier soil conditions (Picon et al. 1996, Poyatos et al. 2008, Klein et al. 2013, Forner et al. 2014). In line with this, in the pot experiment (Chapter IV), pines reached their physiological wilting point earlier than oaks, at values around -1.5 MPa. Once reached these values, xylem and soil water potential in pine pots stabilised, and sap flow stayed minimal until the new irrigation event, suggesting no physiological activity and water consumption during the last days of drought. Conversely, oaks continued water uptake until the last day of drought, reaching values around -2.7 MPa for xylem water potential.

Together with the lower values in midday water potential, in the forest (Chapter III), the oaks showed higher water potential overnight, as measured at pre-dawn. These differences can be mainly associated to root system distribution and water uptake patterns (e.g. Filella and Peñuelas 2003, Moreno-Gutiérrez et al. 2012, West et al. 2012, Barbeta et al. 2015): oaks are generally able to maintain their conservative strategy and continuous physiological activity by relying on stable water pools like groundwater, deep soil layers or more tightly-bound water in the soil, while the pines develop an opportunistic strategy of fast use of precipitation water based in a high root density on top soil layers (e.g. Poyatos et al. 2008, Klein et al. 2013, del Castillo et al. 2016, Grossiord et al. 2016). In this sense, the results on soil and xylem isotopes in Chapter III showed maximum δ^2H correlations for soil water at 50 cm depth for oaks and at 30 cm for pines, reinforcing the general knowledge of oak reliance on deeper soil water pools in comparison to pines (e.g. David et al. 2007, Barbeta et al. 2015). In any case, in the pot trial (Chapter IV), the patterns were not so clear. Only in the soil profile of the last day of drought we could find evidence that pines consumed more surface water, while oaks were showing more homogeneous soil water use along the soil profile, tending slightly towards deeper water. However, the shallow pot length (less than 20 cm) and the restrictions to root growth would have minimized inter-specific differences in root distribution.

Moreover, we found some differences in terms of drought recovery between both studies. In the forest (Chapter III) water potential and isotope data exhibited that oaks recovered faster after the first rains in early autumn in comparison to pines, in line with the access to deeper (and wetter) soil layers than the pines (e.g. Poyatos et al. 2008, Klein et al. 2013, del Castillo et al. 2016, Grossiord et al. 2016). Contrarily, in the pot experiment (Chapter IV) the recovery of xylem water potential was faster in pines than in oaks (in accordance to Poyatos et al. (2008): pines reached their maximum xylem water potential on the next day after watering, whereas oaks needed two days. In contrast, xylem water isotopes in the pot experiment (Chapter IV) evidenced longer residence times of water in pines, taking longer to replace all the water in the xylem with the new irrigation water. The results for the pot experiment, may respond basically to the more isohydric strategy of Scots pine in comparison to oaks (e.g. Irvine et al. 1998, Poyatos et al. 2008, Martínez-Vilalta et al. 2009), but omitting clear differences in depth of water uptake, as explained before.

Concerning this, as we observed in Chapter III, the more dynamic stomatal control of pines does not always imply a faster recovery of physiological activity after drought, since it can be linked to greater xylem vulnerability (Martínez-Vilalta et al. 2004). Therefore, we could presume that after long and severe drought periods, as the summer drought conditions in 2012 (Chapter III), the greater vulnerability to xylem embolism may lead to more limited recovery of transpiration flow in pines, as compared to oaks (e.g. Poyatos et al. 2008, Salmon et al. 2015). Differently, in the pot experiment (Chapter IV) with a shorter drought period, pines may not have developed embolism and in consequence, recovery of xylem water potential once irrigated is faster due to its faster stomatal response. In accordance with these results, the pines in Chapter IV presented maximum conductance after irrigation one day before than oaks, manifesting higher ability to recover the water flux after moderate drought (e.g. Poyatos et al. 2008, Salmon et al. 2015). In any case, in mixed stands (the forest in Chapter III) compared to separated pots in Chapter IV, the risk of xylem embolism in Scots pine could also have been enhanced by the larger ability of oak species to exhaust soil water (Willaarts 2012, Klein et al. 2013).

Gas exchange parameters measured in Chapter IV confirmed the limitation of physiological activity in pines during the first stages of drought and their stagnation once reached the wilting point; contrasting with the constant and continuous activity in oaks despite limited soil hydric conditions (Picon et al. 1996, Poyatos et al. 2008, Klein et al. 2013, Forner et al. 2014, Martín-Gómez et al. 2016). In accordance with previous studies (Gallego et al. 1994, Irvine et al. 1998, Perks et al. 2002, Poyatos et al. 2008, Klein et al. 2013, Salmon et al. 2015, Nardini et al. 2016) we found in the field and in the pot trial (Chapters III and IV) a significant relationship between soil water content and both g_s and A_n in pines, showing that photosynthesis was largely constrained by drought due to stomatal closure. Hence, when pines reached levels of water potential below -1.5 MPa, stomatal conductance was lower than 30 mmol m^{-2} s^{-1}, and consequently carbon assimilation was heavily affected. These relations of soil water status with gas exchange confirmed that drought conditions in Chapter IV may not have been long or strong enough to cause physiological failure in the studied species: Scots pine was rapidly limited by drought but also its recovery was very fast, whereas *Q.subpyrenaica*, remained nearly unaffected by drought conditions and kept their physiological state more or less constant along the irrigation-drought cycles.

Furthermore, in Chapter III we included measures of non-structural carbohydrates to the study of water-carbon relations. Throughout the studied period, we found lower concentrations of both soluble sugars and starch for pines, in comparison to oaks. This is in accordance with general knowledge that stem in conifers has very low content of non-structural carbohydrates, relative to angiosperm species (Martínez-Vilalta et al. 2016), and it is associated to the lower amount of parenchyma in conifer sapwood (Morris et al. 2016, Plavcová et al. 2016). Nevertheless, the values obtained do not suggest a strong depletion in the carbohydrate pools for the studied pines. Nevertheless, given the strong drought-induced limitation of carbon uptake (as measured by gas exchange) in the pines, we still found a positive association between pre-dawn water potential and non-structural carbohydrates for pines, suggesting that drought periods actually imposed certain limitations in carbon storage. Conversely, the oaks did not show any correlation between carbon storage and water potential, and carbohydrate values were generally high (Hoch et al. 2003, Körner 2003, Li et al. 2013, Nardini et al. 2016). This suggests that the particular site conditions allow the oaks to keep sufficient storage pools, minimizing drought effects and allowing a rapid recovery after a severe drought (Tyree et al. 1999, Salleo et al. 2009, Secchi and Zwieniecki 2011, Johnson et al. 2012, Brodersen and McElrone 2013). Along with this, the independence between water potential and carbohydrate pools in oaks, highlights that carbon storage in deciduous oaks is less sensitive to source limitations (e.g. under drought stress) and reflects a more complex dynamic of carbohydrate accumulation and probably starch-soluble sugar conversions (Salleo et al. 2009, Johnson et al. 2012).

In line with this, in Chapter III we observed that in both species radial growth patterns were affected by contrasting climatic years, but *Pinus sylvestris* showed higher variations and a far more pronounced decreasing trend with time. In this sense, as carbon storage has priority over growth (McDowell 2011, Sala et al. 2012, Palacio et al. 2013), we would also expect that an effect of drought on the pool of non-structural carbohydrates would be also translated into growth limitations. In this regard, *Pinus sylvestris* was also the most affected in terms of radial growth, determining that drought events, due to the reduction in stored carbohydrates, may induce long-term physiological disorders, causing a slower recovery of the survival trees (Galiano et al.

2012).

In conclusion, despite the lack of visible symptoms of tree decline, the pines in our field study (Chapter III) are largely limited by drought conditions, and cannot recover easily from severe drought episodes. In this sense, Chapter III and Chapter IV display a number of physiological traits that confer an advantage for survivorship and adaptation to *Quercus subpyrenaica* in comparison to *P. sylvestris.* The isohydric strategy of *P. sylvestris* confers a very sensitive behaviour, where hydraulic and carbon limitations are difficult to separate; soil dryness affects stomatal closure, which in turn influences carbon assimilation and in consequence carbon storage. These results establish evidence for early warning, confirming our initial hypothesis that physiological variables and water stable isotopes could be used to anticipate potential tree decline.

REFERENCES

Araguás-Araguás L, Rozanski K, Gonfiantini R, Louvat D (1995) Isotope effects accompanying vacuum extraction of soil water for stable isotope analyses. J Hydrol 168:159–171.

Barbeta A, Mejía-Chang M, Ogaya R, Voltas J, Dawson TE, Peñuelas J (2015) The combined effects of a long-term experimental drought and an extreme drought on the use of plant-water sources in a Mediterranean forest. Glob Chang Biol 21:1213–1225.

Bertrand G, Masini J, Goldscheider N, Meeks J, Lavastre V, Celle-Jeanton H, Gobat J-M, Hunkeler D (2014) Determination of spatiotemporal variability of tree water uptake using stable isotopes ($\delta^{18}O$, $\delta^{2}H$) in an alluvial system supplied by a high-altitude watershed, Pfyn forest, Switzerland. Ecohydrology 7:319–333.

Brand WA, Geilmann H, Crosson E, Rella C (2009) Cavity ring-down spectroscopy versus high-temperature conversion isotope ratio mass spectrometry; a case study on $\delta^{2}H$ and $\delta^{18}O$ of pure water samples and alcohol/water mixtures. Rapid Commun Mass Spectrom 23:1879–1884.

Brandes E, Wenninger J, Koeniger P, Schindler D, Rennenberg H, Leibundgut C, Mayer H, Gessler A (2007) Assessing environmental and physiological controls over water relations in a Scots pine (*Pinus sylvestris* L.) stand through analyses of stable isotope composition of water and organic matter. Plant Cell Environ 30:113–127.

Brodersen CR, McElrone AJ (2013) Maintenance of xylem network transport capacity: A review of embolism repair in vascular plants. Front Plant Sci 4:108.

Brooks JR, Barnard HR, Coulombe R, McDonnell JJ (2010) Ecohydrologic separation of water between trees and streams in a Mediterranean climate. Nat Geosci 3:100–104.

del Castillo J, Comas C, Voltas J, Ferrio JP (2016) Dynamics of competition over water in a mixed oak-pine Mediterranean forest: Spatio-temporal and physiological components. For Ecol Manage 382:214–224.

Catinon M, Ayrault S, Boudouma O, Asta J, Tissut M, Ravanel P (2012) Atmospheric element deposit on tree barks: The opposite effects of rain and transpiration. Ecol Indic 14:170–177.

Chacko T, Cole D, Horita J (2001) Equilibrium oxygen, hydrogen, and carbon isotope fractionation factors applicable to geologic systems. In: Valley JW CD (eds), Reviews in mineralogy and geochemistry. Stable isotope geochemistry. Mineralogical Society of America. Washington, pp 1–82.

Chen G, Auerswald K, Schnyder H (2016) ^{2}H and ^{18}O depletion of water close to organic surfaces. Biogeosciences 13:3175–3186.

David TS, Henriques MO, Kurz-Besson C, Nunes J, Valente F, Vaz M, Pereira JS, Siegwolf R, Chaves MM, Gazarini LC, David JS (2007) Water-use strategies in two co-occurring Mediterranean evergreen oaks: surviving the summer drought. Tree Physiol 27:793–803.

Dawson TE (1993) Water sources of plants as determined from xylem-water isotopic composition: Perspectives on plant competition, distribution, and water relations. In: Ehleringer JR, Hall AE, Farquhar GD (eds), Stable isotopes and plant carbon-water relations. Academic Press, Inc., New York, pp 465–496.

Dawson T, Ehleringer JR (1993) Isotopic enrichment of water in the ‘woody’ tissues of plants: Implications for plant water source, water uptake, and other studies which use the stable isotopic composition of cellulose. Geochim Cosmochim Acta 57:3487–3492.

Ellsworth PZ, Sternberg LSL (2014) Seasonal water use by deciduous and evergreen woody species in a scrub community is based on water availability and root distribution. Ecohydrology 8:538–551.

Ellsworth PZ, Williams DG (2007) Hydrogen isotope fractionation during water uptake by woody xerophytes. Plant Soil 291:93–107.

Evaristo J, Jasechko S, McDonnell JJ (2015) Global separation of plant transpiration from groundwater and streamflow. Nature 525:91–94.

Filella I, Peñuelas J (1999) Altitudinal differences in UV absorbance, UV reflectance and related morphological traits of *Quercus ilex* and *Rhododendron ferrugineum* in the Mediterranean region. Plant Ecol 145:157–165.

Filella I, Peñuelas J (2003) Partitioning of water and nitrogen in co-occurring Mediterranean woody shrub species of different evolutionary history. Oecologia 137: 51–61.

Forner A, Aranda I, Granier A, Valladares F (2014) Differential impact of the most extreme drought event over the last half century on growth and sap flow in two coexisting Mediterranean trees. Plant Ecol 215: 703–719.

Galiano L, Martínez-Vilalta J, Sabaté S, Lloret F (2012) Determinants of drought effects on crown condition and their relationship with depletion of carbon reserves in a Mediterranean holm oak forest. Tree Physiol 32:478–489.

Gallego HA, Rico M, Moreno G, Santa-Regina I (1994) Leaf water potential and stomatal conductance in *Quercus-Pyrenaica* Willd Forest - Vertical gradients and response to environmental factors. Tree Physiol 14:1039–1047.

Gansert D, Burgdorf M, Lösch R (2001) A novel approach to the in situ measurement of oxygen concentrations in the sapwood of woody plants. Plant Cell Environ 24:1055–1064.

Grossiord C, Sevanto S, Dawson TE, Adams HD, Collins AD, Dickman LT, Newman BD, Stockton EA, McDowell NG (2016) Warming combined with more extreme precipitation regimes modifies the water sources used by trees. New Phytol 213:584-596.

Hillel D., (1982) Introduction to soil physics. Academic Press, San Diego, CA, USA

Hoch G, Richter A, Körner C (2003) Non-structural carbon compounds in temperate forest trees. Plant Cell Environ 26: 1067–1081.

Ingraham NL, Shadel C (1992) A comparison of the toluene distillation and vacuum/heat methods for extracting soil water for stable isotopic analysis. J Hydrol 140:371–387.

Irvine J, Perks MP, Magnani F, Grace J (1998) The response of *Pinus sylvestris* to drought: stomatal control of transpiration and hydraulic conductance. Tree Physiol 18:393–402.

James S, Meinzer F, Goldstein G, Woodruff D, Jones T, Restom T, Mejia M, Clearwater M, Campanello P (2003) Axial and radial water transport and internal water storage in tropical forest canopy trees. Oecologia 134:37–45.

Johnson DM, McCulloh K a, Woodruff DR, Meinzer FC (2012) Hydraulic safety margins and embolism reversal in stems and leaves: why are conifers and angiosperms so different? Plant Sci 195:48–53.

Kelln CJ, Wassenaar LI, Hendry MJ (2001) Stable isotopes (δ^{18}O, δ^{2}H) of pore waters in clay-rich aquitards: A comparison and evaluation of measurement techniques. Gr Water Monit Remediat 21:108–116.

Klein T, Shpringer I, Fikler B, Elbaz G, Cohen S, Yakir D (2013) Relationships between stomatal regulation, water-use, and water-use efficiency of two coexisting key Mediterranean tree species. For Ecol Manage 302:34–42.

Koeniger P, Marshall JD, Link T, Mulch A (2011) An inexpensive, fast, and reliable method for vacuum extraction of soil and plant water for stable isotope analyses by mass spectrometry. Rapid Commun Mass Spectrom 25:3041–3048.

Körner C (2003) Carbon limitation in trees. J Ecol 91:4–17.

Leaney FW, Smettem KRJ, Chittleborough DJ (1993) Estimating the contribution of preferential flow to subsurface runoff from a hillslope using deuterium and chloride. J Hydrol 147:83–103.

Leen JB, Berman ESF, Liebson L, Gupta M (2012) Spectral contaminant identifier for off-axis integrated cavity output spectroscopy measurements of liquid water isotopes. Rev Sci Instrum 83:0445305.

Li MH, Cherubini P, Dobbertin M, Arend M, Xiao WF, Rigling A (2013) Responses of leaf nitrogen and mobile carbohydrates in different *Quercus* species/provenances to moderate climate changes. Plant Biol 15:177–184.

Lin GH, Sternberg LDL, Ehleringer JR, Hall AE, Farquhar GD (1993) Hydrogen isotopic fractionation by plant roots during water uptake in coastal wetland plants. In: JR Ehleringer, AE Hall and GD Farquhar (eds), Stable isotopes and plant carbon–water relations. Academic Press, San Diego, pp 497–510.

Luo YH, Sternberg LDL (1992) Hydrogen and oxygen isotopic fractionation during heterotrophic cellulose synthesis. J Exp Bot 43:47–50.

Mamonov AB, Coalson RD, Zeidel ML, Mathai JC (2007) Water and deuterium oxide permeability through aquaporin 1: MD predictions and experimental verification. J Gen Physiol 130:111–116.

Martín-Gómez P, Serrano L, Ferrio JP (2016) Short-term dynamics of evaporative enrichment of xylem water in woody stems: implications for ecohydrology. Tree Physiol doi: 10.1093/treephys/tpw115.

Martínez-Vilalta J, Cochard H, Mencuccini M, Sterck F, Herrero A, Korhonen JFJ, Llorens P, Nikinmaa E, Nolè A, Poyatos R, Ripullone F, Sass-Klaassen U, Zweifel R (2009) Hydraulic adjustment of Scots pine across Europe. New Phytol 184:353–64.

Martínez-Vilalta J, Sala A, Asensio D, Galiano L, Hoch G, Palacio S, Piper FI, Lloret F (2016) Dynamics of non-structural carbohydrates in terrestrial plants: a global synthesis. Ecol Monogr 86: 495-516.

Martínez-Vilalta J, Sala A, Piñol J (2004) The hydraulic architecture of Pinaceae–a review. Plant Ecol 171: 3–13.

McDonnell JJ (2014) The two water worlds hypothesis: ecohydrological separation of water between streams and trees? WIREs Water 2014, 1: 323-329.

McDowell NG (2011) Mechanisms linking drought, hydraulics, carbon metabolism, and vegetation mortality. Plant Physiol 155:1051–9.

Meißner M, Köhler M, Schwendenmann L, Hölscher D, Dyckmans J (2013) Soil water uptake by trees using water stable isotopes (δ^2H and δ^{18}O) − a method test regarding soil moisture, texture and carbonate. Plant Soil 376:327–335.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI (2012) Isotopes reveal contrasting water use strategies among coexisting plant species in a mediterranean ecosystem. New Phytol 196:489–496.

Morris H, Plavcová L, Cvecko P, Fichtler E, Gillingham MAF, Martínez-Cabrera HI, Mcglinn DJ, Wheeler E, Zheng J, Ziemińska K, Jansen S (2016) A global analysis of parenchyma tissue fractions in secondary xylem of seed plants. New Phytol 209:1553–1565.

Munksgaard NC, Cheesman AW, Wurster CM, Cernusak LA, Bird MI (2014) Microwave extraction-isotope ratio infrared spectroscopy (ME-IRIS): A novel technique for rapid extraction and in-line analysis of ^{18}O and ^{2}H values of water in plants, soils and insects. Rapid Commun Mass Spectrom 28:2151–2161.

Nardini A, Casolo V, Dal Borgo A, Savi T, Stenni B, Bertoncin P, Zini L, Mcdowell NG (2016) Rooting depth, water relations and non-structural carbohydrate dynamics in three woody angiosperms differentially affected by an extreme summer drought. Plant, Cell Environ 39:618–627.

Oerter E, Finstad K, Schaefer J, Goldsmith GR, Dawson T, Amundson R (2014) Oxygen isotope fractionation effects in soil water via interaction with cations (Mg, Ca, K, Na) adsorbed to phyllosilicate clay minerals. J Hydrol 515:1–9.

Orlowski N, Frede H-G, Brüggemann N, Breuer L (2013) Validation and application of a cryogenic vacuum extraction system for soil and plant water extraction for isotope analysis. J Sensors Sens Syst 2:179–193.

Palacio S, Azorín J, Montserrat-Martí G, Ferrio JP (2014) The crystallization water of gypsum rocks is a relevant water source for plants. Nat Commun 5:4660.

Palacio S, Hoch G, Sala A, Körner C, Millard P (2013) Does carbon storage limit tree growth? New Phytol 201: 1096-1100.

Perks MP, Irvine J, Grace J (2002) Canopy stomatal conductance and xylem sap abscisic acid (ABA) in mature Scots pine during a gradually imposed drought. Tree Physiol 22:877–883.

Phillips SL, Ehleringer JR (1995) Limited uptake of summer precipitation by bigtooth maple (*Acer grandidentatum* Nutt) and Gambel's oak (*Quercus gambelii* Nutt). Trees Struct Funct 9:214–219.

Picon C, Guehl JM, Ferhi A (1996) Leaf gas exchange and carbon isotope composition responses to drought in a drought-avoiding (*Pinus pinaster*) and a drought-tolerant (*Quercus petraea*) species under present and elevated atmospheric CO_2 concentrations. Plant Cell and Environment 19: 182 - 190.

Plavcová L, Hoch G, Morris H, Ghiasi S, Jansen S (2016) The amount of parenchyma and living fibers affects storage of nonstructural carbohydrates in young stems and roots of temperate trees. Am J Bot 103:603–612.

Poyatos R, Llorens P, Piñol J, Rubio C (2008) Response of Scots pine (*Pinus sylvestris* L.) and pubescent oak (*Quercus pubescens* Willd.) to soil and atmospheric water deficits under Mediterranean mountain climate. Ann For Sci 65:306.

Resco de Dios V, Díaz-Sierra R, Goulden ML, Barton CVM, Boer MM, Gessler A, Ferrio JP, Pfautsch S, Tissue DT (2013) Woody clockworks: Circadian regulation of night-time water use in *Eucalyptus globulus*. New Phytol 200:743–752.

Sacchi E, Riva Roveda C, Facchinelli A, Defilippi A, Magnoni M, E A (2001) Geochemical and mineralogical evidence of the recent trophic evolution of a small peri-alpine lake (Lake Sirio, Ivrea, Northern Italy). 10th International Symposium on Water-Rock Interaction, Billasimius, Italy.

Sala A, Woodruff DR, Meinzer FC (2012) Carbon dynamics in trees: Feast or famine? Tree Physiol 32:764–775.

Salleo S, Trifiló P, Esposito S, Nardini A, Lo Gullo MA (2009) Starch-to-sugar conversion in wood parenchyma of field-growing *Laurus nobilis* plants: A component of the signal pathway for embolism repair? Funct Plant Biol 36:815–825.

Salleo S, Trifilò P, Lo Gullo MA (2006) Phloem as a possible major determinant of rapid cavitation reversal in stems of *Laurus nobilis* (laurel). Funct Plant Biol 33:1063–1074.

Salmon Y, Torres-Ruiz JM, Poyatos R, Martinez-Vilalta J, Meir P, Cochard H, Mencuccini M (2015) Balancing the risks of hydraulic failure and carbon starvation: a twig scale analysis in declining Scots pine. Plant Cell Environ 38: 2575-88.

Schultz NM, Griffis TJ, Lee X, Baker JM (2011) Identification and correction of spectral contamination in ^{2}H/^{1}H and ^{18}O/^{16}O measured in leaf, stem, and soil water. Rapid Commun Mass Spectrom 25:3360–3368.

Secchi F, Zwieniecki MA (2011) Sensing embolism in xylem vessels: The role of sucrose as a trigger for refilling. Plant, Cell Environ 34:514–524.

Stöhr A, Lösch R (2004) Xylem sap flow and drought stress of *Fraxinus excelsior* saplings. Tree Physiol 24:169–180.

Tang K, Feng X (2001) The effect of soil hydrology on the oxygen and hydrogen isotopic compositions of plants' source water. Earth Planet Sci Lett 185:355–367

Treydte K, Boda S, Graf Pannatier E, Fonti P, Frank D, Ullrich B, Saurer M, Siegwolf R, Battipaglia G, Werner W, Gessler A (2014) Seasonal transfer of oxygen isotopes from precipitation and soil to the tree ring: Source water versus needle water enrichment. New Phytol 202:772–783.

Tyree MT, Salleo S, Nardini A, Gullo MA Lo, Mosca R, Lo Gullo MA, Mosca R, Thomas Tyree M, Salleo S, Nardini A, Lo Gullo MA, Mosca R (1999) Refilling of embolized vessels in young stems of laurel. Do We need a new paradigm? Plant Physiol 120:11–21.

Voltas J, Lucabaugh D, Chambel MR, Ferrio JP (2015) Intraspecific variation in the use of water sources by the circum-Mediterranean conifer *Pinus halepensis*. New Phytol 208:1031–41.

Walker GR, Woods PH, Allison GB, Orlowski N, Frede H-G, Brüggemann N, Breuer L (1994) Interlaboratory comparison of methods to determine the stable isotope composition of soil water. Chem Geol 111:297–306.

Waring RH, Whitehead D, Jarvis PG (1979) The contribution of stored water to transpiration in Scots pine. Plant Cell Environ 2:309–317.

West AG, Dawson TE, February EC, Midgley GF, Bond WJ, Aston TL (2012) Diverse functional responses to drought in a Mediterranean-type shrubland in South Africa. New Phytol 195:396–407.

West AG, Patrickson SJ, Ehleringer JR (2006) Water extraction times for plant and soil materials used in stable isotope analysis. Rapid Commun Mass Spectrom 20:1317–1321.

Willaarts B (2012) Linking land management to water planning: estimating the water consumption of Spanish forests. In: Water, agriculture and the environment in Spain: Can we square the circle?. Lucia De Stefano and M. Ramon Llamas (eds), Water Observatory of the Botin Foundation; Complutense University of Madrid, Spain, pp 139–151.

Zhao L, Wang L, Cernusak LA, Liu X, Xiao H, Zhou M, Zhang S (2016) Significant difference in hydrogen isotope composition between xylem and tissue water in *Populus euphratica*. Plant, Cell Environ. 39: 1848-1857.

CONCLUSIONS

1. Methodological limitations in ecohydrological studies based in water stable isotopes:

1.1. The presence of organic contaminants (methanol, ethanol or more than two carbon alcohols) causes an interference of organic contaminants in water isotopic composition analysed with Isotope-Ratio Infrared Spectroscopy (IRIS).

   1.1.1. The post-processing correction tested improves significantly the performance of IRIS in soil and xylem samples, for concentrations up to 8% for Ethanol, and 0.4% for Methanol.

   1.1.2. Sample pre-treatment through the micro-combustion module (MCM) slightly outperforms post-processing correction in removing methanol interference, but is not effective in removing Ethanol interference: For heavily-contaminated samples with Methanol (up to 1.6%), the best results would be obtained combining both methods.

   1.1.3. We generally recommend to adopt post-processing correction in isotopic analyses of contaminated samples. Only when the main (and mostly unique) contaminant detected is Methanol, the use of MCM (eventually combined with post-processing correction).

1.2. We report evidence of fast and reversible evaporative enrichment in metabolically active stems as a consequence of a temporal decline in sap flow rates.

   1.2.1. Hence, xylem water isotopic composition does not only reflect source water, but also stem hydraulic processes, and observed seasonal or daily fluctuations might not necessarily reflect changes in source water, but a confounding effect of xylem evaporative enrichment under limited sap flow

   1.2.2. Evaporation through the stem surface is the main driver of xylem isotopic enrichment. Indeed, we did not find evidence of a feedback effect of leaf water on stem values, and the dynamics of isotopic enrichment was independent from the original cause for sap flow reductions (*i.e.* limited leaf transpiration or stem hydraulic restrictions), also supporting a physical rather than a physiological regulation of this process.

   1.2.3. Although twig diameter has some effect on stem water losses, it does not seem to affect isotopic composition, and only minor differences can be observed between small branches and twigs in the field measures. However, very large branches or trunk xylem should be avoided, as we found evidence of fractionation in δ^2H (but not $\delta^{18}O$), displacing the values outside the evaporative line of soil, branches and twig samples.

- 1.2.4. We recommend to sample medium-size twigs at the time of maximum transpiration, avoiding periods of water stagnation, such as pre-dawn measurements, or the afternoon depression of stomatal conductance during drought stress.

1.3. We confirm a generalized uncoupling between soil water and previous precipitation or irrigation events, according to our results in the field and in pot experiments.

- 1.3.1. Both in the field and in the pot experiment, soil isotopic composition may remain rather constant despite large variations in the input water, suggesting that not all the water in the soil can be exchanged with new water, despite reaching field capacity, and having enough time for equilibration.
- 1.3.2. Our findings cannot be attributed to incomplete distillation: our tests show that we can ensure complete water retrieval with our established procedure: high temperature (110-120ºC) and vacuum (*ca.* 10^{-2} mbar) and long times (2 h for soil samples and 1.5 h for xylem samples).

1.4. On top of the precipitation-soil uncoupling, xylem water may also display substantial divergences in isotopic signature, as compared to its potential water source:

- 1.4.1. Even in controlled labelling experiments, it is not straightforward to associate the changes in xylem to the use of different soil water pools, due to the confounding effect of evaporative enrichment during drought cycles
- 1.4.2. We evidence the "high complexity of soil water pools" in the field, and even under controlled conditions, against the over-simplified "two water worlds" hypothesis. The key point for future research would be to properly define which soil water fraction (mobile, weakly or tightly bounded) each extraction method captures and which fraction can be used by plants, bearing in mind that plant's ability to uptake the different soil water pools depends on the water-use strategy of the species, its physiological status and environmental conditions.

1.5. Overall, even though it has been shown that stable isotopes are a powerful tool for water tracing on plant, soils and ecosystems, our findings also highlight the need to combine this data with other physiological tools in order to interpret plant water use and plant response during drought and recovery.

2. Submediterranean forests: ecohydrology and ecophysiology in a climate change context:

2.1. Oaks maintain their conservative strategy and continuous physiological activity by relying on stable soil water pools (groundwater, deep soil layers or tightly-bound water), while the pines develop a more opportunistic strategy of fast use of precipitation water based in a high root density on top soil layers, but show a limited ability to cope with drought:

- 2.1.1. The marcescent oaks are able to reach lower midday water potential in comparison to Scots pine, showing their ability to maintain transpiration and physiological activity under drier soil conditions. Conversely, oaks show higher pre-dawn water potential, determining their ability to reach wetter soil layers as a drought avoidance strategy.
- 2.1.2. Field campaigns display maximum δ^2H soil-xylem correlations for soil water at 50 cm depth for oaks and at 30 cm for pines, reinforcing the general knowledge of oak reliance on deeper soil water pools in comparison to pines. This trend is also partly confirmed by the water use patterns in the pot experiment, with pines showing proportionally larger water depletion in upper soil layers than the oaks.
- 2.1.3. In addition to hydraulic limitations, stomatal limitations of photosynthesis in pines reduce physiological activity already during the first stages of drought, and this has negative consequences in carbon assimilation and storage. Conversely, oaks do not show clear relations between seasonal variations in soil water availability and neither carbon assimilation nor storage.

2.2. The physiological advantage of oaks in response to drought is also reflected in the recovery after drought events, although the results may be apparently contradictory in field and pot experiments:

- 2.2.1. After long and severe drought periods as in 2012 summer at field, the greater vulnerability to xylem embolism in pines lead to more limited recovery of transpiration flow after the first rains, as compared to oaks.
- 2.2.2. In pot experiments, with severe but not so long drought period as in field conditions, pines may not develop embolism problems, whereas oaks do not benefit from the advantage of a deep root system. Hence, recovery of xylem water potential once irrigated is faster in the pines due to its non-limited faster stomatal control. Although pot trials are useful to test process-based hypothesis, these findings exemplify the risk of upscaling conclusions derived from pot experiments to the field.

2.3. The observed inter-specific differences in the short-term response to drought have also consequences in their long term performance: the pines show higher variations and far more pronounced decreasing trend with time, indicating that increasing drought events in the near future may induce long-term physiological decay in pines growing in the submediterranean ecotone.

Acknowledgements/Agradecimientos

Llegado el final del viaje resulta inevitable acordarme de todas aquellas personas que me han acompañado, apoyado y alegrado este camino de hacer (y acabar) la tesis doctoral. Espero aquí ser capaz de expresar toda mi gratitud y estima hacia vosotros.

En primer lugar, mi agradecimiento más sincero a Pitter Ferrio, quien se convirtió en amigo y director de tesis "de rebote" y sin el que este trabajo no habría sido posible, ni tan gratificante. Contigo he aprendido a hacer ciencia, a ser creativa, rigurosa, a divertirme y no tenerle miedo a nada. Gracias por estar siempre, desde Lleida hasta Chile, pasando por Japón.

Gracias a Jesús Pemán, por animarme a empezar esta aventura. Por tu entrega y cariño, por tu escucha y perspectiva. Por quererme y cuidarme como una hija.

Gracias a Jordi Voltas, por permitirme unirme al Laboratorio de Silvicultura todo este tiempo y obtener la financiación necesaria. Por ofrecerme las soluciones y una visión práctica cuando lo he necesitado.

Al Laboratorio de Silvicultura, mi casa durante todos estos años. A Mònica, por su lealtad y confianza. Porque fue la primera persona que me enseñó cómo muestrear para obtener datos isotópicos. Por su ojo clínico y buenos consejos. A Pilar y Maria Josep por su ayuda en mis extensos análisis de muestras; sin vosotras no existiría esta tesis. En concreto, gracias a Pilar, por su confianza, interés y total disponibilidad, en el laboratorio, el túnel experimental o en campo; una todoterreno, y a Maria Josep, por su cariño y entrega. A Jorge, por estar siempre acompañando mi izquierda y hacerme reír tanto.

A Luís Serrano, por enseñarme tanta fisiología y filosofía, por estar siempre atento y dispuesto a ayudar. Por enseñarnos a todos la parte más humana de la ciencia; al fin y al cabo todo es ecología.

A Mireia Oromí, por sus explicaciones y colaboración. Por dejarnos meter mano en el Picarro con libertad y curiosidad.

A Matilde Eizaguirre, por prestarme su despacho en el último año de escritura de la tesis. Sin duda, tener mi propio espacio ha contribuido claramente en el resultado final y en mi sentir este tiempo de dedicación absoluta. A Xavier Pons, por ser mi compañero de despacho y compartir fines de semana y festivos. Gracias a los dos por recordarme lo importante que es terminar.

A Alejandro Juárez, por la ayuda técnica, en laboratorio y en los experimentos; por tener soluciones para todo. A Tere Estela, por la ayuda con todo el "complejo" papeleo.

A José Ramóm Olarieta, por estar siempre disponible a ayudar con los suelos, gracias por las explicaciones y las (no pocas) dudas resueltas.

Gracias a Eustaquio Gil y a sus secuaces, Jota y Domingo. Por enseñarme fisiología, por darme buenas ideas y divertirme en cada viaje que hacía a Zaragoza. Seguimos. También gracias a toda la gente de la Unidad de Salud de los Bosques del Gobierno de Aragón, especialmente a José

Luís, Iñaki y Adán, sin los que no tendría muestras de suelo del Boalar. Gracias por vuestra fuerza y entusiasmo.

Gracias al Instituto de Formación Agroambiental de Jaca, por ser mi familia y mi casa. A Pilar García, por su interés (en todo momento) y ayuda logística. A Chema, por valiente, por ofrecerse a recoger el agua de lluvia durante más de un año. A Rubén, por su entusiasmo y por conducir tan bien la retro como para sacar arbolitos del monte con las raíces casi intactas. A Gonzalo, por la ayuda en campo y por no tener pereza en coger la motosierra para abrirnos paso. A todos los profesores, por no dudar en montar una actividad educativa con los alumnos y echarnos una mano en las labores de campo. A los alumnos, por sus manos y fuerza.

A Miguel Ángel Lázaro, por atreverse a escalar los pinos silvestres del Boalar, especialmente al alba. Por los madrugones y los bocatas en el camping. Por su entrega, amabilidad y amistad. A Marcela y Alejandro e Isa y Javi por ofrecerme hogar durante las campañas de muestreo.

A todas las personas con las que he tenido el honor de colaborar en estos años. A Adrià Barbeta, por las largas conversaciones y por la amistad que el Picarro nos ha dado. A Sara Palacio, por dejarme alucinada con sus investigaciones e ideas sobre los yesos y dejarme participar en sus experimentos. A Víctor Resco, quien me enseñó el día y la noche de las plantas permitiéndome participar en el proyecto del Ecotron en Montpellier. Gracias también por los “lab meetings” y las conversaciones de lo más actual en ciencia. A Ulises Rodríguez, por la ayuda que me prestó, por las buenas pláticas y los buenos ratos que pasamos el verano del 2015 en el túnel de malla.

Thanks to Todd Dawson for allowing me to stay for some months in his lab in University of California (Berkeley); also for being the connexion to meet other scientists (Isodopes) and the magical forests in California. Thanks to Stefania Mambelli, for teaching me the importance of being rigorous and taking so much care to me. Thanks to Kevin Simonin for helping me with the experimental design in the fourth chapter, for clearly explain to me the leaf models and for giving me the perspective and confidence I needed to continue with my thesis experimentation. Thank you all for all the support and kindness.

A Larry López, por invitarnos al Summer School en Mongolia, por lo bien que lo pasamos y los lazos que este viaje nos está permitiendo tejer.

A mis amigxs. A las amigas del alma, Laura, Marina, Agnès, Maria y Geo por entenderme como nadie y acompañarme, por hacer el camino más ameno. A las flores de Lleida, Irene, Salomé, Eva y Diana, por lindas. A los chicos del laboratorio de Entomología, por los tuppers compartidos. A mi familia de La Serraneta: Jordi, Manu, Isa, Alberto, Vanessa, Abi y David, por la vida que he podido sentir en las fogatas y paseos perrunos con vosotros.

A mis padres Enrique y Sagrario y a mi hermana Lucía, por su apoyo incondicional, siempre. Os quiero mucho, soy gracias a vosotros.

A Youness, mejor amigo y compañero de vida. Gracias por ponerle pasión y darle sentido a todo; por recordarme cada día que persiga mis sueños.

A Amín, por su vida en la mía; por esperar pacientemente a que tuviera la tesis depositada para nacer.