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ECOHYDROLOGICAL PARTITIONING IN THE CRITICAL ZONE: ANALYSIS OF THE SOIL-PLANT-ATMOSPHERE FLOWS BY MEANS OF STABLE WATER ISOTOPES

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RIPARTIZIONE ECOIDROLOGICA NELLA ZONA CRITICA: ANALISI DEI FLUSSI SUOLO-PIANTA-ATMOSFERA TRAMITE GLI ISOTOPI STABILI DELL'ACQUA

Coordinatore: Prof. Davide Matteo Pettenella

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Dedication

Dedicated to

My Father
MUHAMMAD AMIN

My Mother
ZARINA AMIN

"They are my roots, my foundation as they gave me a clear vision for success. They planted the seed that I base my life on, and that is the belief that the ability to achieve starts in your mind"

&

My Siblings
AAMIR, NIDA, SABA, AHMAD & HAMZA

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Firstly, I want to thank my Creator, God for his countless blessings that He has given me yesterday, today, and for all my tomorrows. With His mercies, grace and favours all the goods can be accomplished.

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To my love, my parents.

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SUMMARY

Tree and small catchment scale studies are important in getting a detailed understanding of ecohydrological processes and the interactions between plants and water fluxes with respect to specific geology, climate, physiographic and topographic settings. However, such studies cannot explain the processes ongoing at large spatial and temporal scales and/or across the globe. Recent global-scale analyses based on water stable isotopes (^{2}H and ^{18}O) quantified the contribution of different water sources to plant transpiration. However, no previous studies have estimated the depth distribution of soil water uptake by plants, and its relation to climate, and plant group at the global scale.

Recently, numerous ecohydrological studies explored the controls of climate and physiographic settings on isotopic composition of plant water. However, the assessment of the critical postulates (i.e., no isotopic fractionation in plant tissues) behind the use of stable water isotopes have not been widely tested. Furthermore, recent literature has raised a concern regarding the lack of standardized methodologies for plant and soil materials sampling and water extraction. The accuracy of different water extraction methodologies and the technical parameters (such as system setup, extraction time and temperature), which may affect the isotopic composition of the soil and plant extracted waters and thus, our interpretation of the results, still need to be thoroughly investigated.

To deal with these issues, this thesis presents an integrated outlook by exploring plant water-relations through use of stable water isotopes. This approach moved from a global scale assessment of the soil water source exploited by plants and the main drivers (i.e., climate and plant functional type) controlling the isotopic compositions of xylem water. This analysis revealed soil water as main water source for plants. A new graphical inference method has been developed for the quantification of soil water depths contribution. The results indicated the largest overlap (up to 100%) for shallow soil water and xylem water in cold zone, while the overlap between deep soil water and xylem water was largest for arid and tropical climate zones (i.e. >75%). This method has a great prospective to be tested and applied at other study sites around the globe.

Such global or catchment scale water source quantification by stable water isotopes is based on fundamental underlying postulation, henceforth, as a second objective in this thesis, it was attempted to test the isotopic fractionation of plant water along the pathway from roots to leaves

under a greenhouse setup. Besides this, the measured isotopic compositions were also evaluated towards their sensitivity to the plant water extraction method via an interlaboratory comparison of commonly used cryogenic vacuum distillation system for plant materials. The resulting isotopic composition of different plant tissue water (i.e., stem and core) showed no fractionation with some exception to the root water and soil water samples at depths >15 cm, which were isotopically enriched as compared to the irrigation water. The samples extracted in two labs were comparable, nevertheless this controlled experiment further highlights the urgency of such comparisons to be performed for other existing plant water extraction methodologies and across other plant species.

Thereupon a subsequent comparison of different plant water extraction techniques (Scholander-type pressure chamber vs. cryogenic vacuum distillation) was performed. Results indicated a significant difference in the isotopic values obtained by the two extraction methods and this difference was smaller for beech samples compared to the chestnut samples. In addition, different results were observed for $\delta^{2}H$ and $\delta^{18}O$, i.e. larger differences between the plant water extraction methods and the samples were found for $\delta^{2}H$ compared to $\delta^{18}O$.

1 INTRODUCTION

The water supply decline around the globe calls out for advancements in ecohydrological research to improve the management of water resources, especially in those areas where climate and land use changes potentially have a strong impact on evapotranspiration fluxes. Indeed, plants play an important role in the regulation of hydrological cycle by carrying out 60-80% evapotranspiration fluxes on land (Schlaepfer et al., 2014; Schlesinger and Jasechko, 2014). Transpiration determine precipitation patterns and local microclimate and have great impacts on water source partitioning. Given the significant role plants play in the water cycle, a more detailed understanding of plant water uptake and interactions between plants and the potential water sources is central towards the development of effective strategies for forest management and sustainable use of water resources (NRC-NAS, 2012).

Ecohydrology is coming up as an integrative discipline by linking water-related research with ecological, social and physical sciences. Ecohydrology deals with the interaction between vegetation and water, and the movement of water fluxes through ecosystem in the critical zone of earth. This critical zone (CZ) defined as *"an open system extending from the canopy top to the groundwater extremity* (Figure 1.1)*"* (Brooks et al., 2015) exerts long term feedbacks on ecosystems (Jenerette et al., 2012; Turnbull et al., 2012), and therefore, has recently become a purview of ecohydrology. CZ is facing ever growing pressure due to increasing population, wealth and global warming (NRC-NAS, 2012). CZ has significant controls on plant water uptake, especially in dry climates (Asbjornsen et al., 2011; Newman et al., 2006). CZ acts like a continuously evolving nonlinear system with connection to energy and water fluxes (Chorover et al., 2011; Rasmussen et al., 2011; Zapata-Ríos, 2016). A more detailed understanding of the CZ evolution, formation, structure, and functioning is extremely important for the prediction of CZ response to the changes in land use, climate and water resources (National Research Council, 2001). Climate and energy gradients greatly influence the biogeochemistry, ecohydrology and soil formation in the CZ and have been considered as ideal areas to study the variability of water fluxes (Chorover et al., 2011). Ecohydrological study is concerned with the interactions between climate, topography, vegetation and soil and their impacts on the hydrological systems over a long period of time (Ehret et al., 2014; Hopp et al., 2009; Troch et al., 2009; Moore et al., 2015). Hydroclimatic variability related to latitude and elevation together with landscape changes (such as vegetation and geology) affects the partitioning of water and energy fluxes (Gustafson et al., 2010; Molotch et al., 2009; Veatch et

al., 2009). In the CZ, plants act as primary conduits that return water to the atmosphere (Chapin et al., 2002). Accounting for about 39% of precipitation on land and up to 80% of evapotranspiration globally (Schlaepfer et al., 2014; Schlesinger and Jasechko, 2014), plants transpiration represents one of the most important components of the terrestrial freshwater fluxes. Thus, the quantification of water sources accessible to and exploited by plants is still a key issue in ecohydrological research.

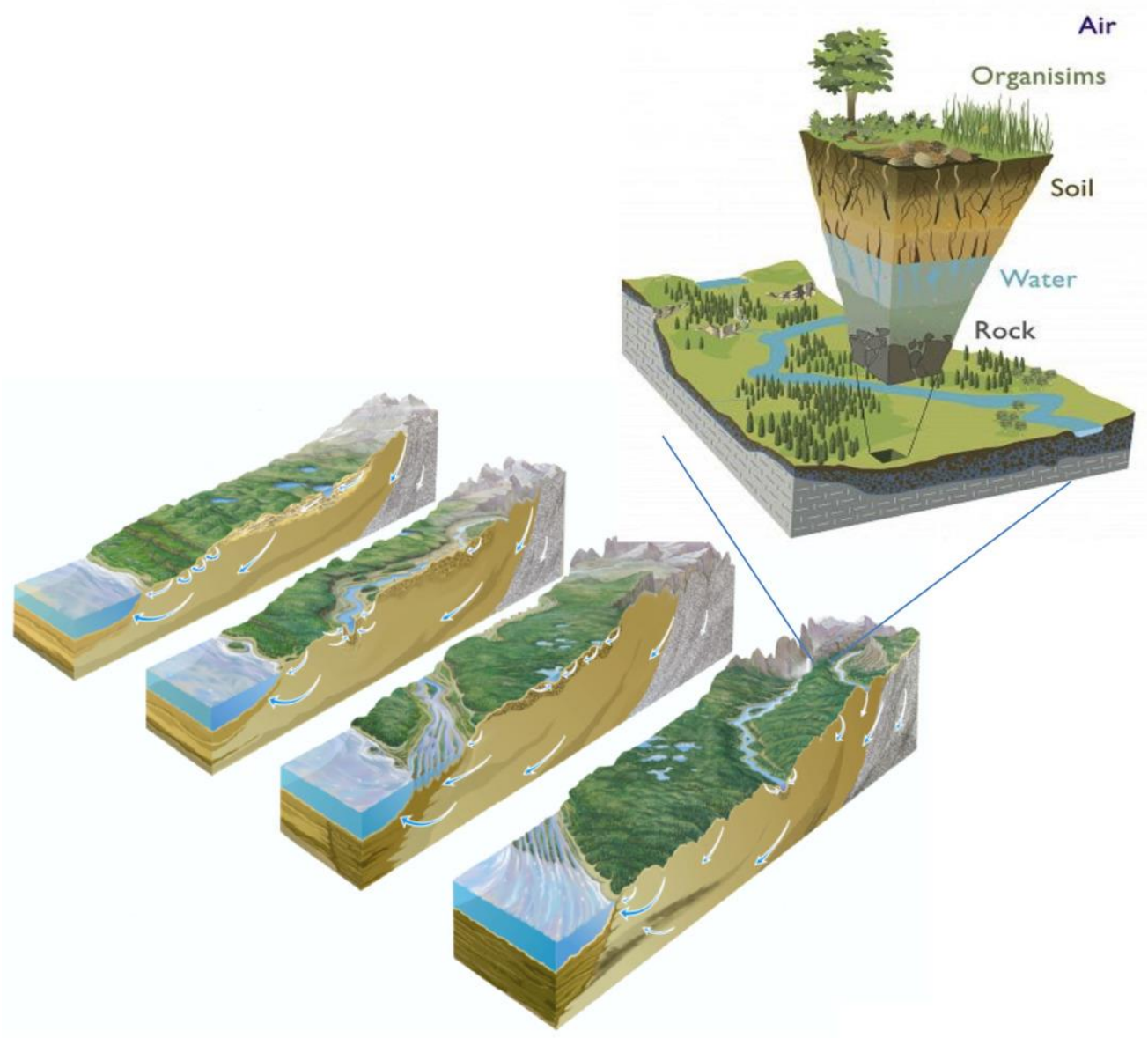


Figure 1.1. A sketch showing the interaction of life, air, water, soil and rock in the critical zone. The right inset shows the vertically deeper and longer temporal scale focal points of CZ science and their relation to ecological and hydrological research. The multiscale nature of the CZ processes is illustrated by the four transects from mountains to the sea (taken from *Brooks et al., 2015*).

Water stable isotopes (^{2}H and ^{18}O) proved to be powerful tools for the quantification of proportions of water sources to plant transpiration and for the determination of climatic and physiographic controls on water source partitioning at the soil-plant interface (Meißner et al., 2014). Stable isotopes have a conservative nature and their existence in water molecule makes them unique tracers (Penna et al., 2018; Scandellari and Penna, 2017). The stable isotopic tracing approach is very important to answer some basic questions in ecohydrology, e.g. "Where do plants uptake their water from?", "What are the controlling factors leading the plants to select a particular water source?", and "What are the main sources of plant transpiration?".

Soil water is often considered as the main plant water source. However, soil water is link to surface water and groundwater due to infiltration and evaporation processes. Soil water migrates via preferential and piston type flow in the unsaturated soil zone (vadose zone) (Zhang et al., 2017), and the soil contribution to plants at different depths can be investigated by a comparison of xylem water with soil water. The vertical isotopic profile of soil water is significantly affected by evaporation and infiltration processes resulted from the mixing of different precipitation events (Che et al., 2019). Furthermore, the rain reaches the groundwater via infiltration through the soil profile and for shallow groundwater zones, it then recharges the unsaturated soil profile through the capillary fringe. In this way, waters in different parts of soil-plant-and-atmosphere continuum have different isotopic signatures (Dawson 1993; Dawson and Pate 1996). As water is available from both deep and shallow zones in the soil profile, some plants develop dimorphic root system representing two active root zones, i.e. one in the upper soil profile for taking up soil water recharged by summer precipitation and one in the deeper soil zone to utilize groundwater during the dry conditions (Ehleringer and Dawson, 1992).

There has been a rapid increase in the number of ecohydrological studies quantifying the potential water sources for plant uptake and some of these reported soil water as a primary source for transpiration (Asbjornsen et al., 2011; Gardner and Ehlig, 1963). Recent studies have observed similarities in the isotopic signal of xylem and soil water implying that plants more likely take up soil water than groundwater (e.g., Bowling et al., 2017; Brooks et al., 2010; Evaristo et al., 2019; Grossiord et al., 2016; Gu et al., 2015; Wei et al., 2013; Yang and Fu, 2017). Several site and climate zone specific studies using water stable isotopes have been conducted so far (Anderegg et al., 2013; Barbeta et al., 2019, 2015; Brum et al., 2017; Daniels

et al., 2017; Dudley et al., 2017; Evaristo et al., 2016) assessing the discrimination among various water sources by plants from the tree to the catchment scale. Studies have analysed soil water uptake at different soil depths (Asbjornsen et al., 2008; Barnard et al., 2010; Le Roux et al., 1995; Schwendenmann et al., 2015), under different conditions of soil types (e.g., Geris et al., 2017; Meißner et al., 2014) and across different climatic regimes of the world and on different plant species (e.g., few of them are as follow: Allen et al., 2019; Bertrand et al., 2014; Bijoor et al., 2012; Brum et al., 2017; Chi et al., 2019; Dudley et al., 2017; Evaristo et al., 2019; Nie et al., 2019; Oerter and Bowen, 2017; Wang et al., 2017). However, a global-scale analysis able to investigate if observed differences in xylem and soil water isotopic composition are related to climate, and/or vegetation type is still missing.

Meta-analysis is a good approach to complement the information obtained from individual studies. As such, by a proper assessment of publication bias, primary information obtained from individual study sites can be put together in a large context to answer some global level research questions. Basic field studies even though provide some important discoveries, but we have to acknowledge that these studies represent only a part of global scale information. Perhaps this partial information will remain insufficient until accompanied by a large-scale research (in other words, meta-analyses) (Evaristo and McDonnell, 2017a). Recently, global scale analyses based on isotopic data have been performed for soil water, groundwater, stream water, as well as plant water, but despite that the xylem water measurements remained dispersed throughout the whole primary specialist literature. A global scale analysis by McDonnell (2014) suggested that plants uptake soil water, which neither contribute to groundwater recharge nor to the streamflow, thus supporting the hypothesis of ecohydrological separation given by Brooks et al. (2010). Subsequently, other meta-analyses by Evaristo and McDonnell (2017b) and Barbeta and Peñuelas (2017) quantified groundwater contributions across different biomes, plant species, seasons, soil types and landscape locations. Barbeta and Peñuelas (2017) found that groundwater contributions were more likely at sites with a pronounced dry condition, and represents, on average, 49% of transpired water in dry seasons and 28% in wet seasons, and higher groundwater fractions were observed on rocky substrates (Barbeta and Peñuelas, 2017). However, none of the global meta-analyses have quantified the contributions of soil water to plants at different soil depths. Indeed, this depth distribution of soil water source from soil surface to water table is a key missing element to link some of the inconsistencies in the results from various research studies. Therefore, a global analysis of the

isotopic composition of xylem and soil waters is required to get insights into plant-soil water interactions in different climatic, physiographic and geological settings.

Global analyses through stable isotope tracing are powerful tools in quantifying different water sources to plants, nevertheless, there are some underlying assumptions behind the use of isotopic analysis approach. First, there are significant differences in isotopic composition between water sources potentially exploited by plants (soil water at different depths and groundwater). Second, there is no isotopic fractionation by a plant during water uptake. For instance, the fractionation of isotopic composition in the soil (potential plant water source) or within the plant tissues can lead to a "false" interpretation of the results. Isotopic signatures are shaped by the fractionation and mixing processes occurring in the atmosphere-plants-soil compartments, either simultaneously or at different times as water flows through the system.

For the reason that we still miss a proper understanding of the mixing, as well as fractionation processes occurring at spatial and temporal scales, difficulties remain in inferring the isotopic values measured in our water samples (Penna et al., 2018). The water fluxes partitioning by vegetation occurs at different spatial and temporal scales making the system heterogeneous. Such heterogeneity of our natural systems makes it difficult to sample completely, while the analytical processes on the other hand add further uncertainty to the collected data. The isotopic variability that exists in the various water compartments either within a catchment or an ecosystem or even within a tree are the result of many concurrent processes. As known from the literature, climate is the main driver of heterogeneity characterizing different water compartments. Atmospheric parameters (such as temperature, radiation and humidity) significantly affect the isotopic signatures of soil and plant water source (i.e., precipitation) (Figure 1.2) over large temporal and spatial scales (Allen et al., 2018; Bowen, 2008; Coplen et al., 2008; Dansgaard, 1964; Fischer et al., 2017; Ingraham, 1998; Rozanski et al., 2013). Current studies have drawn special attention towards the climatic controls on the isotopic signatures of the precipitation. These controls mainly include moisture source (e.g., Breitenbach et al., 2010; Sengupta and Sarkar, 2006), amount of precipitation (Vuille et al., 2005; Kurita et al., 2009), temperature (Tian et al., 2007 and the studies there after), and other mixing, and condensation processes occurring during the transport (Brunello et al., 2019).

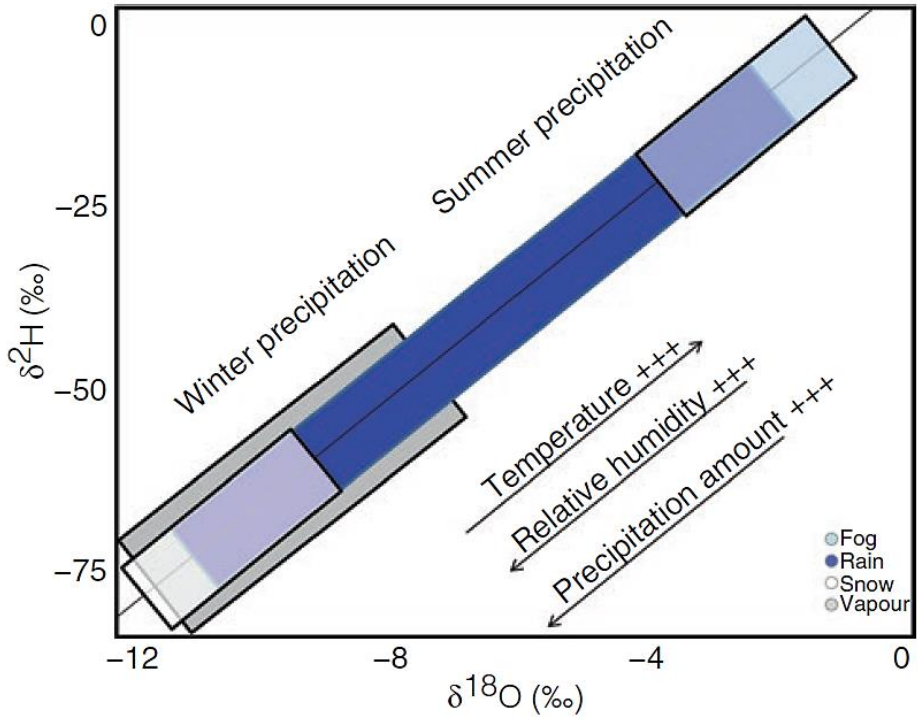


Figure 1.2. Schematic dual-isotope plot showing different types of precipitation water samples and showing the effects of various environmental factors on the isotopic compositions (*Barbeta et al., 2018*).

Plant water partitioning is driven by diverse climate-soil-vegetation relations (Verstraeten et al., 2008) and can be well observed in the isotopic composition of xylem and its source water in several studies (Brandes et al., 2007; Goldsmith, 2012).

In addition, not only the temporal and spatial scale variability of precipitation, but there are other factors such as soil water availability, rooting depth, uptake strategies, and topography leading plants to change water uptake patterns across seasons (Brum et al., 2017; Dawson and Pate, 1996; Eggemeyer et al., 2009; Oliveira et al., 2005; Rodriguez-Iturbe, 2000; Rossatto et al., 2012; Schwendenmann et al., 2015). Change in water uptake pattern can also occur at tree level on much shorter timescales (e.g., within a few days; Stahl et al., 2013). For example, trees with a dimorphic rooting system (i.e., roots are distributed laterally in shallow soil and a tap root growing towards deeper soil layers) are able to extract water from shallow and deeper soil layers (Dawson and Pate, 1996; Ehleringer and Dawson, 1992; Scholz et al., 2008). Other studies found that trees in environments that lack a deep soil (Canadell et al., 1996; Fan et al., 2017) or at locations where dry seasons are less pronounced, invest in a shallow and dense horizontal root system (Brum et al., 2019; Meinzer et al., 1999).

Furthermore, this spatial and temporal scales water source variation based on the plant functional rooting systems can result in distinct and highly variable isotopic compositions among different species and individuals (Bertrand et al., 2014; Bijoor et al., 2012; Ehleringer and Dawson, 1992; Schwendenmann et al., 2015; Volkmann et al., 2016). A vertical isotopic gradient in xylem water isotopic signature may be observed due to the temporal variations in water source within the plant resulting from increase in the travel time with the plant height (Cernusak et al., 2005; Zhao et al., 2016). However, the xylem water isotopic signature can only be used to trace back the water source under the assumption of *"no isotopic fractionation within plants during the water uptake by roots and distribution to twigs"* (Dawson et al., 2002; Dawson and Ehleringer, 1991), and therefore, the isotopic composition of xylem water should be identical to the soil water isotopic composition at the site from where it is taken by roots (Bowling et al., 2017; Dawson, 1993; Dawson and Ehleringer, 1993). However, recently, some studies have shown that this is not always the case and there are physiological processes and soil-plant interaction processes that can lead to a divergence of xylem water isotopic signal from the original source soil water (Ellsworth and Sternberg, 2015; Ellsworth and Williams, 2007; Poca et al., 2019; Vargas et al., 2017; Zhao et al., 2016). Some plants related factors such as storage vessels, conduits dimensions and even the direct absorption of water from the surrounding atmosphere (e.g., in the form of fog and dew) can affect the xylem water isotopic signature. The differences in the stem to roots connectivity and the mixing of xylem-phloem fluids are some of the proposed mechanisms of xylem water enrichments along stem, branches and young plant tissues (Cernusak et al., 2005; Dawson and Ehleringer, 1993; Thorburn et al., 1993). Subsequently, it is not certain yet if the observed fractionation occurred during the water movement from roots to stem or the distribution in the stem, or roots and/or whole plant.

In addition to the possible fractionation of the water source within the plants, there are other technical issues that need a special attention from the ecohydrologic community. Soil and xylem water extraction methods and procedures are one of the bottle necks in the application of stable water isotopes in plant-water relation studies.

There exist an array of plant and soil water extraction methods, like squeezing or heating the plant materials. Such techniques have been in use for thousands of years to obtain essential oils and other chemical materials from plants (Kockmann, 2014). However, today, such squeezing or heating of plant materials is carried based on their high tech versions, e.g., cryogenic vacuum distillation (Koeniger et al., 2011; West et al., 2006), microwave extraction (Munksgaard et al.,

2014), azeotropic distillation (Swaffer et al., 2014; Twining et al., 2006), centrifugation (Peters and Yakir, 2008), in situ monitoring via using direct vapor equilibration (Volkmann et al., 2016; Wassenaar et al., 2008). Nonetheless, each of the underlined method has a related challenge concerning the accuracy, repeatability and uncertainty (Millar et al., 2018; Orlowski et al., 2018a; Orlowski et al., 2016a). Cryogenic vacuum distillation is a widely used extraction method for plants and soil materials, however, recent studies have raised some issues related to cryogenic extraction parameters (such as extraction temperatures and times of vacuum) and physiochemical properties of the soil media as the factors affecting the isotopic compositions of soil water extracts (Orlowski et al., 2018a). Correspondingly, other commonly used methods have likelihood of coextraction of various organic compounds, which can later affect the isotopic analysis via laser spectroscopes (Millar et al., 2018; West et al., 2010).

Lately, the interlaboratory comparison of soil water extraction methods carried out by Orlowski et al. (2018a) critically discussed the accuracy of widely used cryogenic vacuum distillation as a standard method for plant-water research (Newberry et al., 2017; Orlowski et al., 2013). Some of these studies have shown a significant effect of the extraction technique on the isotopic signal of the soil water extract. For example, Sprenger et al. (2015) reported variability in the pore water isotopic value based on soil texture and organic content. They showed that these protocols and applied methods are not comparable sometimes, e.g., when soils are dried at high temperatures (105°C), then the soil material properties change, which would not occur in field soils in which the plants are rooted. Furthermore, we are still lacking the knowledge about the range of cryogenic extraction parameters (such as temperature, time, and pressure threshold) and the effects of soil physicochemical properties and the possible isotopic fractionation processes in plants which may have a significant effect on the isotopic composition of plant and soil water extracts (Gaj et al., 2017a, 2017b; Orlowski et al., 2018a; Orlowski et al., 2016a, 2016b). Previous soil water extraction studies found that different soil types need different extraction temperatures to extract the same pool of water (Gaj et al., 2017b), however, further experiments to find a range of temperatures for different soil types are still absent.

Detailed interlaboratory comparisons of plant and soil water extraction techniques (such as the cryogenic extraction method, centrifugation, microwave extraction, mechanical squeezing, direct vapor equilibration), soil-water sampling methods (for instance, tension lysimeters) and specifically some of xylem water extraction approaches (such as Scholander-type pressure chambers) are urgently needed to develop efficient and standard soil and plant sampling and

extraction procedures (Millar et al., 2018). More explicitly, it is important to carry out experiments that will allow us to understand whether the isotopic variability of a certain water source is happening in the real world or it is just an artefact of the sampling and extraction methodologies (Penna et al., 2018) because this chain of processes from sampling to extraction of water result into an increase in cumulative error (Figure 1.3). A proper understanding is required to know if a particular water type obtained from soil and plant materials is relevant to answer the research questions that we asked. For instance, different methods sample different water pools in the soil and plants (as such the cryogenic extraction system is extracting nearly all the water from the soils even the water held at high soil tensions) while on the other side, tension lysimeters can sample only the mobile soil water held at <200 kPa (Geris et al., 2015) and do not collect all the water from the soil material that plants can uptake and thus, leading to potential differences in the interpretation of results (Sprenger et al., 2017). Similarly, in case of plants, different extraction methodologies do exist which lead to the extraction of entire plant tissue including intracellular water while in contrast, the Scholander-type pressure chambers (Figure 1.4) and the direct vapour equilibration techniques (Volkmann et al., 2016) are able to extract xylem water only. While new in situ measurement techniques are continuously evolving for both plant and soil water isotopic composition, however, to date no such interlaboratory comparison has been performed for plant water extraction methods. Such interlaboratory comparisons should be carried in future for other existing plant water extraction methodologies to account for any possible differences and effects on the isotopic compositions of the water extracts with special consideration to the extraction method parameters and uncertainty.

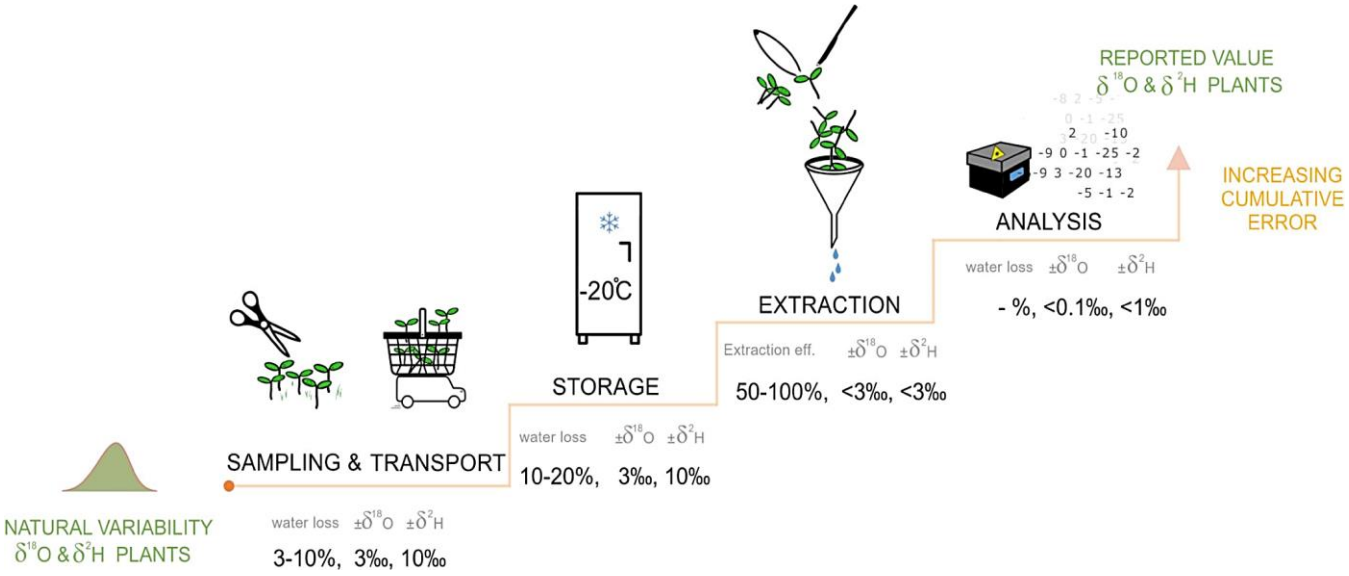
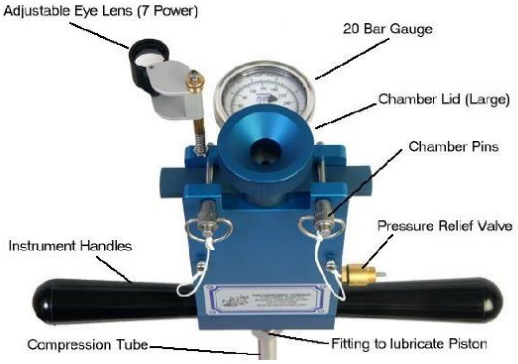
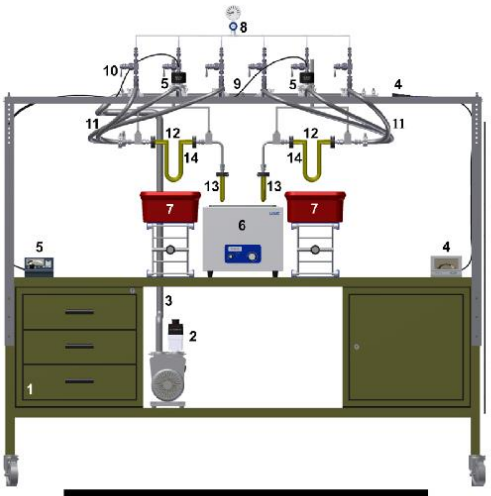


Figure 1.3. Chain of processes starting from plant sampling to the extraction of water in the laboratory and then isotopic analysis of water to obtain the final isotopic composition. A potential risk of water loss and the resulting error in the isotopic composition for both ^{18}O and ^{2}H (in permil) is shown. At each step ahead, cumulative error in the measurement of isotopic composition increases, highlighting the need to focus on the entire chain of the processes instead of just the extraction methodology (from *Fischer et al., 2019*).

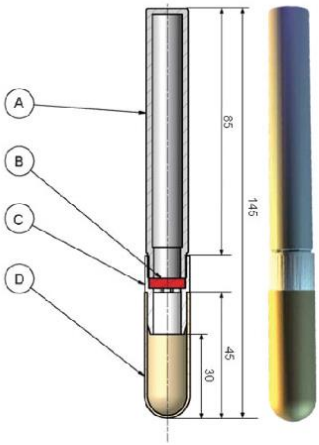
Cryogenic Vacuum Extraction systems



Pressure Chambers



Cryogenic Vacuum line



Centrifugation

Figure 1.4. Examples of some plant water extraction techniques.

1.1 Research questions and objectives

Research questions

This work specifically aims to answer the following questions:

- ✓ To what extent does the isotopic composition of xylem water reflect that of soil water (its potential water source) at different soil depths and how does climate and plant type control these isotopic patterns?
- ✓ In the light of the recent advancements in isotope hydrology and ecohydrology, can we still assume that no isotopic fractionation occurs in the pathway from the roots to the upper twigs?
- ✓ Is the widely used cryogenic vacuum distillation methodology reliable to extract plant water samples?
- ✓ Do cryogenic vacuum distillation and the Scholander-type pressure chamber extract the same xylem water?

Research objectives

Based on the recent needs, this work proposes an integrated outlook to explore plant-water relations in vegetated environments by a global scale assessment of xylem water isotopic compositions, and through a comparative analysis of plant water extraction methodologies. The specific research objectives are:

i) the quantification of the soil water (sampled at different depths) contribution to plant water uptake and the analysis of the role of climate and plant type on the isotopic composition of soil and xylem waters

ii) the investigation of xylem water isotopic composition along the pathway from roots to leaves

iii) testing whether the measured isotopic compositions are sensitive to the cryogenic vacuum distillation developed in two different laboratories

iv) testing whether the cryogenic vacuum distillation and the Scholander-type pressure chamber extract xylem water with similar isotopic composition.

1.2 Structure of the thesis

This thesis comprises three main sections (Chapter 2 to 4) reporting the main activities carried out during the PhD.

The first paper (Chapter 2) is a global analysis that quantifies the depth distribution of soil water contributions to vegetation (by a new direct inference approach) and evaluates the controls of climate and plant functional type on the isotopic composition of xylem water.

The second paper (Chapter 3) proposes the assessment of the hypothesis of "no isotopic fractionation in plant water", which is fundamental for the use of stable water isotopes in ecohydrological applications.

The third paper (Chapter 4) focuses on the comparison of Scholander-type pressure chamber and cryogenic vacuum distillation methods for the extraction of water from plant materials in an experimental catchment in the Italian pre-Alps.

The thesis concludes with the *Conclusions* (Chapter 5) and *References* (Chapter 6) and then *List of publications* (Chapter 7). All the references from Chapters 1 to 5 are integrated at the end of the thesis in Chapter 6.

2 Depth distribution of soil water sourced by plants at the global scale: a new direct inference approach[1]

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2.1 Introduction

Transpiration of terrestrial vegetation is a dominant force in the global water cycle, accounting for 60-80% of total evapotranspiration fluxes on land (Schlaepfer et al., 2014; Schlesinger and Jasechko, 2014). Transpiration is a major determinant of local microclimate and precipitation patterns and has a direct impact on water balance and streamflow regimes . Given the important role of plant transpiration in the hydrological cycle, a more detailed understanding of plant water uptake and ecohydrological interactions between plants and soil water is crucial for developing effective land surface models and sustainable water use strategies.

The stable isotopes of hydrogen and oxygen (^{2}H and ^{18}O) are effective tools to determine the proportions of water sources to plant transpiration. Due to their conservative nature through soils and their occurrence in the water molecule, stable isotopes are increasingly used for tracing water fluxes in ecohydrological and other interdisciplinary studies (Penna et al., 2018; Scandellari and Penna, 2017). The quantification of the main water sources for plant transpiration based on isotopic tracers is typically carried out through a graphical inference method (Brunel et al., 1995), two-end-member mixing models (e.g., Thorburn and Walker, 1993) or statistically-based multi-source mixing models (e.g., Schwendenmann et al., 2015). The graphical inference method defines the mean root water uptake depth as the soil depth where the isotopic composition of soil water is most similar to or equals the one of xylem water. Basically, this approach represents the plant root system as one unique root (Rothfuss and Javaux, 2017). IsoSource (Phillips and Gregg, 2003) is a widely used linear mixing model based on a mass balance equation (recent examples are: Jia et al., 2017; Zhu et al., 2016). Nowadays, statistical Bayesian mixing models such as SIAR (Parnell et al., 2013), MixSir, and MixSIAR (Moore and Semmens, 2008) are gaining popularity (e.g., Beyer et al., 2018). For a review and intercomparison of these methods the reader is referred to Rothfuss and Javaux (2017). Bayesian isotope mixing models have the advantage of providing uncertainties of the estimated fractions of water sources, and provide an optimal solution rather than a range of feasible solutions (Rothfuss and Javaux, 2017). However, a common underlying assumption of these approaches is that all water sources accessed by plant roots are adequately sampled (Rothfuss and Javaux, 2017) and that the tracer signature is conserved through the mixing processes (Phillips et al., 2014). But in field studies, sampling all potential water sources is not always practical or possible, creating potential bias in the mixing model estimation of a given

plant water source. Therefore, new methods that allow for robust quantification of water sources accessed by plants and that can be distinctly sampled are needed.

In many cases, the primary source of plant transpiration is soil water extracted from different depths by vegetation through roots (Asbjornsen et al., 2011; Gardner and Ehlig, 1963). Plants can access shallow and deep soil water, as well as groundwater with a tendency to prioritize the use of stable and continuous water sources (Zhao and Wang, 2018), at least in regions where some sources are continuously available. Several studies based on an isotope approach and focusing on the discrimination among different water sources accessed by plants have been conducted at individual sites in many regions of the world and on different species (e.g., to name a few recent studies, Allen et al., 2019; Chi et al., 2019; Dubbert et al., 2019; Evaristo et al., 2019; Nie et al., 2011; Oerter et al., 2019; Qiu et al., 2019). Recent meta-analyses assessed plant water sources across different biomes and species (Evaristo et al., 2015; Evaristo and McDonnell, 2017b; Barbeta and Peñuelas, 2017).

Despite these meta-analyses and the notable number of studies focusing on the quantification of water sources for tree transpiration in different parts of the world, knowledge on global scale estimates of soil water sources at different depths is still missing. Thus far, global meta-analyses have not quantified the depths of soil water contributions to plant water uptake. Indeed, this depth distribution (from the soil surface to the water table) is the key missing link to perhaps reconcile some of the disparity in results from different groups. Here we analyse isotope data extracted from 65 peer-reviewed papers published between 1990 and 2017. We propose a new direct inference method that can approximate the proportion of water sources to root water uptake even if one or more sources are missing. We use this new approach to evaluate the main water sources used by plants around the world and to quantify explicitly the depth distribution of soil water uptake and its relation to climate and plant group.

Specifically, our work aims to answer the following questions:
1) To what extent does the isotopic composition of xylem water reflect that of soil water for different plant groups across the globe?
2) What is the depth distribution of this source water?
3) How does climate and plant group control these patterns?

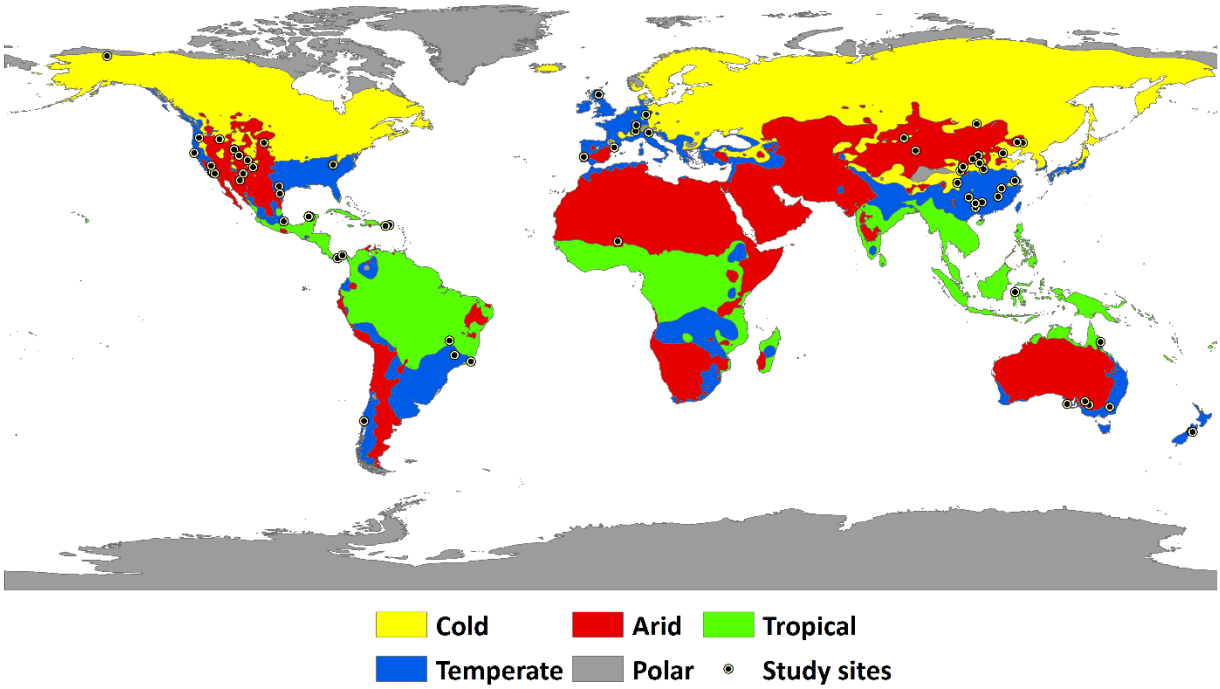


Figure 2.1. Köppen climate classification (*Peel et al., 2007*) for all study sites (n=77) included in this global analysis.

2.2 Materials and methods

2.2.1 Literature selection and data extraction

We based our global meta-analysis on isotope data extracted from 65 peer-reviewed papers published between 1990 and the end of 2017 that used both stable isotopes of water (δ^2H and $\delta^{18}O$) for ecohydrological studies. We performed searches in Web of Science, Scopus, and Google Scholar using different combinations of the following keywords: "water uptake", "xylem", "soil water", "stable isotopes", "hydrogen", "oxygen". Of the returned papers, we considered only those that reported both $\delta^{18}O$ and δ^2H data in soil water at different soil depths, and in xylem water. Our returns included 25 and 37 of the 47 and 138 papers in Evaristo et al. (2015) and Evaristo and McDonnell (2017b) and 7 of the 35 papers examined in Barbeta and Peñuelas (2017).

We excluded studies that did not report both isotopes or did not include soil water. Stable isotope data reported in the original papers were extracted either directly from tables or the text, or through the data extraction tool Graph Data Extractor (Matthews, 2017) or obtained from digital repositories. The database includes isotope data from soil water (n=5328) and

xylem water (n=2579) from 77 study sites (some papers reported data from more than one site, so the number of study sites is larger than the numbers of screened papers) belonging to four different climate zones of the world, according to Köppen classification (Peel et al., 2007) (Figure 2.1 and Table S2.1). Groundwater was excluded from the analysis because only 28 papers (37 study sites) did report the groundwater data sampled either from wells or springs (later one considered as representative of groundwater at the study site by the authors of the paper). For each paper we collected additional information including the geographical coordinates of the study area, elevation, plant group or species, and soil depths reported by authors of the original papers. In case some of this information was missing, we obtained it through online resources. Coordinates were extracted via Google Earth, elevation via GPS Visualizer (Schneider, 2017), and the distinction between gymnosperms and angiosperms via The Plant list (2013).

To evaluate the effects of different environmental factors on the isotopic composition of soil and xylem water, we compiled the following information in a GIS environment: Mean Annual Temperature (MAT) in °C obtained from Esri ArcGIS online map derived from WorldClim version 1.4 (Hijmans et al., 2005) for the period 1950-2000 (30 arc seconds or approx. 1 km at equator); Global Aridity Index (UNEP, 1997) values extracted from a dataset provided by Consortium for Spatial Information (CGIAR-CSI) (Trabucco and Zomer, 2009) at 0.0083° spatial resolution. We chose to retrieve MAT and Global Aridity Index values of the study sites from global databases because not all the papers reported all the characteristics and we wanted to have a consistent database avoiding different classifications.

2.2.2 Statistical tests and direct inference method

To compare xylem water and soil water samples from the four climate zones we computed the deuterium-excess (d-excess) for each data point following (Dansgaard, 1964):

$$\mathrm{d-excess} = \delta D - 8 \times \delta^{18}O \qquad \text{(Eq. 1)}$$

d-excess is interpreted as an index for the characterization of non-equilibrium conditions during global evaporation-condensation process. We used d-excess instead of line-conditioned excess (Landwehr and Coplen, 2006) because Local Meteoric Water Lines and/or the isotopic

composition of local precipitation were not available for all the individual study sites. Therefore, for consistency in the data analysis we computed d-excess.

All extracted isotopic data of soil and xylem waters were tested for normality and revealed that the data were not-normally distributed (Shapiro-Wilk normality test, $p<0.01$). All subsequent statistical analyses to compare samples were thus performed using non-parametric tests. These included the Mann–Whitney rank sum test (Hollander and Wolfe, 1999) to compare the isotopic values of xylem water and soil water within each climate zone, and the Kruskal-Wallis one-way analysis of variance test to assess differences in the isotopic composition of xylem and soil waters across the four climate zones, plant groups, soil depths classes. Spearman rank correlation analyses were used to assess the strength of the correlation between MAT, elevation of the study sites and Global Aridity Index with $\delta^{18}O$ and d-excess of soil water (Table 2.2) and xylem water (Table 2.3).

We quantified the contributions of water sources (e.g., soil water at different depths) to root water uptake at the global scale using a new isotope-based direct inference method. The only assumption behind this method is that the isotopic signature of xylem water is conserved during the water uptake (i.e., no fractionation during transport) and reflects the contributions of the different water sources (Dawson et al., 2002; Dawson and Ehleringer, 1991). For each study site, we plotted all the data in a dual-isotope space, and drew ellipses based on 99% confidence intervals (Friendly et al., 2013) for each source (soil water at different depths and xylem water; Figure 2.2). For the determination of the ellipses parameters, we applied two algorithms to remove outliers, including the minimum volume ellipsoid (MVE; Van Aelst and Rousseeuw, 2009) and the minimum covariance determinant (MCD; Croux and Haesbroeck, 1999; Rousseeuw and Van Driessen, 1999) in order to provide information about the uncertainty of estimates. Furthermore, the application of the two different methods for outliers detection allowed for the analysis of the sensitivity of our results to the chosen algorithm. We then counted the number of xylem water samples falling in the intersection space (graphically overlapping) between the ellipse of the xylem water samples and the ellipses of the different potential water sources as follows:

$$overlap\ (\%) = \frac{n_{water\ source}}{n_{total} - n_{out}} \times 100 \qquad \text{(Eq. 2)}$$

where $n_{water\ source}$ represents the number of xylem water samples falling in the intersection space with a given water source, n_{total} represents the total number of xylem water samples, and n_{out} is the number of xylem water samples falling outside the xylem water ellipse (Figure 2.2). As sample size for some study sites was small, we considered only those water sources with a minimum of four data points for the application of our inference method. We found that applying this method when less than four data points were available led to biased results compared to the ones obtained when more than four data points were available. This reduced the number of study sites from 77 to 56 for the computation of the overlap of soil water and xylem water. The key difference between the new isotope-based direct inference method and more commonly used mixing models is that the contributions here do not add up to one (or 100%) of the water uptake. Hence, the results do not provide proportional water use directly, but instead offer a more transparent approach towards similarities between different source waters and xylem water. As it eliminates issues with potentially unsampled water sources and the uncertainties associated with best-solution mixing models, comparisons between sites are therefore more straightforward.

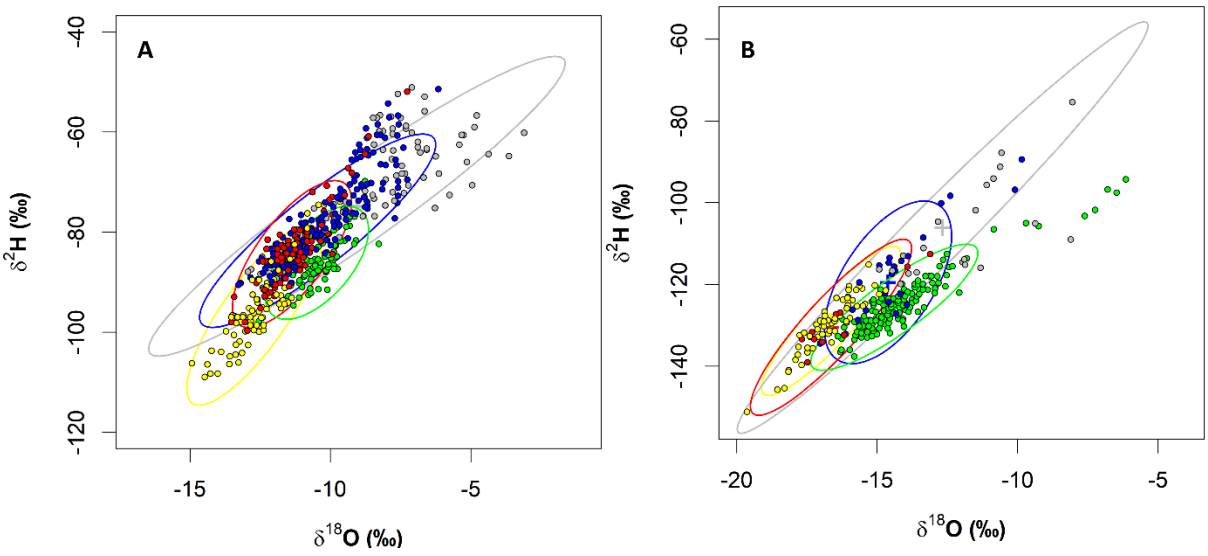


Figure 2.2. Examples of the computation of the degree of overlap between xylem water (green 99% confidence ellipse), 0-10 cm soil water (grey ellipse), 10-30 cm soil water (blue ellipse), 30-50 cm soil water (red ellipse) and > 50 cm soil water (yellow ellipse) plotted in the dual isotope space. Study site A (left panel, xylem water: n=93; 0-10 cm: n=85, 10-30 cm: n=160; 30-50 cm: n=85, and > 50 cm: n=77) and study site B (right panel, xylem water: n=137; 0-10 cm: n=21, 10-30 cm: n=24; 30-50 cm: n=19, and > 50 cm: n=58) respectively.

2.3 Results

2.3.1 Isotopic composition of xylem and soil waters in different climate zones

Soil and xylem waters had a broad isotopic variability in all four climate zones (Figure 2.3). The median isotopic compositions of soil and xylem waters were very enriched in heavy isotopes in the tropical zone (δ^2H=-34.7‰ (soil), -28.1‰ (xylem); $\delta^{18}O$=-5.20‰ (soil), -3.88‰ (xylem)), and the most depleted in the cold zone (δ^2H=-78.2‰ (soil), -95.6‰ (xylem); $\delta^{18}O$ =-10.27‰ (soil), -11.74‰ (xylem)) (Table S2.2). Soil and xylem waters in the arid and the temperate zones had similar median isotopic compositions, and intermediate between the tropical and the arid zones. The isotopic composition of xylem water reflected that of soil water quite well in all climates, especially in the tropical zone (Figure 2.3 and Figure S2.1).

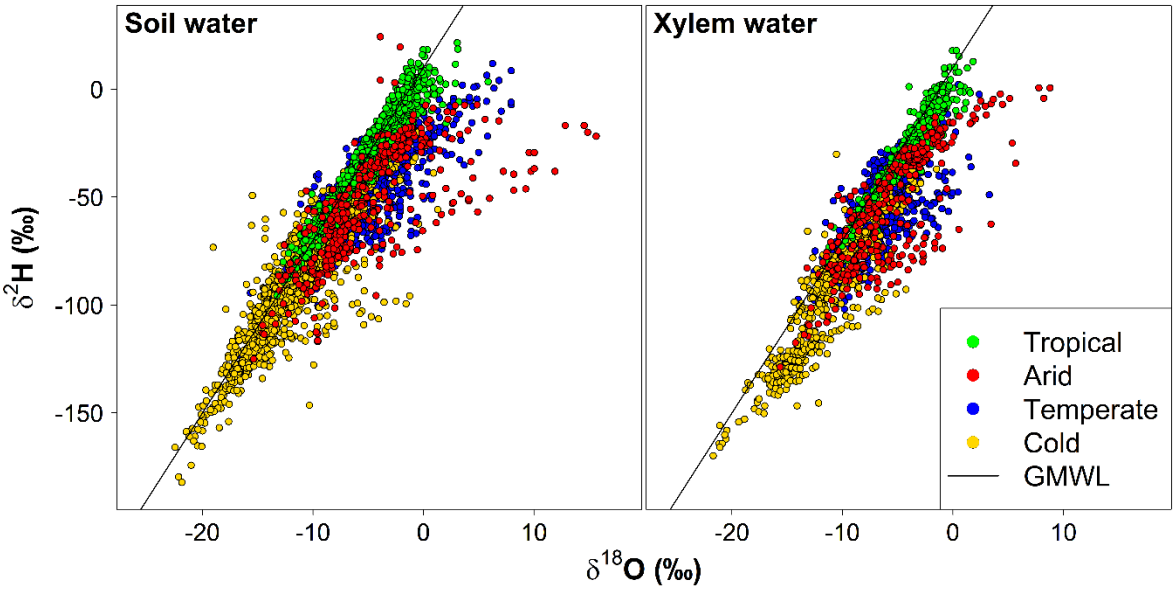


Figure 2.3. Dual-isotope plot of soil water (tropical, n=1284; arid, n=383; temperate, n=2075; cold, n=1586) and xylem water (tropical, n=442; arid, n=308; temperate, n=1237; cold, n=592) samples in different climate zones according to Köppen climate classification.

Deviations of soil water from the GMWL were particularly large in the arid zone, followed by the temperate and cold zone, and very small in the tropical zone. In addition, in the temperate and the arid zones, soil water was often more evaporated than xylem water, particularly for soil water samples which were very enriched in heavy isotopes (Figure 2.3 and Figure S2.1). The deviation of soil water from the GMWL was related to the sampling depth, but there were differences across the climate zones (Figure 2.4). In the tropical zone, all the soil water samples

plotted together along the GMWL, except for few samples of soil water at the 0-10 cm depth probably affected by evaporation (very low d-excess values). In the temperate and the cold zones, soil water sampled at 30-50 cm and >50 cm depths plotted together and quite close to the GMWL, while shallower soil water tended to deviate more from the GMWL. In the arid zone, the soil water sampled at >50 cm depth was the least affected by evaporation, while almost all the soil water from shallower layers plotted well below the GMWL (Figure 2.4). However, some discrepancies were observed due to the isotopic variability among the study sites.

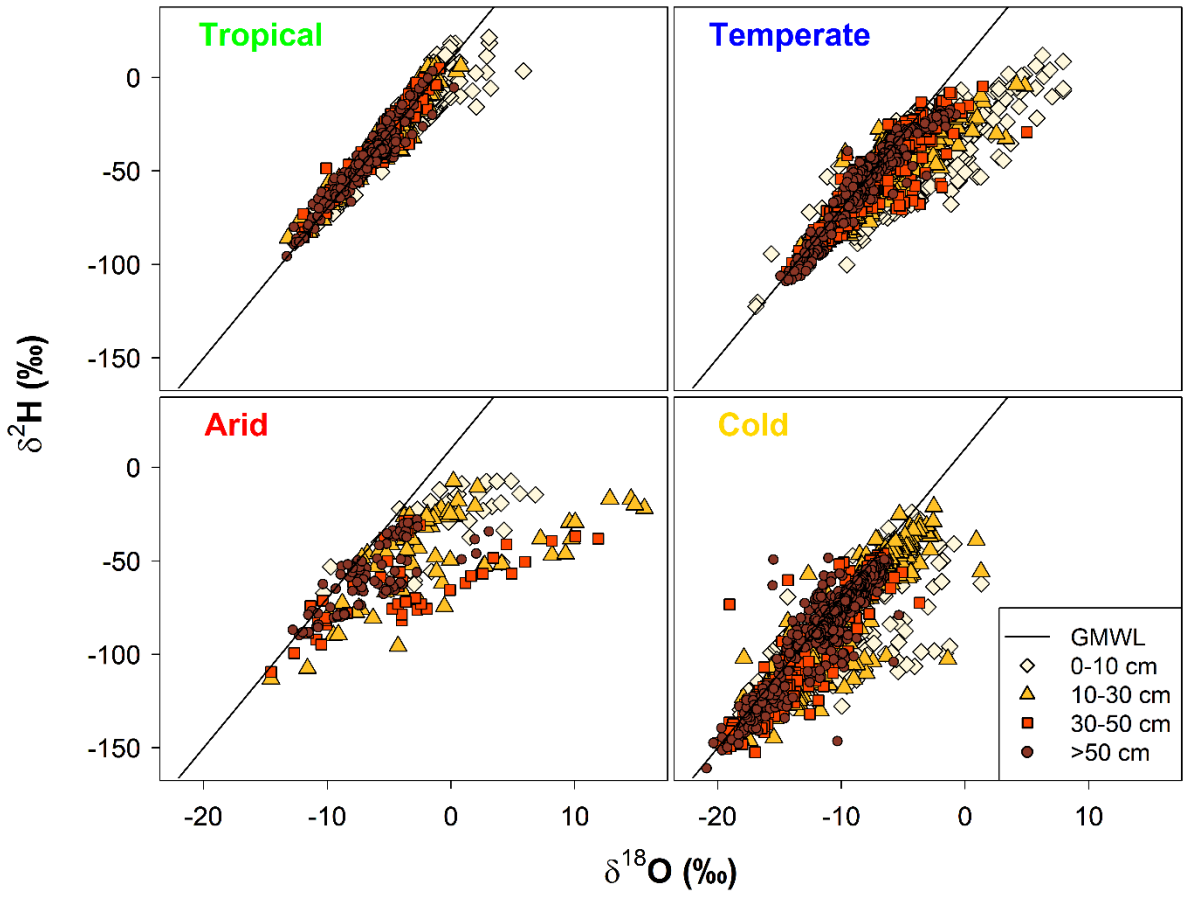


Figure 2.4. Dual-isotope plot of soil water at different depths in different climates, i.e. tropical (0-10 cm, n=294; 10-30 cm, n=296; 30-50 cm, n=281; >50 cm, n=323), arid (0-10 cm, n=39; 10-30 cm, n=70; 30-50 cm, n=54; >50 cm, n=94), temperate (0-10 cm, n=583; 10-30 cm, n=387; 30-50 cm, n=511; >50 cm, n=335) and cold zone (0-10 cm, n=236; 10-30 cm, n=393; 30-50 cm, n=231; >50 cm, n=684).

We found a significant difference in the isotopic composition (for both isotopes) of soil water at different depths separately tested for the four climate zones (Kruskal-Wallis one-way analysis of variance test, $p<0.001$ and $p<0.001$ for both δ^2H and $\delta^{18}O$).

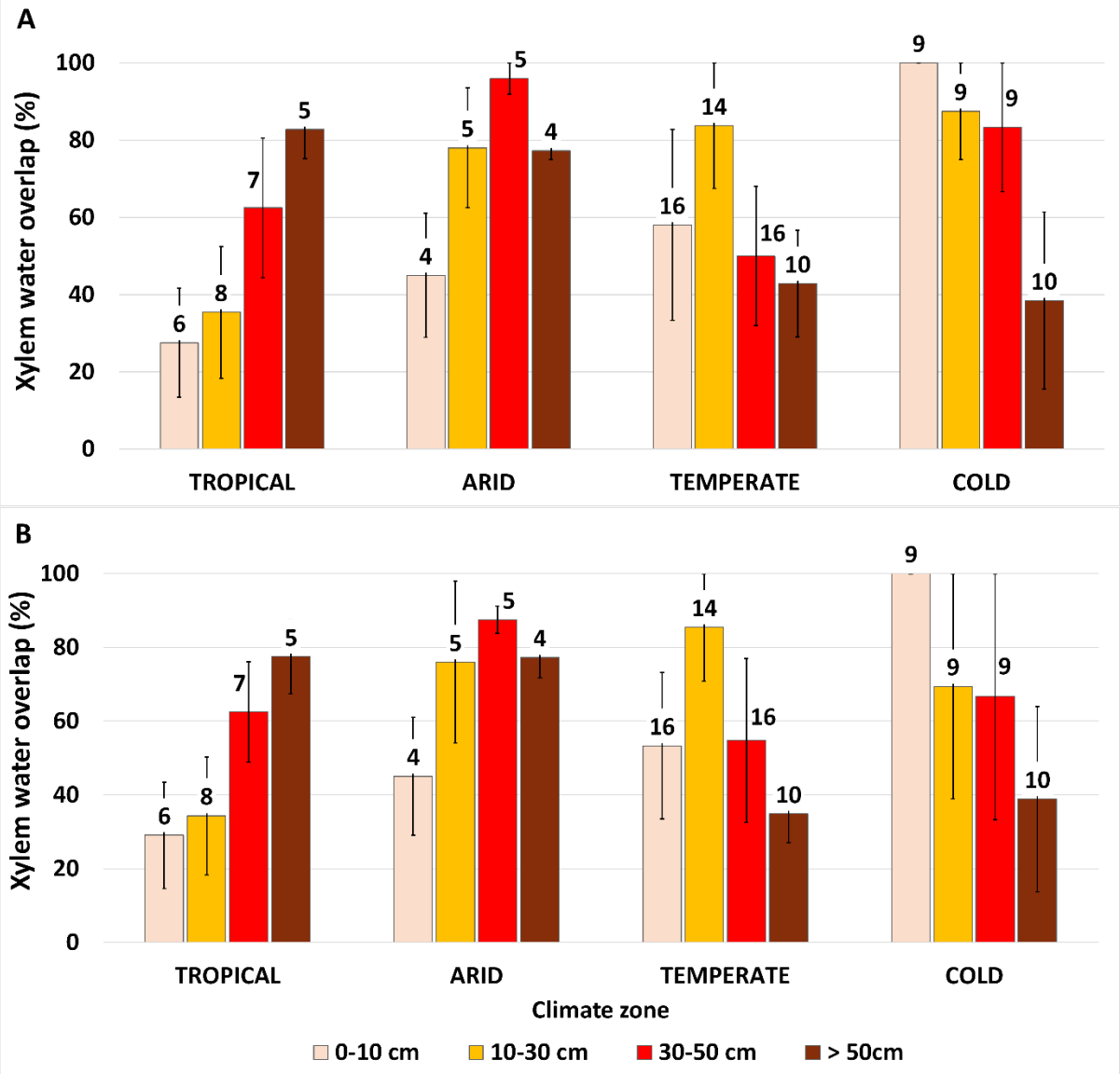
Table 2.1. Median d-excess (‰) of xylem water and soil water for different plant groups and soil water depths in different climate zones. Number of samples are reported in parentheses.

	Tropical	**Arid**	**Temperate**	**Cold**
Xylem water				
Angiosperms	4.3 (407)	-4.1 (196)	-1.4 (625)	-6.4 (395)
Gymnosperms	-	-16.7 (112)	0.5 (587)	-0.7 (197)
Soil water				
0-10 cm	6.1 (294)	-27.6 (39)	-2.7 (583)	-1.5 (236)
10-30 cm	8.9 (296)	-18.4 (70)	3.1 (387)	1.1 (393)
30-50 cm	9.4 (281)	-10.0 (54)	6.3 (511)	4.1 (231)
> 50 cm	9.3 (323)	-2.6 (94)	7.6 (335)	4.4 (684)

2.3.2 Overlap between xylem water and water sources

The dual-isotope plots in the four climate zones highlighted the large overlap between the isotopic composition of soil water and xylem water (Figure 2.3 and Figure S2.1). The median overlap between xylem water and soil water (Figure 2.5) at 0-10 cm depth decreased from the cold zone (100±0%, representing median±median absolute deviation, for the computations with both the MCD and MVE algorithms) to the temperate (58±25% and 53±20% for MCD and MVE, respectively), the arid (45±16% for both MCD and MVE) and the tropical zone (28±14% and 29±14% for MCD and MVE, respectively). The median overlap between xylem and soil waters at 10-30 cm varied between 35±17% using MCD (or 34±16% for MVE) in the tropical zone and 84±16% for MCD (85±15% for MVE) in the temperate zone. The median overlap between xylem and soil waters at 30-50 cm depth was particularly large in the arid zone (96±4% for MCD, 88±4% for MVE), while the overlap between xylem and soil waters at >50 cm depth was the largest in the tropical zone (83±8% for MCD, 77±10% for MVE) followed by the arid climate (77±2% for MCD, 77±6% for MVE).

These results suggest that the isotopic composition of xylem water tends to be very similar to that of shallow soil water (0-10 and 10-30 cm depths) in the temperate and the cold zones, while in the arid and the tropical zones the isotopic signature of xylem water reflects more the composition of deep soil water (30-50 and >50 cm depths).



Climate zone	No. of xylem samples	No. of soil samples at 0-10 cm	No. of soil samples at 10-30 cm	No. of soil samples at 30-50 cm	No. of soil samples > 50 cm
TROPICAL	406	289	296	287	321
ARID	254	36	67	51	85
TEMPERATE	1105	577	380	506	329
COLD	437	233	391	227	688

Figure 2.5. Median overlap (%) of xylem water with soil water at different depths (0-10 cm, 10-30 cm, 30-50 cm and > 50 cm) in different climate zones. The number of samples considered for each climate zone is shown in a table given below the bar plot. The number reported above each bar indicates the number of study sites. Error bars represent median absolute deviations. Robust covariance ellipses by using two methods for outliers detection: A) minimum covariance determinant (MCD), B) minimum volume ellipsoid (MVE).

The median absolute deviations of the overlaps were quite variable across the climate zones and water sources (Figure 2.5). They were quite large especially in the cold zone, indicating a high variability in the overlap across the few study sites. However, the variability in the overlap across the study sites did not significantly affect the overall interpretation of the strong similarity in the isotopic composition of xylem water and soil water and the differences found across the climate zones. The differences in the overlaps between xylem water and soil water at different depths resulting from the application of the two algorithms (MCD and MVE) varied between -1% and +1% for 61% of the cases, suggesting that there was a small sensitivity of the results to the choice of the algorithm used for detecting outliers and drawing the ellipses.

2.3.3 Variability in the isotopic composition of xylem and soil waters among different climates

We found a significant difference in the isotopic composition (for both δ^2H and $\delta^{18}O$) of xylem and soil waters across the four climate zones (Kruskal-Wallis one-way analysis of variance test, $p<0.001$ and $p<0.001$ for both δ^2H and $\delta^{18}O$ of xylem water and soil water, respectively) (Figure 2.3).

The median d-excess of soil water increased (i.e. became less negative) with increasing soil depth in all climate zones (Table 2.1). Except for the tropical zone, the soil water at 0-10 cm depth had a negative median d-excess, while the deeper soil water had a positive median d-excess in all the climates, but the arid zone. This indicates that evaporative fractionation processes were strongest in the arid zone, where even the median d-excess of soil water at >50 cm depth was negative and lower than the median d-excess of shallow soil water in the tropical and the cold zones (Table 2.1).

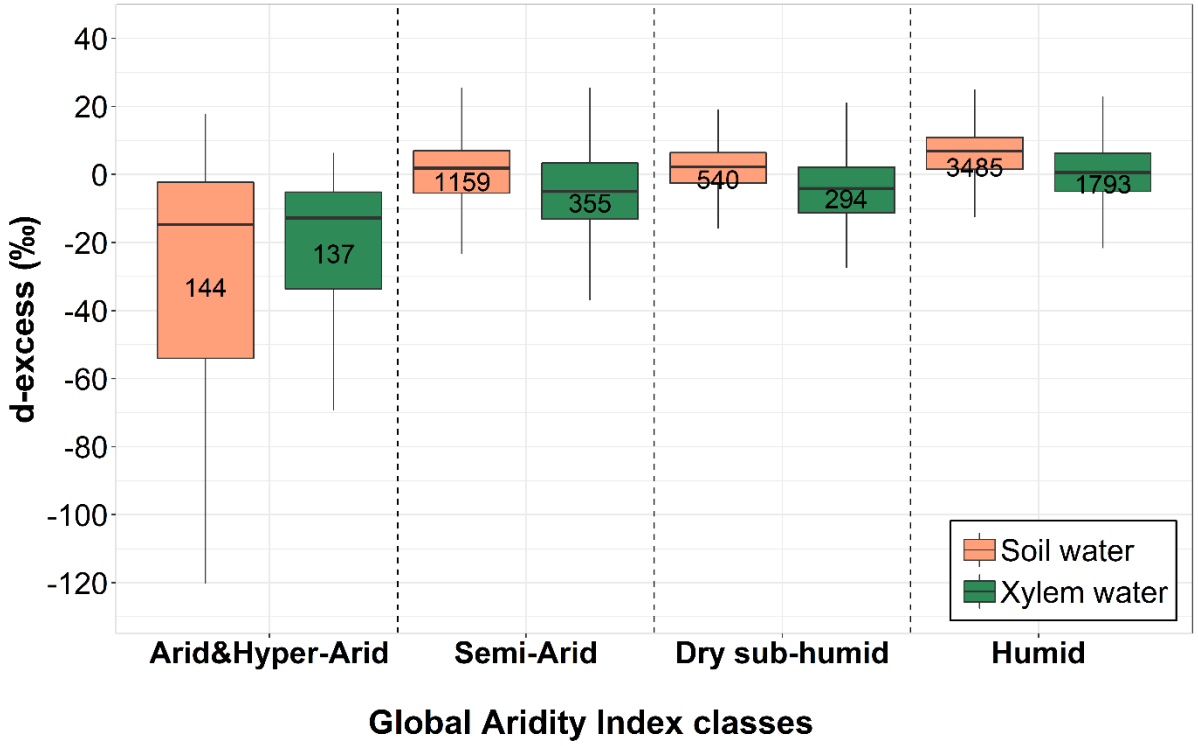


Figure 2.6. Boxplots of soil water and xylem water d-excess grouped as a function of Global Aridity Index classes (Aridity Index (*UNEP, 1997*); aridity index values increase for more humid conditions and decrease with more arid conditions). Boxes represent the 25th and 75th percentiles, while whiskers indicate the minimum and maximum values excluding the outliers. The number inside each box indicates the sample size. The horizontal solid line within boxes represents the median.

The classification of the study sites based on the Global Aridity Index confirms that xylem and soil waters in the most arid study sites had the most negative d-excess, suggesting the influence of relatively stronger evaporation processes (Figure 2.6). An overall increasing trend in d-excess was observed for both xylem and soil waters from the arid&hyper-arid class to the humid class. Xylem water had lower d-excess than soil water in most of the Global Aridity Index classes. However, for the arid&hyper-arid class, where sample size was smaller and variability generally larger, this pattern was not observed. Only in the humid class more than 50% of soil water and xylem water samples had positive d-excess values (Figure 2.6).

Table 2.2. Spearman rank correlation coefficient for the relation between characteristics of the study sites and $\delta^{18}O$, $\delta^{2}H$ and d-excess of soil water (no. of soil water samples=5328). All the correlations are significant ($p < 0.001$).

	$\delta^{18}O$ **(‰)**	$\delta^{2}H$ **(‰)**	**d-excess (‰)**
MAT (°C)	0.58	0.67	0.16
Elevation (m a.s.l.)	-0.38	-0.49	-0.15
Global aridity index	0.17	0.31	0.36

Table 2.3. Spearman rank correlation coefficient for the relation between characteristics of the study sites and $\delta^{18}O$, $\delta^{2}H$ and d-excess of xylem water (no. of xylem water samples=2579). All the correlations are significant ($p < 0.001$).

	$\delta^{18}O$ **(‰)**	$\delta^{2}H$ **(‰)**	**d-excess (‰)**
MAT (°C)	0.65	0.73	0.28
Elevation (m a.s.l.)	-0.39	-0.49	-0.27
Global aridity index	0.12	0.25	0.35

Both soil water (Table 2.2) and xylem water (Table 2.3) $\delta^{2}H$ and $\delta^{18}O$ values were strongly positively correlated with mean annual temperature. $\delta^{2}H$ and $\delta^{18}O$ of both xylem and soil waters also had a significant negative correlation with elevation of the study sites (Table 2.2 and Table 2.3), and a positive but weaker correlation with the Global Aridity Index.

d-excess of soil and xylem waters had the strongest positive correlation with the Global Aridity Index (Table 2.2 and Table 2.3), confirming that evaporative fractionation was stronger in the arid than the humid study sites. MAT and elevation also had significant correlations with d-excess of soil water (Table 2.2) and xylem water (Table 2.3), but they were weak particularly for soil water.

2.3.4 Variability in the isotopic composition of xylem water for different plant groups

The isotopic compositions of xylem water in angiosperms and gymnosperms were similar in the cold zone (Mann-Whitney rank sum test, $p>0.05$ for both $\delta^{18}O$ and $\delta^{2}H$). We found a significant difference in the isotopic composition of xylem water of angiosperms and

gymnosperms in the arid zone (Mann-Whitney rank sum test, $p<0.001$ for both δ^2H and $\delta^{18}O$) and the temperate zone (Mann-Whitney rank sum test, $p<0.001$ for $\delta^{18}O$ and $p<0.05$ for δ^2H). The deviation of xylem water samples from the GMWL was evident and different for angiosperms and gymnosperms in the arid zone, while xylem water samples of angiosperms and gymnosperms plotted together and deviated similarly from the GMWL in the temperate and the cold zones (Figure 2.7). The median d-excess was very negative for xylem water samples in the arid zone, with lower values for gymnosperms compared to angiosperms (Table 2.1). In the temperate zone, the median d-excess of xylem water was slightly lower for angiosperms than gymnosperms, while in the cold zone, stronger evaporation processes determined the lower median d-excess for angiosperms than gymnosperms.

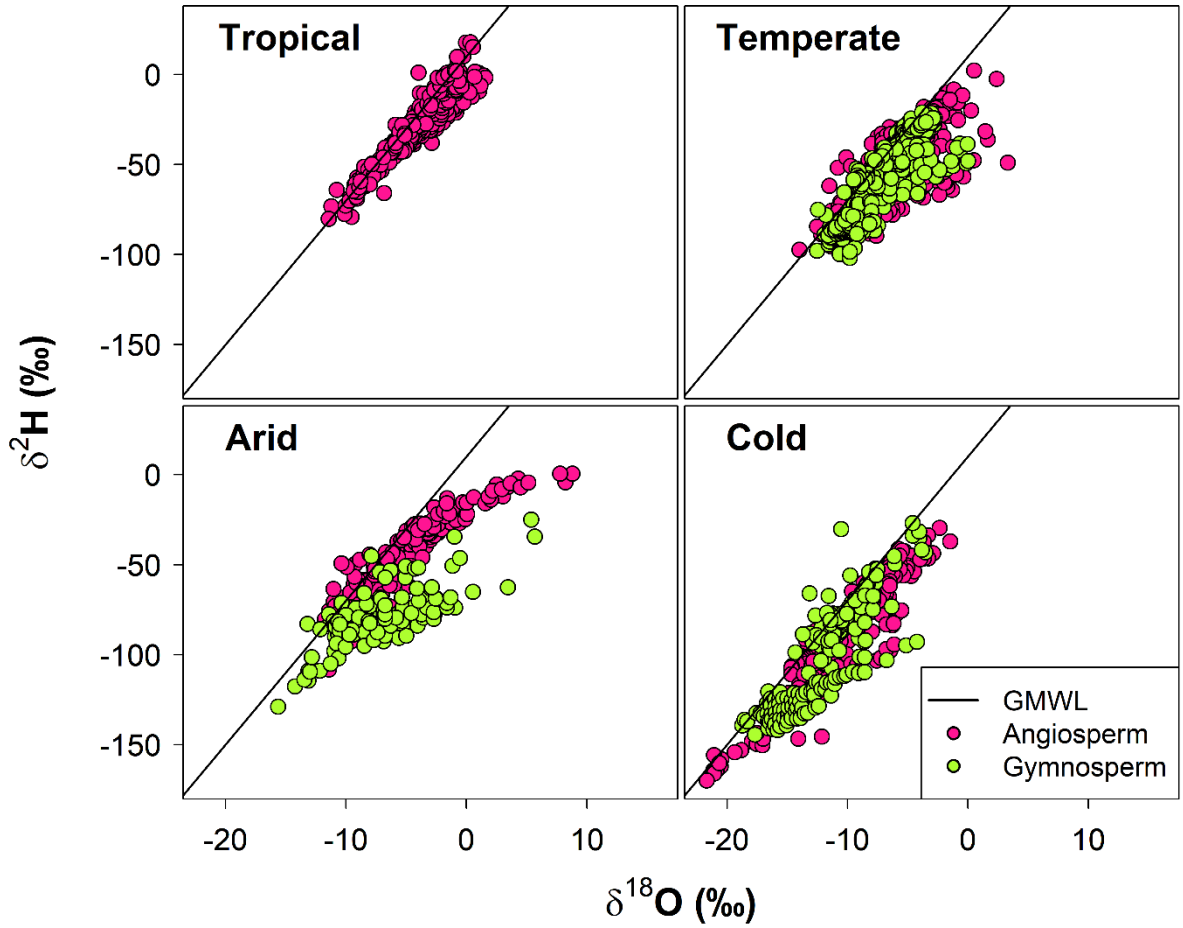


Figure 2.7. Dual-isotope plot of xylem water for different plant groups in the different climate zones. Tropical zone, angiosperms: n=407. Temperate zone, angiosperms: n=625; gymnosperms: n=587. Arid zone, angiosperms: n=196; gymnosperms: n=112. Cold zone, angiosperms: n=395; gymnosperms: n=197.

We found statistically significant differences in the d-excess of xylem water of angiosperms and gymnosperms in all the three climates (Mann-Whitney rank sum test, $p<0.001$ in the arid and the temperate zones, $p<0.01$ in the cold zone). Our choice in grouping of plants did not allow for exploring their role in the tropics as all the samples contained data for angiosperms only (Figure 2.7 and Table 2.1).

2.4 Discussion

2.4.1 Xylem water isotopic composition reflects soil water uptake from different depths across the globe

Dual-isotope plots (Figure 2.3) and the direct inference approach (Figure 2.5) revealed a strong overlap of xylem water and soil water isotopic composition across climate zones. This implies that trees and shrubs across the globe obtain most of their water from soil water. It has to be noted that our findings might be slightly biased towards soil water uptake as groundwater data were not considered in our study (Figure 2.5). However, our findings are in line with previous studies and meta-analyses showing that trees across most climate zones predominantly rely on soil water (Bowling et al., 2017; Brooks et al., 2010; Evaristo et al., 2019; Geris et al., 2017; Grossiord et al., 2016; Gu et al., 2015; Rose et al., 2003; Rossatto et al., 2012; Wei et al., 2013; Yang and Fu, 2017).

Our results also suggest that trees take up most of their water from the upper soil layers (here: 0-50 cm, Figure 2.5). Despite strong fluctuations in soil water availability, several studies have shown that trees obtain a considerable proportion of water from shallower soil layers (Barnard et al., 2010), although the extent of this proportion is highly variable depending on tree species, soil type and environmental conditions. The reliance on water from upper soil layers has been related to higher nutrient availability (Goldsmith et al., 2012; Schwendenmann et al., 2015) and root biomass (February and Higgins, 2010; Jobbágy and Jackson, 2001) in the upper soil layers and rehydration of upper soil due to hydraulic lift (often termed as hydraulic redistribution) under dry conditions (Caldwell et al., 1998). The vertical mixing due to uplift is obvious in our dataset where the soil water at depths (i.e., 10-30 cm, 30-50 cm and >50 cm) tends to be similar isotopically except the topsoil layer which seems to be enriched in all the climate zones (Figure 2.4). However, some discrepancies were observed due to the isotopic variability among the study sites especially in the arid climate zone.

Studies have shown that plant sustain the root water uptake via the hydraulic redistribution of water in unsaturated zone during the dry conditions and due to soil water passive transport through the rooting system following a hydraulic gradient (Richards and Caldwell, 1987). However, it is not possible to quantify this hydraulic redistribution volume based only on the isotopes (Emerman and Dawson, 1996), so the measurements of matric potential have been applied to show the amounts of water moved by the hydraulic redistribution. For instance, Kurz-Besson el al. (2006) found a redistribution of 17 to 18% of daily transpiration water in Mediterranean oak species. Similarly, a redistribution of 247 litres/day by a dry savannah adapted tree species (Bayala et al., 2008) has been reported. Jackson et al. (1999) found a shift in the isotopic signal of soil water at 0.5 m depth toward the deep soil water and explained this shift by hydraulic lift, since such shift was missing in bare soil. Other studies by Kulmatiski et al. (2010) and Brooks et al. (2006) have also shown a horizontal hydraulic redistribution of water in sprinkling experiments using the deuterated water.

To minimize energy use, plants are likely to extract water from soil layers with highest rooting density assuming the soil is uniformly wet (Adiku et al., 2000), and at the highest available water potential (i.e., easiest to withdraw; Gardner, 1960). Moreover, plants can often take up relatively "new" water (Sprenger et al., 2019) although some recent studies showed that water transpired from trees during summer originated from rain that fell during the previous winter (Allen et al., 2019; Brinkmann et al., 2018).

In line with other studies, our findings show that plants extract water from multiple sources, including different soil layers (Figure 2.5) (Asbjornsen et al., 2008; Le Roux et al., 1995; Schwendenmann et al., 2015). Water uptake from deeper soil layers (here: below 50 cm, Ma and Song, 2016) is often reported from arid/semiarid regions (Evaristo and McDonnell, 2017b) but is also found in areas characterised by pronounced dry seasons (Barbeta and Peñuelas, 2017). For example, deep rooting (Davidson et al., 2011; Markewitz et al., 2010) and deep soil water extraction in tropical forests are important mechanisms to sustain growth during the dry season (Restrepo-Coupe et al., 2013; Wu et al., 2016). A number of studies suggest that deep root systems are not restricted to trees and shrubs in arid/semiarid and seasonally dry forests (e.g., Pierret et al., 2016). Thus, the role of deep root water uptake across ecosystems may have been underestimated (Pierret et al., 2016). Studies have shown that in very deep soil, some trees can develop deep roots that may access "old" waters (Zhang et al., 2017; Sprenger et al., 2019).

2.4.2 Climate has first order effect on the isotopic compositions of xylem and soil waters

The observed major control exerted by climate on the isotopic composition of xylem water and soil water confirmed several ecological studies across different regions (see Werner et al., 2012, and references therein). This was evident via climate zone grouping (Figure 2.3-2.5), and exploring variations in isotopic signatures with climate forcing proxies including aridity index (Figure 2.6) and mean annual temperature (Figure S2.2) and elevation (Table 2.2 and Table 2.3; Figure S2.3). Stable water isotope composition of precipitation is strongly related to altitude, temperature and other climate factors (Dansgaard, 1964; Gat, 1996). Therefore, to some extent, the relative differences between isotopic signatures in the various climates found in xylem water and soil water samples reflected those in precipitation. d-excess values for soil and xylem waters both decreased as a function of aridity index values, whereby most negative d-excess values were found in the arid climate zone while least negative or even positive values were found in humid regions (Figure 2.6; Table 2.2). High potential evapotranspiration, relatively low soil water content (Allison et al., 1983a) and low relative humidity (Cappa et al., 2003; Gibson et al., 2008) are all factors that enhance non-equilibrium fractionation during evaporation and are typically most pronounced for arid regions.

The evaporation front in the soil profile was also markedly different between climate zones and again most distinct for the arid regions. Our results showed that overall, deep (>50 cm) soil water was less deviated from the GMWL (Figure 2.4). The d-excess profile changes with depth were most marked in the arid, then temperate and cold zone, while little variations were found for the tropical zone (Table 2.1). Using data from 25 sites across the world, Sprenger et al. (2016) revealed that the evaporative fractionation effects were generally limited to the upper 30 cm of the soil, but that this effect was climate dependent. Deep progression fronts up to 2-3 m have been reported for sites in arid climates (e.g., Beyer et al., 2018; Singleton et al., 2004), while in tropical climates a clear vertical gradient in the soil water isotopic signal is usually only observed under pronounced dry seasons (Querejeta et al., 2007). In tropical regions, the high humidity (Goller et al., 2005; Good et al., 2015) and typically dense vegetation cover (Dubbert et al., 2013) can both contribute to relatively low soil evaporation.

Xylem water in the arid and cold zones have lower d-excess (Table 2.1). This is consistent with Bertrand et al. (2014), Yang and Fu (2017) and Zhu et al. (2014) and values reported for the tropical and temperate zone (Goldsmith et al., 2012; Hervé-Fernández et al., 2016a; Rosado et al., 2013). The patterns between climate zones largely reflect those found in the soil water and indicate more fractionation with aridity. However, the result for the cold zone is quite different, with a more extreme difference between the soil and xylem water found in this region. As observed elsewhere and across climate zones (e.g., Evaristo et al., 2015) soil water often shows more fractionated isotope signatures than xylem water, with xylem water reflecting water uptake from a blend of sources. However, in our analyses for the cold climate zone, none of the soil water depths showed similarly strong negative median d-excess values as the xylem water (Table 2.1). In addition to possible improper sampling, i.e., not sampling the right water pool (Penna et al., 2018), one explanation could be that plant source water in cold regions might not be adequately represented by the soil water samples alone, or that soil water in cold climates is recharged by (non-fractionated, and isotopically light) snowmelt (Bowling et al., 2017; Maurer and Bowling, 2014). Isotope signatures in soil and xylem water always reflect the combined effects of source variation, mixing, and fractionation (Benettin et al., 2018). By bulking all soil water across depths and plant water across groups, some of the patterns may have also come out more extreme than as for a per-site comparison of samples across soil depth and plant groups.

2.4.3 Effect of plant group on isotopic composition of xylem water

We found significantly higher xylem water δ^2H and $\delta^{18}O$ values in angiosperms than gymnosperms in the arid zone (Table 2.1 and Figure 2.7). The most enriched xylem water across all studies was measured in *Guiera senegalensis*, a perennial woody shrub found across the Sahel (Brunel et al., 1997). Xylem water of *Guiera senegalensis* was often higher than the highest soil water δ^2H and $\delta^{18}O$ values (Brunel et al., 1997). Evaporative enrichment of xylem water has been associated with leaflessness (Ellsworth and Sternberg, 2015; Phillips and Ehleringer, 1995) and periods of limited sap flow (Martín-Gómez et al., 2017) which may partly explain higher xylem water δ^2H and $\delta^{18}O$ values in angiosperms across the arid zone.

Furthermore, differences in plant functional traits between angiosperms and gymnosperms (e.g., photosynthetic capacity, leaf phenology, transpiration rate, hydraulic capacity, water use efficiency, rooting pattern) (Augusto et al., 2014; Cernusak et al., 2008) may contribute to

differences in xylem water isotopic composition. For example, angiosperms tend to have higher leaves transpiration rates (Hetherington and Woodward, 2003) and are less drought resistant than gymnosperms (Choat et al., 2012). To meet the demand of water, plants in arid systems often rely on water from upper soil layers which is characterised by higher $\delta^{18}O$ and $\delta^{2}H$ values due to high evaporation especially during summer (West et al., 2007; Rose et al., 2003).

However, most studies show that water isotope composition and water uptake patterns tend to be species- and ecosystem- specific (e.g., Asbjornsen et al., 2008; Goldstein et al., 2008; Phillips and Ehleringer, 1995; Weltzin and McPherson, 1997; Williams and Ehleringer, 2000). For example, various gymnosperms growing in a woodland in southern Utah showed differences in their water uptake. *Pinus edulis* acquired water from both shallow and deep water sources, whereas the shrub *Juniperus osteosperma* used shallow water when water was available in the spring and shifted to deeper sources for the remainder of the growing season, and *Pinus taeda* obtained water predominantly from the upper soil profile (Retzlaff et al., 2001; West et al., 2007).

2.5 Limitations of the study

Our findings can be considered statistically robust due to the large number of samples (> 5000 for soil water and > 2500 for xylem water, Table S2.1) and of plant species (> 170) we considered. Nevertheless, we acknowledge some limitations that may impact the interpretation of the results. First, the study sites are unevenly distributed among the four climatic zones, with the highest number in the temperate zone and the smallest number in the tropical zone. Hence, some areas characterized by particular climatic conditions within the arid, tropical and cold zones might be underrepresented, and more studies would be necessary to include in order to make the global analysis of plant water uptake more generalizable.

Second, coexisting plant species may have different ecohydrological niches that we were not able to adequately represent in this study, other than simply separating angiosperms from gymnosperms. Similarly, different species, or even the same species but of different age, size and/or growing in diverse environmental conditions, are likely characterized by different root depths that might reach different soil depths and access different water sources, therefore hampering an equal comparison in the analysis of plant water uptake (e.g., Bargués Tobella et

al., 2017). However, the large sample size of our study may make the general pattern reasonably valid.

Third, most papers did not collect samples of soil and xylem waters at the same time, or did not specify the collection time: this limits the assessment of the possible differences between the isotopic composition of xylem water and of its potential water sources in the light of the lag time between root water absorption and transport to the leaves, that can take days to weeks or even months (e.g., Allen et al., 2019; Brinkmann et al., 2018). However, given the large number of samples taken from different species and different climate regions these possible differences are likely smoothed out.

Fourth, some issues intrinsic in meta-analyses, particularly at the global scale, exist, and they can limit more vigorous comparison of results. Of particular importance is the adoption of different sampling protocols for xylem water (e.g., samples collected from twigs or from the stem or from wood cores), and soil material (e.g., Goldsmith et al. (2019) showed heterogeneity of isotopic signal due to spatial variability of soil water samples) and different water extraction methods both for xylem water and soil water (Table 2.4). Several studies have reported that different water extraction techniques can return different isotopic composition from the same sample (see the comprehensive review by Millar et al. (2018) for plant water samples, and comments in Penna et al., 2018), and that even the same technique carried out in different laboratories can have a strong impact in determining the isotopic composition of soil water (Orlowski et al., 2018a). Therefore, uncertainties associated to the different water extraction techniques can possibly impact our findings. However, this uncertainty is difficult to quantify due to the variety of extraction methods (Table 2.4) and settings reported in the collected papers, and the lack, in many papers, of any information on the uncertainty related to the application of the extraction method, and, more in general, the lack of a common procedure. With these conditions, it is almost impossible to incorporate a reliable uncertainty estimate into the algorithms used in our direct inference approach (Figure 2.2 and Figure 2.5). Moreover, there might be other factors that could alter the isotopic composition of soil water and that we were not able to consider, often because not reported in the reviewed papers. For instance, nutrients tend to be concentrated in the upper soil and can lead to temporally plastic root water uptake behaviour and hence variations in the isotopic composition of xylem water even in

plants with access to groundwater (Dubbert et al., 2019). Organic matter can prevent or reduce soil evaporation and increase water retention (Ankenbauer and Loheide, 2017; Saxton and Rawls, 2006; Schoonover and Crim, 2015) and influence fractionation effect of soil water as well as soil microbial respiration (Stoll, 2014).

Table 2.4. Percentage of papers based on water extraction techniques.

Extraction technique	**No. of papers**	**Percentage (%) of papers**	**No. of samples per climate zone***			
			Tropical	**Arid**	**Temperate**	**Cold**
Azeotropic distillation	8	12.1	0	187	257	200
Cryogenic vacuum distillation	56	84.8	1726	504	2937	1724
Liquid-vapor equilibration	1	1.5	0	0	0	254
Pressure chamber	1	1.5	0	0	86	0

**The number of samples reported in the table are the sum of both xylem water and soil water samples.*

Finally, we must note that differences exist in the estimates of soil water uptake reported in the original studies included in the database and the estimates derived from our analysis. Differences are not surprising due to intrinsic uncertainty in the different methods applied. The mixing models typically used in the papers we analysed (e.g., IsoSource, MixSIAR) are based on the assumption that all water sources accessed by plant roots are adequately sampled and that the tracer signature is conserved through the mixing processes. However, in field studies, sampling all potential water sources is not always practical or possible, creating potential bias in the mixing model estimation of plant water source. The direct inference method we proposed here is a simple statistically-based method that we applied to compare all data included in the global database. Based on its assumptions, this approach has the advantage to quantitatively assess the contribution of each of the identified water sources even if one or more sources are missing. However, this approach relies on the assumption that no-fractionation occurs at the soil-root interface or within plant woody tissues which is being increasingly questioned (Barbeta et al., 2019). Hence, these results should be used with caution, and extended analyses

on various plant species and in different climatic contexts are needed to further test this method and to evaluate the differences compared to widely used mixing model results.

2.6 Concluding remarks and how to move forward

Previous global meta-analyses studies have assessed the relative contributions of soil water and groundwater used by various plant species, but have not yet provided estimates of soil water depth contributions to water uptake. This study evaluated the main water sources used by plants globally and explored the effect of climate, and plant group on water uptake variability. Our meta-analysis was based on the extraction of isotopic data (both δ^2H and $\delta^{18}O$) from 65 peer-reviewed papers published between 1990 and the end of 2017. The database included isotopic compositions of soil water and xylem water from 77 study sites belonging to four climate zones (i.e., tropical, arid, temperate and cold zone).

The analysis of dual-isotope plots showed that there was a wide overlap between the isotopic composition of xylem water with that of soil water, indicating soil water as the main water source for plant transpiration. We developed and applied a new direct inference method to quantitatively assess the overlapping proportions between xylem water and water sources potentially exploited by plants. The median overlaps between xylem and soil waters at different depths were generally above 50%. We also found that climate acts as the main driver of the isotopic composition of soil water. Our results suggest that the isotopic composition of xylem water tends to be very similar to that of shallow soil water (0-10 and 10-30 cm depths) in the temperate and the cold zones, while in the arid and the tropical zones the isotopic signature of xylem water reflects more the composition of deep soil water (30-50 and >50 cm depths).

The proposed new direct inference method to quantify overlaps between xylem water and various water sources has a high potential due to its intrinsic ease of application and because it is an information-based method that can be used to determine the main water sources exploited by plants for transpiration. However, future research should aim at testing the new direct inference method across more study sites and comparing it to other methods (e.g., Bayesian mixing models) for the quantification of the contribution of water sources to transpiration.

Finally, our research suggests that further ecohydrological research should be performed in tropical and arid zones because of the few studies published in these regions so far. Collection of soil water samples at different depths to connect in time and space to corresponding xylem

water samples is urgently needed to build a more robust dataset for future analysis of transport processes within the soil-plant-atmosphere continuum.

SUPPLEMENTARY TABLES AND FIGURES

Table S2.1. Basic information on the compiled database.

	Tropical	**Arid**	**Temperate**	**Cold**	**Total**
No. of papers	8	12	31	15	65*
No. of study sites	11	14	37	15	77

**The total number is different because one paper represents study sites in two different climate zones.*

Table S2.2. The mean, median and measures of dispersion of $\delta^{18}O$ and $\delta^{2}H$ values (expressed in ‰) of xylem water and soil water across different climatic zones. MAD: Median absolute deviation.

	SOIL WATER								**XYLEM WATER**							
Climate	**Tropical**		**Temperate**		**Arid**		**Cold**		**Tropical**		**Temperate**		**Arid**		**Cold**	
No. of samples	1284		2075		383		1586		442		1237		308		592	
Isotope	$\delta^{18}O$	$\delta^{2}H$	$\delta^{18}O$	$\delta^{2}H$	$\delta^{18}O$	$\delta^{2}H$	$\delta^{18}O$	$\delta^{2}H$	$\delta^{18}O$	$\delta^{2}H$	$\delta^{18}O$	$\delta^{2}H$	$\delta^{18}O$	$\delta^{2}H$	$\delta^{18}O$	$\delta^{2}H$
Mean	-4.97	-31.9	-7.12	-55.5	-4.77	-53.7	-10.75	-83.4	-3.88	-27.1	-6.34	-52.8	-5.35	-54.2	-11.57	-97.9
Median	-5.20	-34.7	-7.65	-55.4	-5.50	-55.4	-10.27	-78.2	-3.88	-28.1	-6.24	-51.2	-5.26	-55.0	-11.74	-95.6
Standard deviation	2.76	20.0	3.44	21.6	5.02	25.4	3.67	27.4	2.44	17.7	2.50	19.6	4.06	26.4	3.54	29.3
MAD	1.72	12.1	2.05	16.2	2.80	19.8	2.29	17.7	1.60	10.9	1.78	14.6	2.60	22.5	2.55	25.7

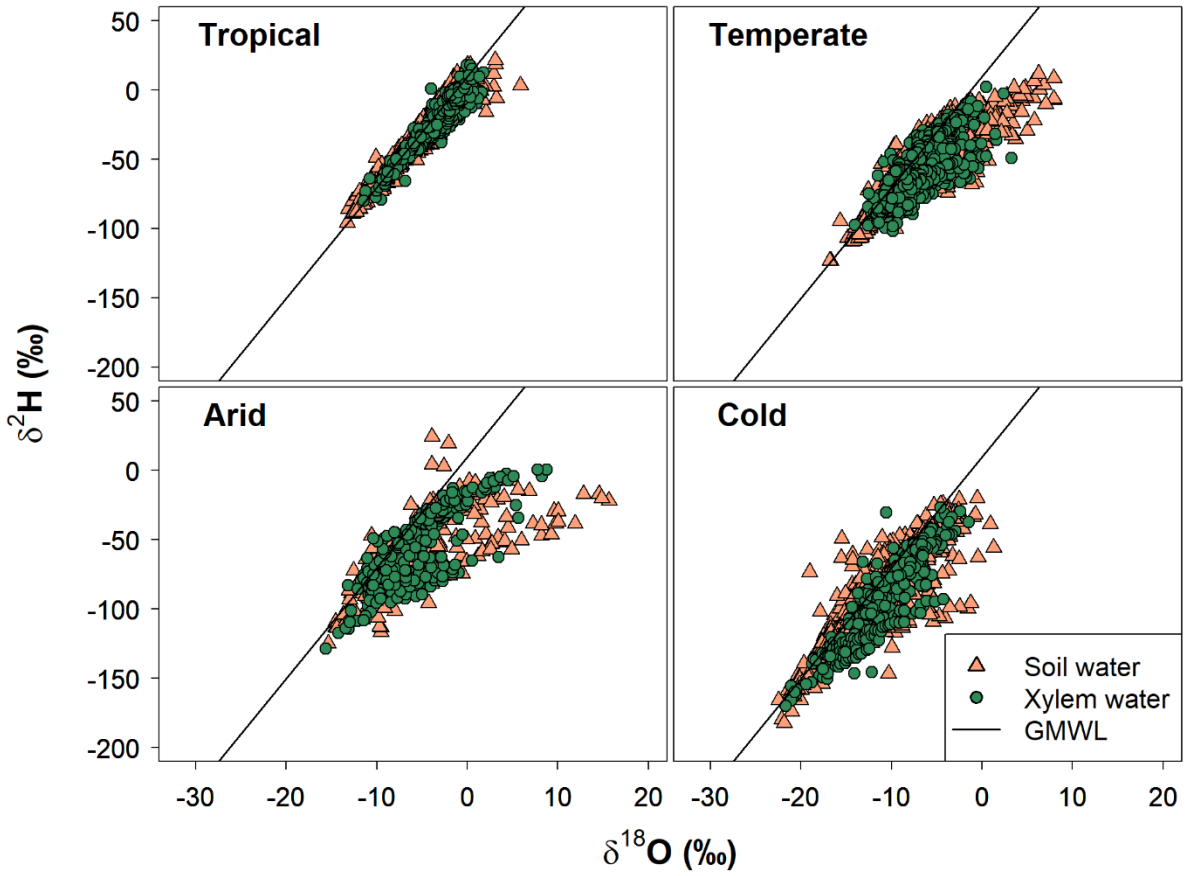


Figure S2.1. Dual-isotope plot of soil water (tropical, n=1284; arid, n=383; temperate, n=2075; cold, n=1586) and xylem water (tropical, n=442; arid, n=308; temperate, n=1237; cold, n=592) in different climate zones.

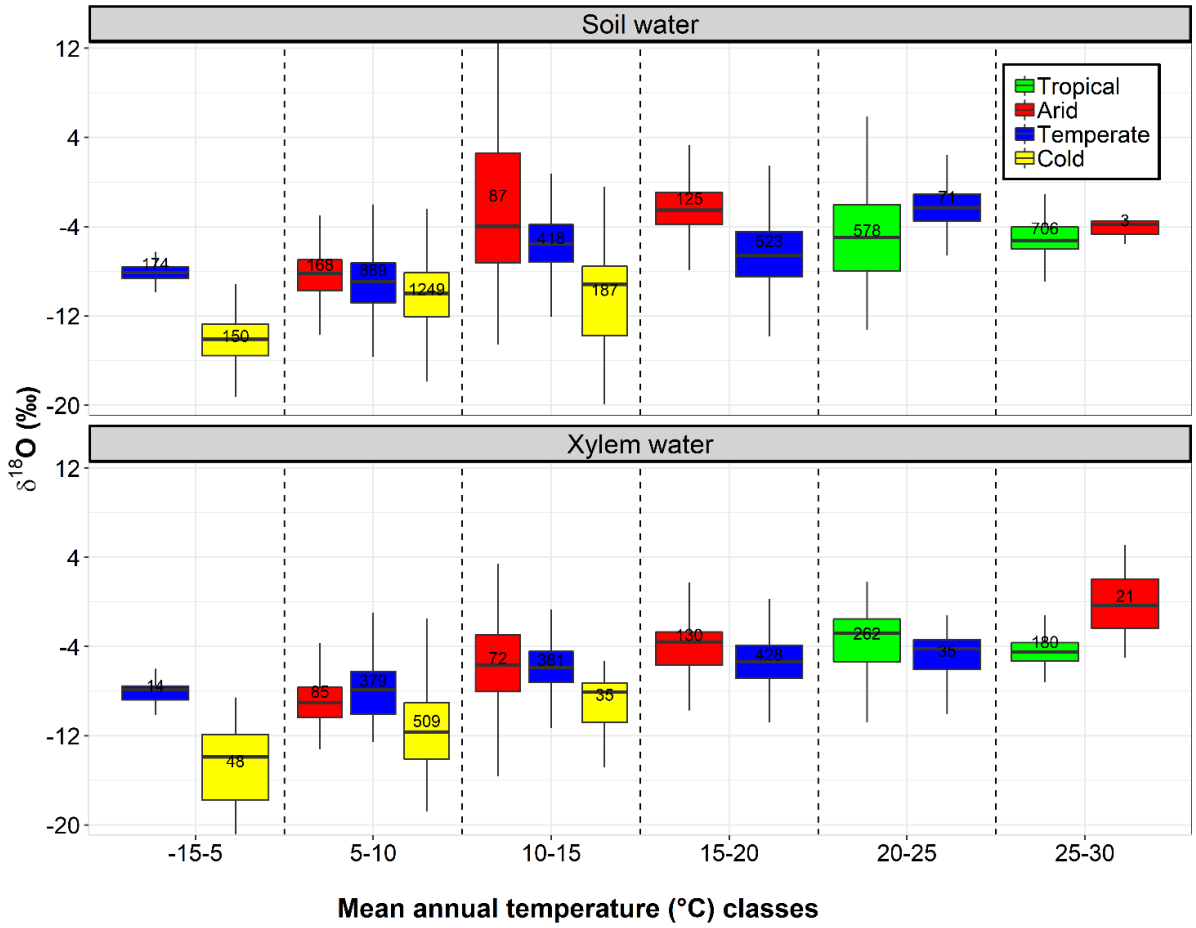


Figure S2.2. Boxplots of soil water and xylem water oxygen isotope composition of different climate zones grouped as a function of mean annual temperature where the colours correspond to the climate zone. Boxes represent the 25th and 75th percentiles, while whiskers indicate the minimum and maximum values excluding the outliers. The number inside each box indicates the sample size. The horizontal solid line within boxes represents the median.

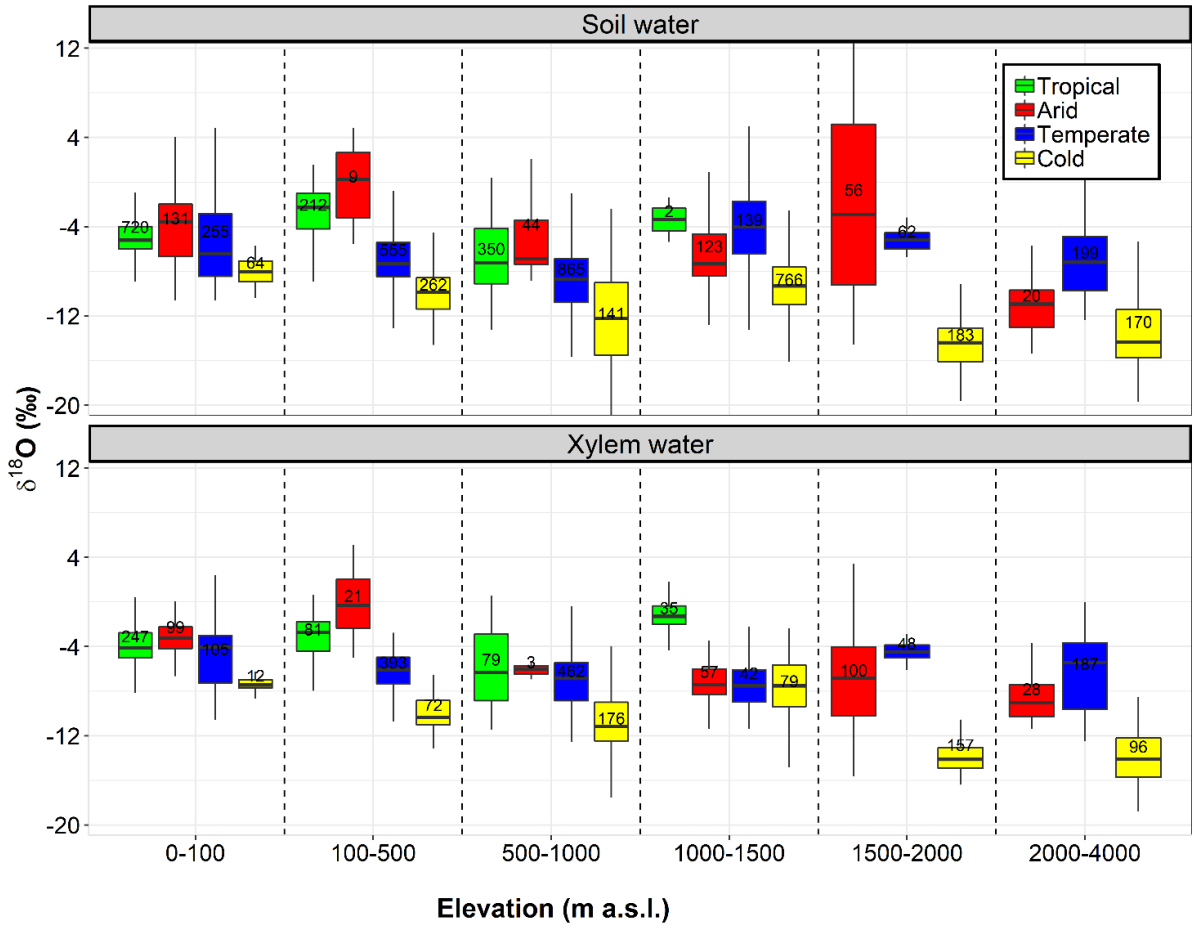


Figure S2.3. Boxplots of soil water and xylem water oxygen isotope composition of different climate zones grouped as a function of elevation (m a.s.l.) where the colours correspond to the climate zone. Boxes represent the 25th and 75th percentiles, while whiskers indicate the minimum and maximum values excluding the outliers. The number inside each box indicates the sample size. The horizontal solid line within boxes represents the median.

3 Isotope fractionation in plants: A greenhouse tracing experiment[2]

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3.1 Introduction

Stable water isotopes (^{2}H and ^{18}O) offer a unique opportunity to investigate water pathways and determine the water sources potentially used by plants for water uptake (Barbeta et al., 2019; Beyer et al., 2016; Hervé-Fernández et al., 2016; Orlowski et al., 2018b; Volkmann et al., 2016). Recently, many studies focused on the quantification of the proportion of soil water and groundwater exploited by plants for transpiration at the plot, catchment and global scale (e.g., Barbeta et al., 2019; Barbeta and Peñuelas, 2017; Dudley et al., 2017; Evaristo et al., 2019, 2016, 2015; Evaristo and McDonnell, 2017). Most of the studies applying stable water isotopes assume that isotopic fractionation does not occur during the water uptake by plants and along the pathway from the roots up to the leaves (Dawson et al., 2002; Dawson and Ehleringer, 1991). Isotopic fractionation is regarded as a change in the ratio of heavy to light isotopes of water relative to a set standard, which occurs during a phase change, i.e. from solid to liquid to vapor and contrariwise (Michener and Lajtha, 2008). Due to the difference in the physical properties of water isotopologues, isotopic fractionation naturally happens during the phase changes (e.g., evaporation and condensation), and its magnitude is determined by variations in temperature and humidity conditions (Gat and Matsui, 1991). At the soil-vegetation interface, isotopic fractionation during transpiration varies with the soil water content and type of the soil. As such, the soil drying due to direct evaporation and plant water uptake creates small gaps between soil and roots, leading the water with lighter isotopes to make their way to plants. Studies by Allison et al. (1983b) and Allison and Hughes (1983) suggested an alteration of soil water isotopic composition due to transpiration, and a recent study by Vargas et al. (2017) reported soil water enrichment (in ^{2}H and ^{18}O) due to plant preferential use of lighter isotopes (^{1}H and ^{16}O). Also, some plants related factors such as storage vessels, conduits dimensions and even the direct absorption of atmospheric water can affect the xylem water isotopic signature. The mixing of xylem-phloem fluids across membranes can also result in xylem water enrichment along stem, branches and young plant tissues (Cernusak et al., 2005; Dawson and Ehleringer, 1993; Thorburn et al., 1993).

Despite the large number of ecohydrologic studies using stable water isotopes, there is limited research testing the assumption of no or negligible isotopic fractionation in controlled or controlled vegetated environments (i.e., Dawson et al., 2002; Dawson and Ehleringer, 1993; Ellsworth and Sternberg, 2015; Ellsworth and Williams, 2007; Lin and Sternberg, 1993; Thorburn et al., 1993; Vargas et al., 2017; Walker and Richardson, 1991; Washburn and Smith,

1934; Zhao et al., 2016). Recently, Zhao et al. (2016) showed remarkable δ^2H differences (up to 26‰) between xylem sap, stem water, core water and root tissue water. Similarly, in a more recent work by Poca et al. (2019) tested the effect of mycorrhizal fungi on the xylem water isotopic signal and reporting a significant depletion for both δ^2H (up to -24.6‰) and $\delta^{18}O$ (up to -2.9‰) isotopes in xerophytic *Acacia caven*.

In addition, Ellsworth and Williams (2007) observed fractionation throughout the root water uptake pathway, e.g. 3 to 9‰ fractionation of δ^2H recorded at the soil-root interface in 12 xerophytic species. Lin and Sternberg (1993) in a work carried out under greenhouse hydroponic system found significant depletion of δ^2H of about 10 to 11‰ in stem water relative to the source water in salt excluding halophyte tree species and about 3‰ in salt secreting species (*A. germinans*). In contrast under field conditions none of the studied plants showed any significant oxygen isotope fractionation. They found a strong correlation of stem water δ^2H depletion (relative to water source) with plant growth occurring due to different environmental conditions (*R. mangle* spp.) (Lin and Sternberg, 1993).

Nowadays, potential issues are associated to the methods used for the extraction of soil and plant waters, which can alter the original isotopic composition of the sample (Penna et al., 2018). Despite the technological advancements in laser spectroscopy and water extraction methods, testing isotopic fractionation in soil and plant materials can still be challenging due to several problems, such as extraction times and temperatures leading to incomplete extraction (Orlowski et al., 2016a, 2013; Gaj et al., 2017b), physiochemical soil properties (Orlowski et al., 2018a) , soil water content (Oerter et al., 2014; Orlowski et al., 2018b), and correspondingly for plant materials, likelihood of coextraction of various organic compounds, which can later affect the isotopic analysis via laser spectroscopes (Millar et al., 2018; West et al., 2010). A clear example of these challenging issues is reported by Orlowski et al. (2018a) who carried out an interlaboratory comparison of soil water extraction methods finding that large differences observed in isotopic compositions of the extracted soil water among the laboratories involved in the comparison. The differences were linked to multiple factors, such as soil water content, soil type, extraction efficiency, system setup and accuracy, and to the isotopic analysis techniques (i.e. isotopic data obtained from IRMS and OA-ICOS respectively).

Particularly, when focussing on the extraction of xylem water from plant material, it must be noted that the extracted water may contain organic contaminations when the process occurs at high temperatures (Millar et al., 2018). Large amounts of organic compounds present in the xylem water extracts can potentially affect the isotopic measurements by laser spectroscopes (West et al., 2010). For instance differences observed in the isotopic values of the xylem water extracts obtained from the direct vapor equilibration and cryogenic vacuum distillation system (Millar et al., 2019, 2018). Despite the number of works comparing, testing new, or improving already established extraction methods (Fischer et al., 2019; Koeniger et al., 2011; Millar et al., 2019, 2018), we have not found research studies comparing the isotopic composition of plant waters extracted by various cryogenic vacuum distillation systems developed in different laboratories.

Here we present a greenhouse experiment based on the injection of isotopically - labelled water in the soil close to the root system of two olive plants (*Olea europaea*) to investigate fractionation of plant water along the transport pathway, from roots up to the twigs and to test the isotopic variability based on an interlaboratory comparison of widely used cryogenic water extraction system.
Plant water was extracted from different tissues (i.e., roots, twigs along branch and cores) of olive plants by means of cryogenic vacuum distillation performed in two different laboratories.

The specific research questions that we addressed were:

i) How much the isotopic composition of plant water varies at different locations along the stem/twigs within an olive plant?
ii) Does the isotopic composition of plant water undergo fractionation along the pathway from roots to leaves?
iii) Is the measured isotopic composition of plant water sensitive to water extraction process carried out using the same method (cryogenic vacuum distillation) in two different labs?

3.2 Materials and Methods

3.2.1 Set up of the experiment

Two 6-year-old olive plants (from here therein: OvA and OvB) with a 6-cm diameter and a 185-cm height were set in a greenhouse managed by Dept. of Land, Environment, Agriculture and Forestry of the University of Padova. The two plants were positioned in 70-L pots (Figure 3.1) containing a soil mix (80% of agriculture soil with a sandy loam texture and 20% pumice). Both pots were tightly covered with transparent plastic sheet to avoid evaporation from the soil surface. Three holes were made in each pot and covered with aluminium trays for recording daily soil moisture and irrigating the plants. Shallow soil moisture (at 0-6 cm depth) measurements were taken manually twice a day by a soil moisture probe (ML2x type 2 Thetaprobe, Delta-T devices Cambridge, UK) at three different locations in each pot. The two pots were also equipped with two suction cups installed at 15 and 30 cm depth to extract mobile soil water. Granier sensors were installed in the stem of the two plants to record sapflow continuously at a 5-min interval (Figure 3.1). Air temperature, relative humidity and global solar radiation were measured continuously at a 5-minutes interval by a weather station installed inside the greenhouse 1 m away from the two olive plants. Temporal dynamics of sapflow, air temperature, relative humidity and solar radiation were used to infer the evapotranspiration dynamics throughout the experiment.

The experiment lasted seven weeks (Table 3.1). The period was split into a 2-weeks conditioning period (starting on 14 May 2018) during which the plants were irrigated with tap water and a 5-week period where both plants were irrigated with isotopically labelled water (Table 3.2).

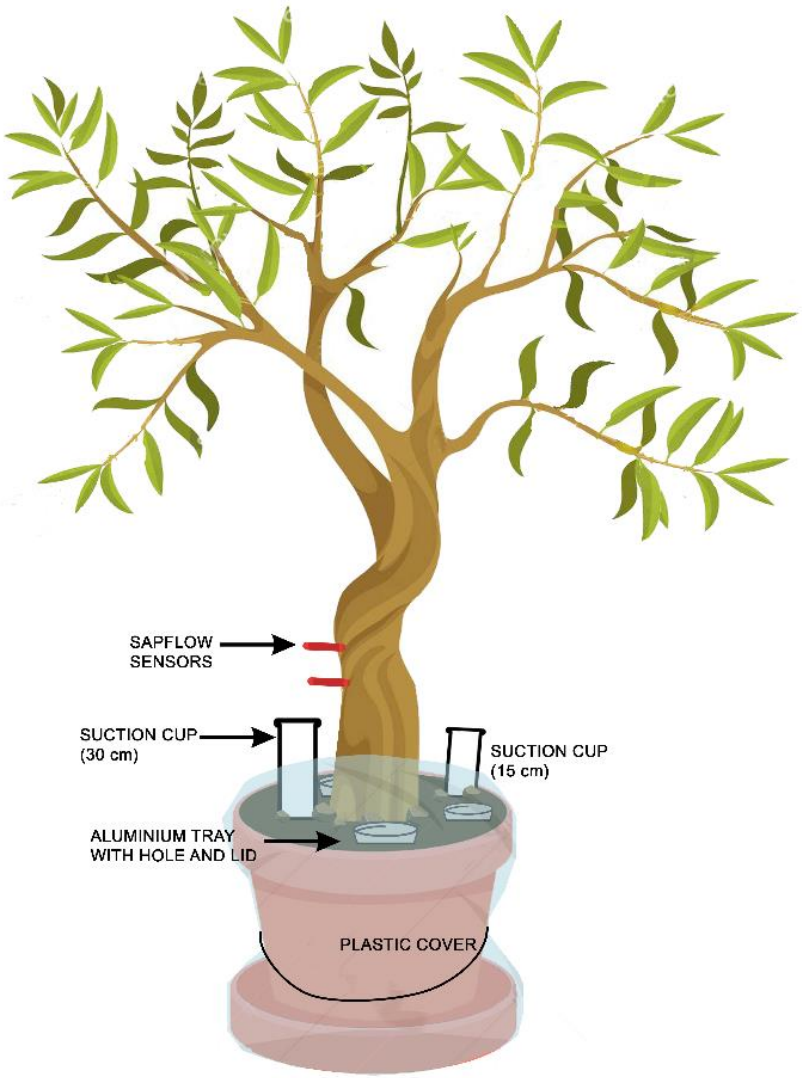

Figure 3.1. Sketch of the experimental setup for one olive plant with sapflow sensors, suction cups and holes for irrigation and soil moisture measurements.

3.2.2 The tracer experiment and sample collection

The two olive plants were irrigated with tap water during the set-up of the experiment (from late April until 13 May 2018) and the conditioning period (14-27 May 2018; Table 3.1). During the conditioning period the two olive plants were irrigated with 3 L of water every two day. On the first day of tracer experiment (28 May 2018), 3 L of isotopically labelled water was added to each pot, but, due to the high transpiration rates, the amount of water was increased from 3 to 5 L later-on. 5 L of labelled water was provided to each plant on Monday, Wednesday and then Friday each week until the end of the experiment. The total amount of water added to each plant per week was kept equal to 15 L/pot every week.

Table 3.1. Number of samples collected throughout the sampling rounds (R_0-R_6) during the set-up, the conditioning period and the experiment. Samples were grouped based on their type, olive plant (OvA and OvB) and laboratory for soil and plant waters extractions.

	Sample size							
	LAB 1				**LAB 2**			
Sampling rounds	**OvA**		**OvB**		**OvA**		**OvB**	
	Soil	**Plant**	**Soil**	**Plant**	**Soil**	**Plant**	**Soil**	**Plant**
Preliminary samplings	2	7	-	9	3	2	-	-
Conditioning period	7	5	7	5	7	7	5	5
Tracer experiment	22	31	21	21	24	33	23	22
Last sampling (*after cutting down plant OvA entirely*)	8	22	6	5	12	25	9	5
Total number of samples	**39**	**65**	**34**	**40**	**46**	**67**	**37**	**32**

Table 3.2. The isotopic compositions (mean±standard deviation) of tap water and isotopically labelled water used for irrigation.

	Sample size	$\delta^{18}O$ **(‰)**	$\delta^{2}H$ **(‰)**
Tap water	3	-7.97±0.8	-52.3±1.8
Labelled water	4	-12.75±0.5	-93.3±1.8

A first round of samples was collected during the preliminary sampling (R_0), then soil and vegetation samples were taken at the end of each week during the conditioning period (R_1) and the experiment (R_2-R_6) (Table 3.1). Soil water extracted by suction cups was collected at 15 cm and 30 cm depths. Replicate samples were collected for twigs along the entire length of each sampled branch (Figure S3.1), the soil (at three different depths, i.e. 0-5 cm, 5-15 cm and 15-25 cm) and roots (during different sampling rounds when possible). All the twigs samples (6-7 cm long) were debarked quickly before storing them in 12-ml Labco Exetainer® glass vials. At the end of the experiment (06 July 2018) all the soil and plant materials were retrieved completely from OvA (considered as the treated plant) while usual sampling was carried out from OvB (considered as a control plant). The materials collected after completely cutting down OvA included debarked twigs, roots (at two different depths, i.e. 0-15 cm and 15-35 cm), and stem cores (at four different location along the entire length of the trunk, i.e., close to the ground, at 0.8 m, at 1.2 m and just below the branches (at 1.7 m).

3.2.3 Soil and plant water extraction by cryogenic vacuum distillation

The extraction of soil water (except for that collected by suction cups) and plant water samples was done by cryogenic vacuum distillation performed in two different laboratories, at the Faculty of Science and Technology (Lab 1), Free University of Bozen-Bolzano (Italy) and at the McDonnell Hillslope Hydrology Lab (Lab 2), Global Institute for Water Security, University of Saskatchewan (Canada). Both laboratories adopted the cryogenic vacuum distillation system developed by Koeniger et al. (2011).

The apparatus comprised of a pair of two Labco Exetainer® (Labco Ltd, Lampeter, UK) vials (12 ml each) serve as independent extraction-collection units connected by a thin stainless-steel capillary tube (2.00 x 0.95 mm) (Figure 3.2). The first step after labelling and the preparation of extraction-collection vials unit for each sample, was to freeze the sample vials by immersing

them in liquid nitrogen (approx. –196 °C) to prevent the loss of water vapor during evacuation. Prior to heating the sample vials for water extraction, the collection vials were evacuated to a baseline vacuum pressure. Samples were then loaded in an aluminium container and heated to a temperature of 200°C. All the individual soil and plant samples were extracted at a temperature of 200°C for an extraction time of 15 mins per sample. After the water in the sample had been quantitatively transferred to the collection vial, were removed from the cold trap, defrosted in perfect sealed conditions at room temperature, wrapped tightly with Parafilm®, labelled and stored in a refrigerator until the plant water samples were shipped to InnoTech Alberta (Canada) for isotopic analysis via Isotope Ratio Mass Spectrometry (IRMS) and soil water samples to Faculty of Science and Technology (Bolzano) for isotopic analysis via Picarro isotope analyzer.
During the cryogenic extraction process in both labs, soil and plant samples were weighted pre and post water extraction and after the oven-drying (24 hours, at 100°C) for the computation of extraction efficiencies.

Both cryogenic extraction systems (Lab 1 and Lab 2; Figure 3.2) developed on the same design and have the same characteristics. However, differences were observed in handling i.e. amount of soil and plant materials considered for extraction, sample preparation time, vacuum pressure, calculations of extraction efficiencies, post-extraction filtering method for soil and plant water extracts and cleaning of capillary tubes. The collections vials in Lab 1 were evacuated to a pressure of 0.1 kPa while to 87.0 Pa in Lab 2. In Lab 2 the post-extraction filtrations of plant water samples were carried out on 0.45-µm Nylon disk filters and the filtered samples were always transferred to 2 ml amber glass vials covered by polypropylene screw-thread caps with septa and sealed tightly with Parafilm®. On the other hand, no such filtration was done for plant water samples extracted in Lab 1.

For the subsequent rounds of the extractions, the capillary tubes were cleaned each time by acetone and dried on the hot plate in Lab 1. While, in Lab 2 before the next round of the cryogenic extraction, the capillary tubes were cleaned firstly by blowing air and then leaving them in a mixture of water + sulphuric acid (50%) over a vibrating platform (Fisher Scientific) for 40 mins and then oven dried for 1-2 hours.

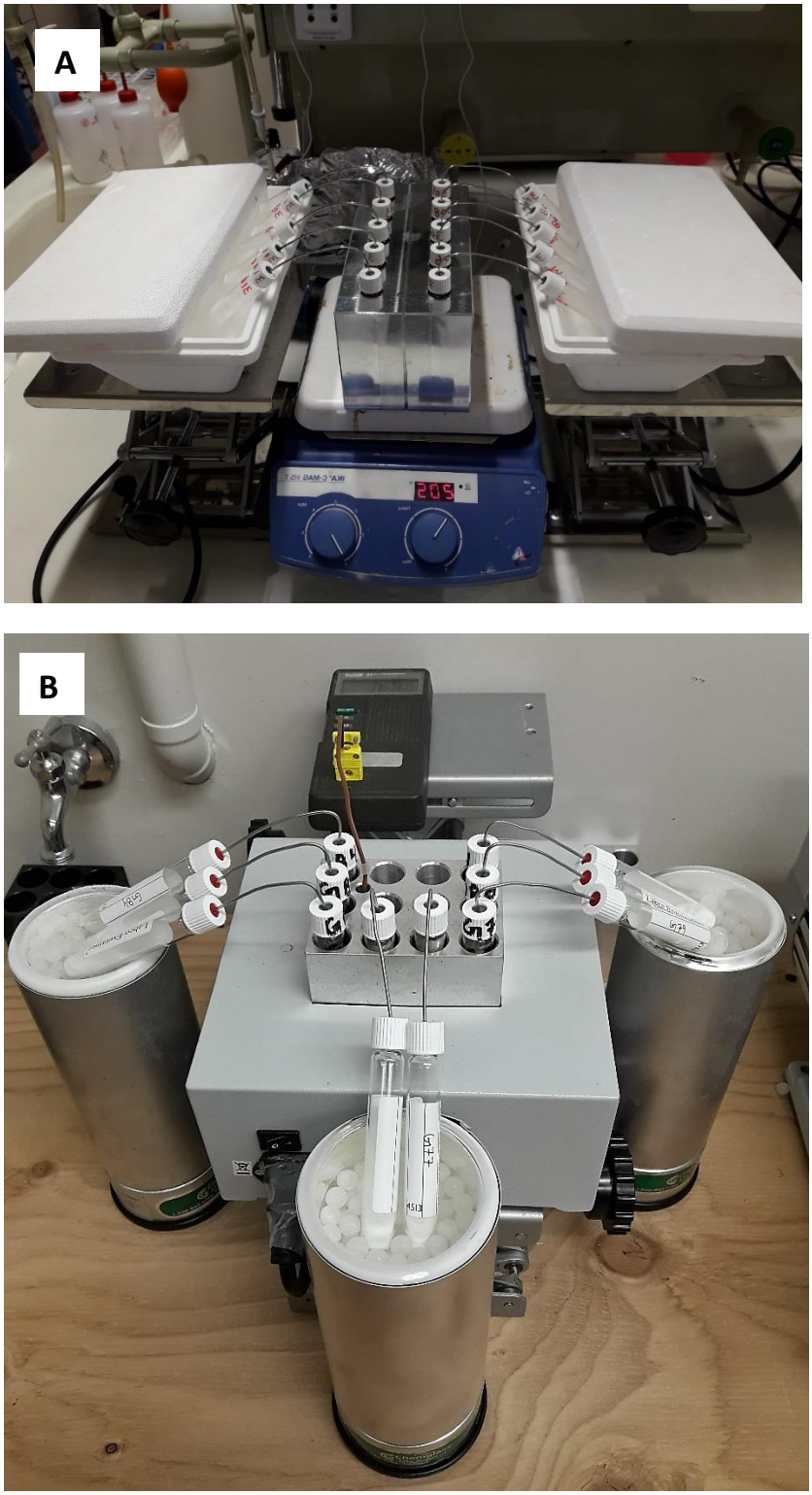

Figure 3.2. Cryogenic vacuum distillation system in Lab 1 (a) and Lab 2 (b).

3.2.4 Isotopic analyses

The isotopic compositions ($\delta^{2}H$ and $\delta^{18}O$) of plant water samples were measured by isotope ratio mass spectrometer (IRMS) at InnoTech Alberta, Edmonton (Alberta, Canada). The soil

water samples (from both labs) were transferred to Faculty of Science and Technology (Bolzano) for isotopic analysis (L2130-I, Picarro Inc., USA) at the Free University of Bozen-Bolzano (Italy). The precision of Picarro analyzer for $\delta^{18}O$ was 0.25‰ and 0.5‰ for $\delta^{2}H$.

$\delta^{2}H$ of plant water samples was determined on a Delta V advantage mass spectrometer and an HDevice peripheral. Briefly, a CTC Analytics autosampler inject water sample (1uL) into a HDevice septum and a dual inlet peripheral produce H_2 gas from H_2O using chromium metal at 900°C. The H_2 gas of the water sample introduced to mass spectrometer by dual inlet bellows, and the isotopic composition was measured relative to pure hydrogen gas. The resulting isotopic composition were reported vs Vienna Standard Mean Ocean Water (VSMOW). The accuracy of the results is +/- 1 per mil.

$\delta^{18}O$ of plant water samples was determined on a Delta V advantage mass spectrometer and GasBench II peripheral. Samples were equilibrated in sealed vial and flushed with CO_2 (0.3%), as a result, the oxygen in the water sample exchange with oxygen of CO_2.
Then a CTC Analytics autosampler sampled the headspace onto the GasBench II where multiple injections of the sample oxygen (as CO_2) are measured versus a pure CO_2 gas. Likewise, hydrogen isotopic ratio, the resulting isotopic compositions of oxygen were reported vs Vienna Standard Mean Ocean Water (VSMOW). Results are accurate to +/- 0.2 permil.
A more detailed description of this methodology used at InnoTech Alberta (Edmonton, Canada) can be found in Nelson (2000).

3.2.5 Data analysis

Plant and soil water isotopic data (both hydrogen and oxygen) followed a non-normal distribution ($p<0.01$, using Shapiro-Wilk normality test). Thereafter, non-parametric tests were performed, i.e. Kruskal-Wallis test for multiple comparison of treatments ($\alpha=0.05$) for assessing the differences in the isotopic composition of plant and soil waters for tap water and labelled water treatments across different plant tissues and soil water depths and between the two labs.

3.3 Results

3.3.1 Time series of meteorological variables, sapflow and shallow soil moisture

The daily dynamics of the meteorological variables did not change significantly during the experiment (Figure 3.3). The average relative humidity inside the greenhouse was 48% (with humidity varying between 14% and 88%), with an average temperature of 28 °C (with a minimum temperature of 13°C and a maximum of 47°C) (Figure 3.3a).

The sapflow monitored in the plant called OvB was more stable during the entire period compared to OvA (Figure 3.3b). Sapflow measured in OvA was lower during the conditioning period than during the experiment, suggesting that OvA had lower transpiration rates than OvB especially before starting the 5-weeks experiment. Daily sapflow dynamics were very pronounced for both plants, indicating that they were transpiring efficiently the irrigation water throughout the whole duration of the experiment.

During the conditioning period, we observed that shallow soil moisture differed in the two pots, with OvA having a surface soil with higher moisture than the soil of OvB (Figure 3.3c). However, during the experiment the measured soil moisture was much more similar in the two pots. Shallow soil moisture had clear temporal dynamics of wetting up due to the input of irrigation water and then drying up until the following irrigation time.

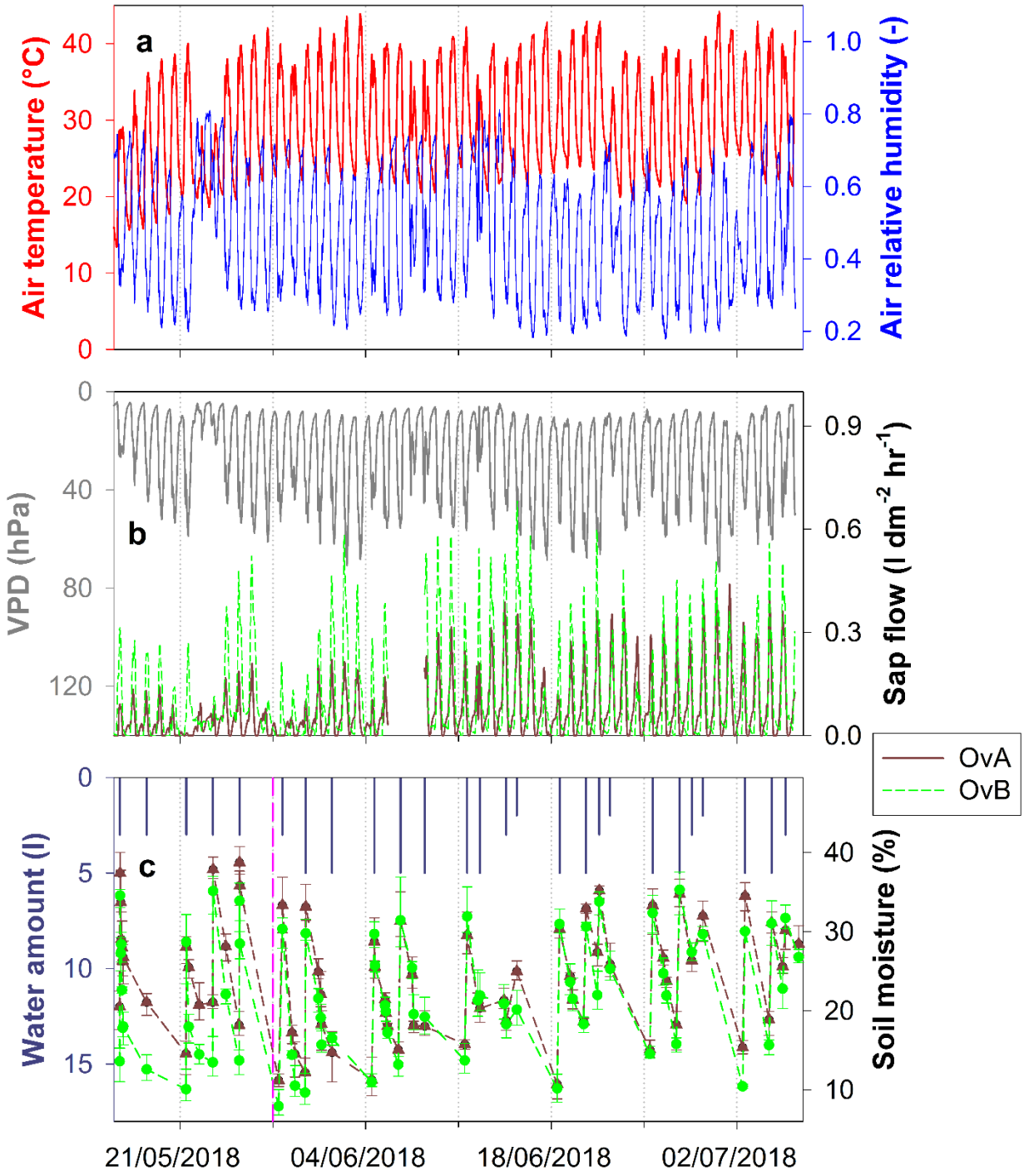

Figure 3.3. Time series of (a) relative humidity and air temperature in the greenhouse; (b) vapour pressure deficit (VPD) and sap flow rates and (c) average soil moisture (%) of plant A (OvA) and plant B (OvB). Range bars represent the relative minimum and maximum soil moisture readings. Red colour corresponds to plant A and light green colour to plant B.

3.3.2 Isotopic response of soil and plant waters to labelled water

At the end of the conditioning period, δ^2H and $\delta^{18}O$ values of soil and plant waters were similar to the isotopic signature of tap water, but some samples (particularly soil water) were more enriched in heavy isotopes than tap water (Figure 3.4 and Table 3.2). We found no significant differences in the isotopic composition of soil (Mann-Whitney rank sum test, $p>0.05$ for $\delta^{18}O$ and $p>0.001$ for δ^2H) and plant water (Mann-Whitney rank sum test, $p>0.05$ for both $\delta^{18}O$ and δ^2H) in the two olive plants before the start of the experiment. Indeed, absolute differences in the median isotopic composition of plant water collected from twigs of OvA and OvB were 0.10‰ (Lab 1) and 0.19‰ (Lab 2) for $\delta^{18}O$, 2.1‰ (Lab 1) and 1.0‰ (Lab 2) for δ^2H (Table S3.1). Absolute differences in the median isotopic composition of soil water taken from OvA and OvB pots were 2.27‰ (Lab 1) and 0.34‰ (Lab 2) for $\delta^{18}O$, 8.1‰ (Lab 1) and 1.6‰ (Lab 2) for δ^2H (Table S3.1).

We found that just after a week from the start of the experiment, plant water retrieved from twigs had a more negative isotopic composition than during the conditioning period and it plotted close (δ^2H) or together ($\delta^{18}O$) with the labelled irrigation water (Figure 3.4 and Figure 3.5). Plant water kept an isotopic composition similar to the labelled water throughout the experiment; median $\delta^{18}O$ of plant water taken from twigs was -12.43 (Lab 1) and -12.71 (Lab 2), while median δ^2H was -92.6 and -93.7 Throughout the experiment, we did not detect large and systematic differences in the isotopic composition of twigs collected from different location along the branches (Table S3.2). The temporal dynamics of the plant water isotopic composition were quite similar for OvA and OvB and for the samples extracted in the two laboratories.

Soil water extracted by suction cups showed temporal dynamics in the isotopic composition quite similar to those of plant water, except for a sample collected on 20 June 2018 which was more enriched in heavy isotopes compare to labelled water, previous and following soil water samples (Figure 3.4 and Figure 3.5). Unlike plant water, soil water sampled at different depths has not shown an immediate change in isotopic composition (i.e., were not as negative as that of the labelled water). Besides, the isotopic compositions of soil water do vary significantly at different soil depths after the start of the experiment and soil water isotopic compositions showed more similarity with labelled water towards the end of the experiment. During the experiment, the absolute differences in the median isotopic composition of soil water of OvA

and OvB were 0.19‰ (Lab 1) and 0.55‰ (Lab 2) for $\delta^{18}O$, 0.5‰ (Lab 1) and 3.8‰ (Lab 2) for $\delta^{2}H$ (Table S3.2). Throughout the experiment, more negative values of median $\delta^{18}O$ of -11.46 (Lab 1) and -11.40 (Lab 2) and median $\delta^{2}H$ of -83.7 (Lab 1) and -85.5 (Lab 2) were observed for soil water taken at 0-5 cm soil depth while less negative values of median $\delta^{18}O$ of -9.61 (Lab 1) and -9.30 (Lab 2) and median $\delta^{2}H$ of -78.1 (Lab 1) and -77.1 (Lab 2) were shown by soil water at 15-25 cm soil depth. Thus, indicating that deeper soil water was a bit enriched in heavy isotopes than the upper soil layer water (at 0-5 cm depths).

Corresponding to plant water isotopic composition, we did not detect large and systematic differences in the isotopic composition of soil water collected at different soil depths (Table S3.2). Also, the temporal dynamics of the soil water isotopic compositions during the experiment were quite similar for OvA and OvB and for the samples extracted in the two laboratories.

d-excess values (Figure 3.6) showed that most of the plant water samples (e.g., twig water taken at different locations) fall within the range of tap water (Table S3.1) and labelled water (Table S3.2) thus providing an evidence of no isotopic enrichment of twig water samples throughout the experiment. While on the other hand, soil water samples at depths (5-15 and 15-25 cm) were found to be very enriched in heavy isotopes (i.e., quite negative d-excess values) particularly at the end of the conditioning period with d-excess values of soil water found to be -8.2 and 1.7 for OvA and OvB (Lab 1) and be -9.9 and -8.9 for OvA and OvB (Lab 2).

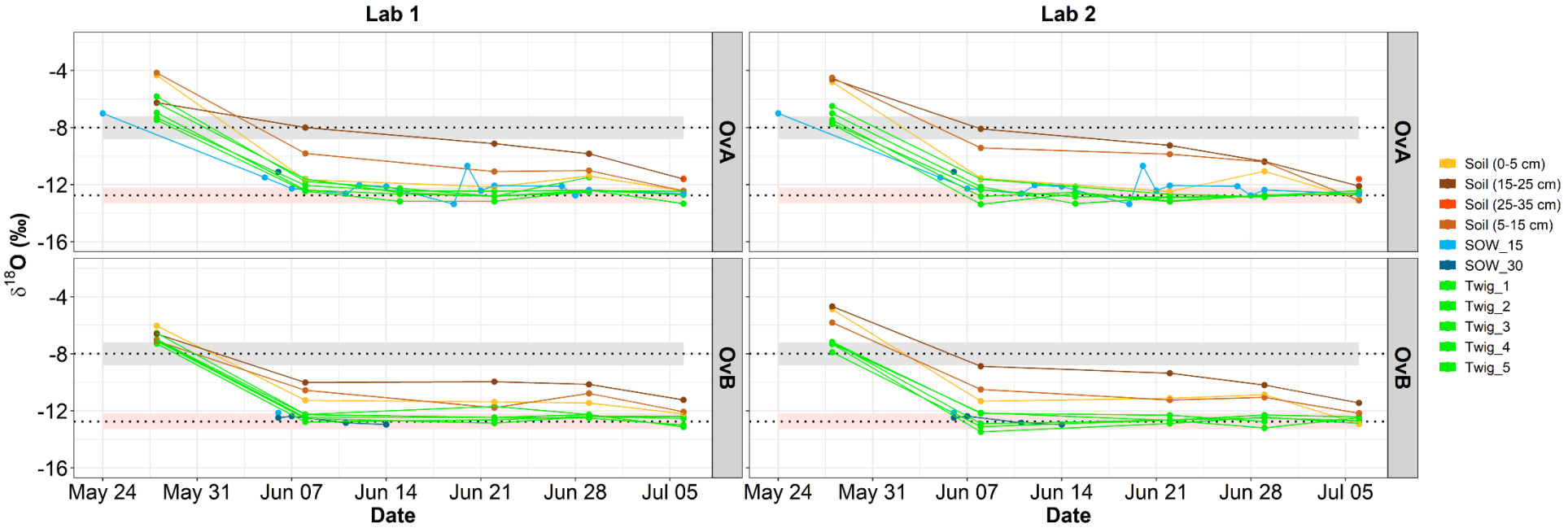

Figure 3.4. Time series for $\delta^{18}O$ measured in soil and plant waters for samples extracted in the two laboratories (Lab 1 and Lab 2). SOW_15 and SOW_30 represent the mobile soil water obtained via suction cups. The two horizontal dotted lines with their respective bands indicate the average and the standard deviation, respectively, of the isotopic composition of irrigation waters (grey for tap water, pink for the labelled water).

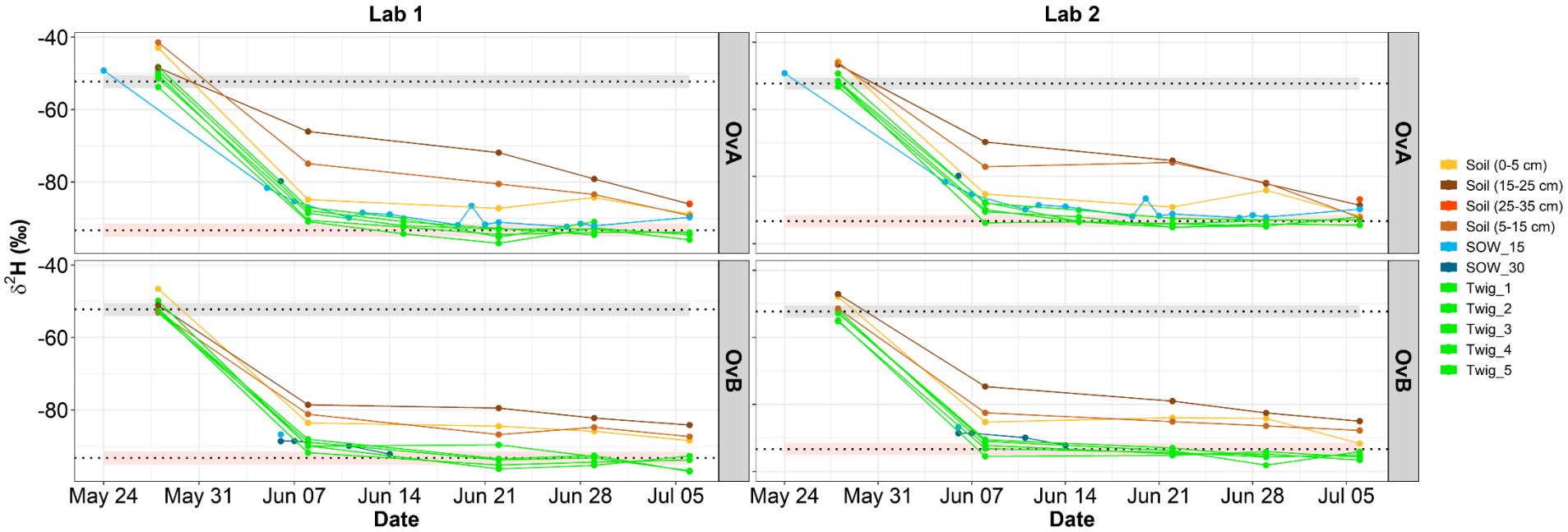

Figure 3.5. Time series for δ^2H measured in soil and plant waters for samples extracted in the two laboratories (Lab 1 and Lab 2). SOW_15 and SOW_30 represent the mobile soil water obtained via suction cups. The two horizontal dotted lines with their respective bands indicate the average and the standard deviation, respectively, of the isotopic composition of irrigation waters (grey for tap water, pink for the labelled water).

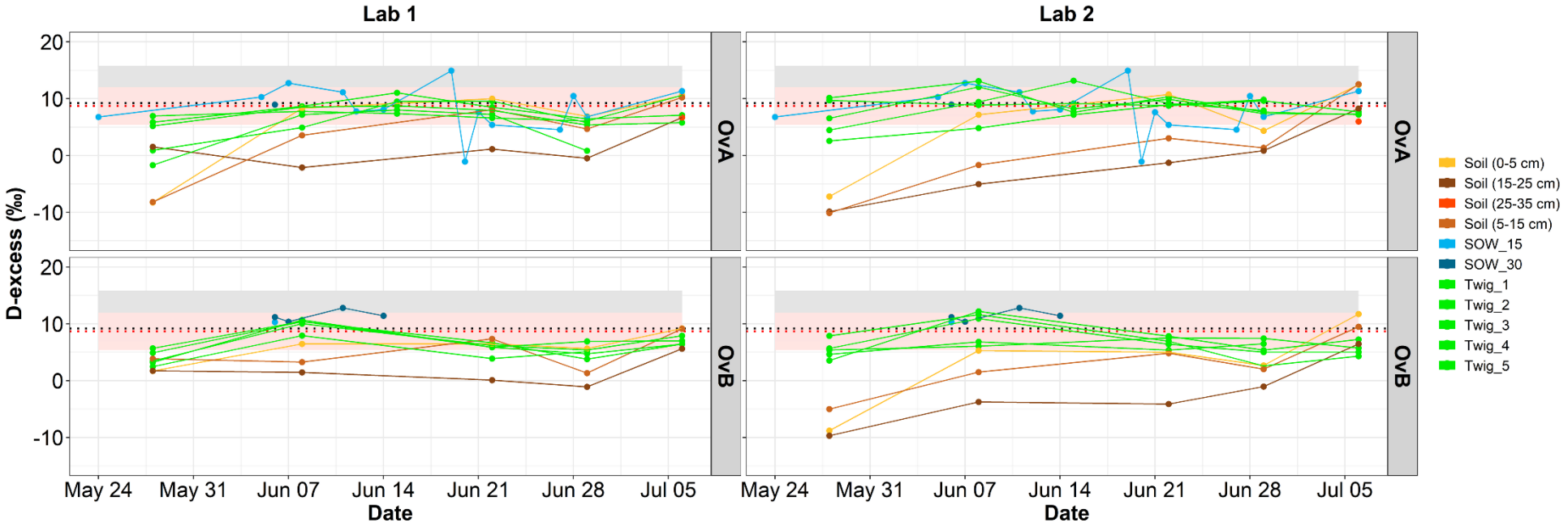

Figure 3.6. Time series for d-excess determined in soil and plant waters for samples extracted in the two laboratories (Lab 1 and Lab 2). SOW_15 and SOW_30 represent the mobile soil water obtained via suction cups. The two horizontal dotted lines with their respective bands indicate the average and the standard deviation, respectively, of the d-excess of irrigation waters (grey for tap water, pink for the labelled water).

3.3.3 Variability in the isotopic composition across plant tissues and soil water

To find out if the isotopic compositions of plant water varies from roots to leaves pathway, an extensive sampling round was carried out at the end of the experiment after cutting down OvA completely while the usual sampling was done for OvB. The core samples obtained at different locations along the trunk and the twigs taken close to the leaves and trunk of OvA (Figure 3.7) showed that the isotopic compositions were identical to labelled water isotopic composition with exception for the root water samples and soil water samples at depth (> 15 cm). However, no such differences were observed in the trends of $\delta^{18}O$ and $\delta^{2}H$ of the soil and plant water extracts obtained from Lab 1 and Lab 2 (Figure 3.7).

Specifically, plant water samples had a small variability (ranges in $\delta^{18}O$ and $\delta^{2}H$ were 0.8‰ and 2.3‰ (core water), and 0.6‰ and 1.4‰ (twig water) for Lab 1). Similarly, for Lab 2, the ranges in $\delta^{18}O$ and $\delta^{2}H$ were 1.1‰ and 5.0‰ (core water), and 0.5‰ and 3.1‰ (twig water).

Results of Kruskal-Wallis one-way analysis of variance test assessed no significant differences in the isotopic composition of plant water at different locations of the stem between the two labs for both $\delta^{2}H$ (at $P > 0.05$) and $\delta^{18}O$ (at $P > 0.05$) of OvA for the last sampling round. Significant differences were found between twig water and root water and/or soil water samples at >15 cm depth (Kruskal-Wallis one-way analysis of variance test, $p<0.001$ for both $\delta^{18}O$ and $\delta^{2}H$).

D-excess values (Figure 3.8) of plant water samples (e.g., twigs water and core water) showed similarity with d-excess of labelled water. In addition, our results revealed similarity between the isotopic composition (both $\delta^{2}H$ and $\delta^{18}O$) of soil water and plant water between Lab 1 and Lab 2 at the end of the experiment (Mann-Whitney rank sum test, $p>0.10$ for both $\delta^{18}O$ and $\delta^{2}H$).

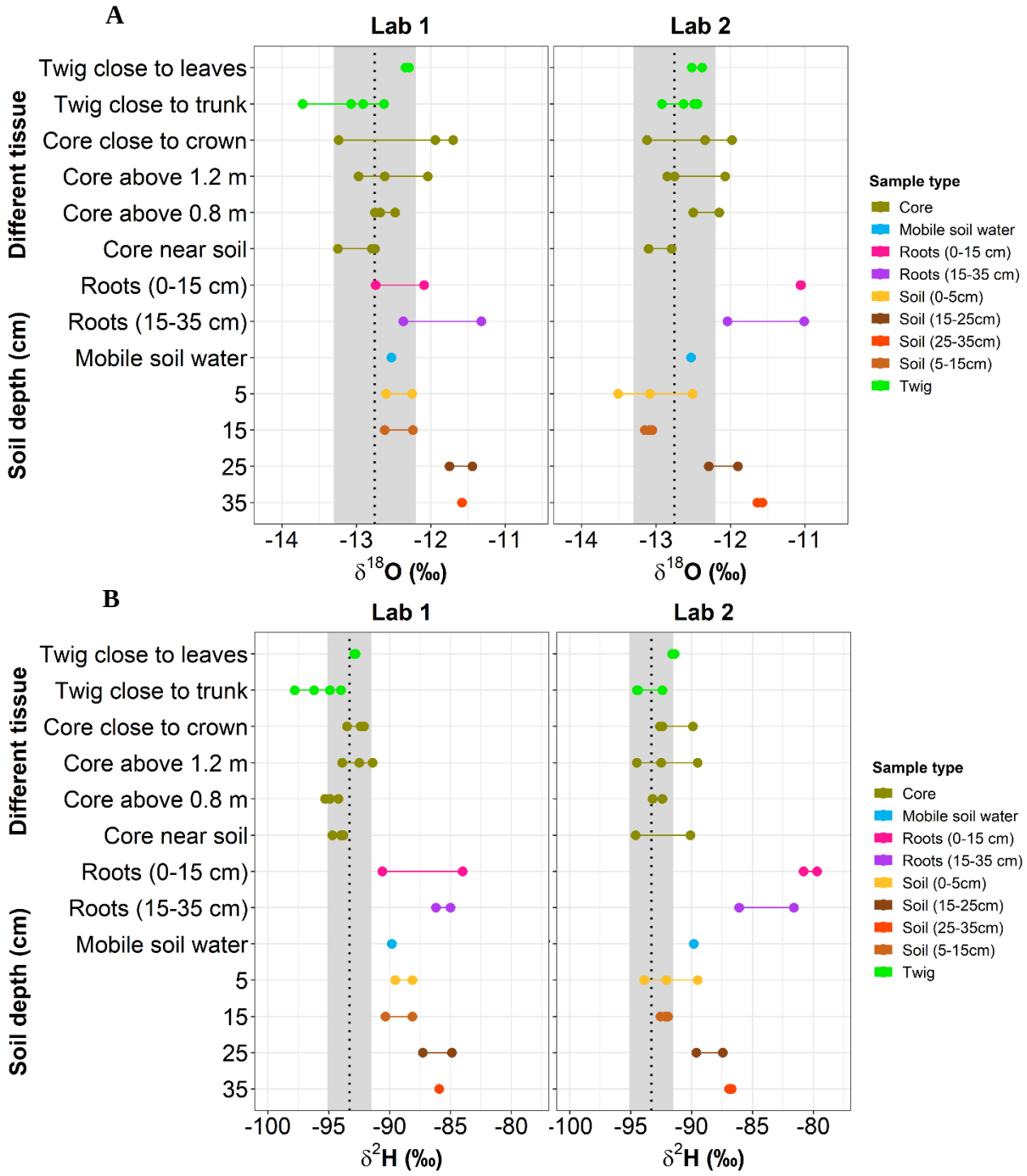

Figure 3.7. The spatial variation of A) $\delta^{18}O$ and B) $\delta^{2}H$ of twig water, core water (different cores at various heights above the ground), root (at different depths), soil water (at 0-5 cm, 5-15 cm, 15-25 cm and 25-35 cm depths: cryogenically extracted soil water) and mobile soil water of only experimental plant i.e. OvA (olive plant A) after cutting it down completely. The grey bands shown is the isotopic composition of labelled water (mean±SD).

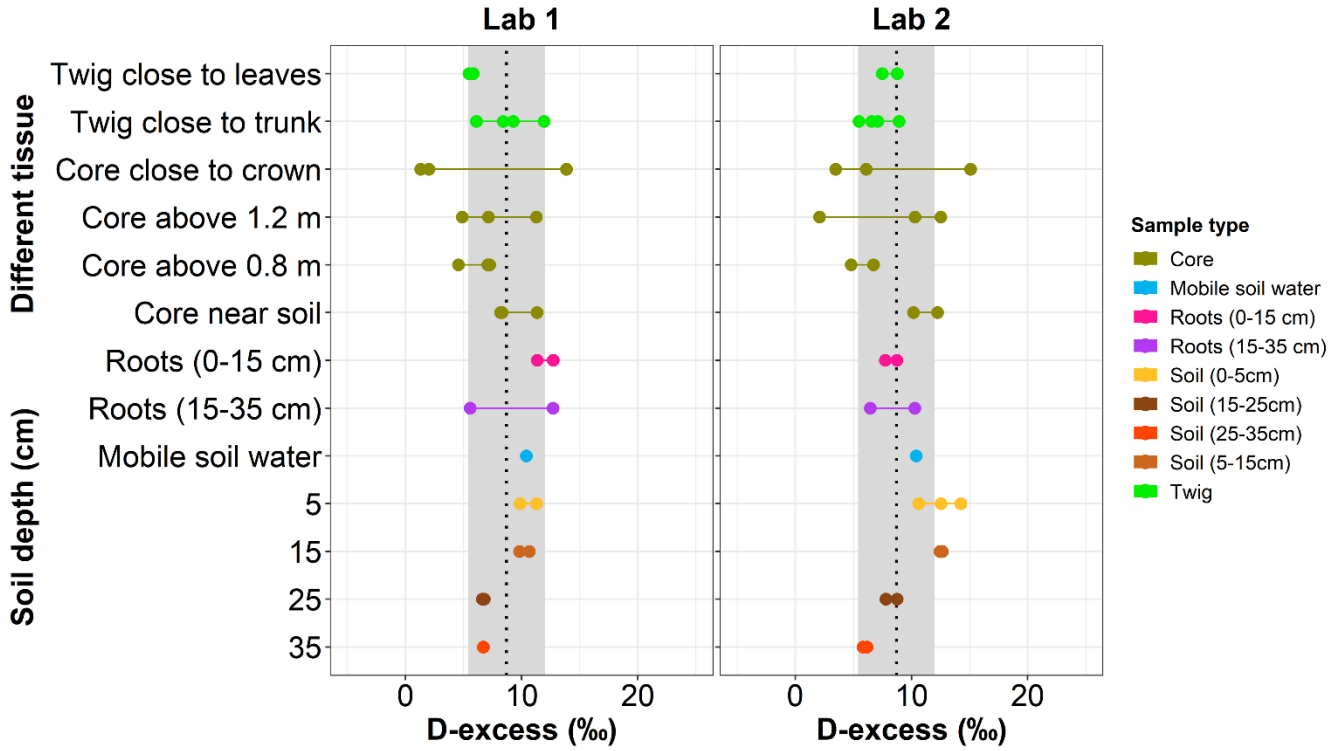

Figure 3.8. The spatial variation of d-excess of twig water, core water (different cores at various heights above the ground), root (at different depths), soil water (at 0-5 cm, 5-15 cm, 15-25 cm and 25-35 cm depths: cryogenically extracted soil water) and mobile soil water of only experimental plant i.e. OvA (olive plant A) after cutting it down completely. Shown are the data for both Lab 1 and Lab 2. The grey band shown is the d-excess of labelled water (mean±SD).

3.3.4 Differences in the isotopic compositions of soil and plant waters extracted in the two laboratories

Scatterplots of δ^2H and $\delta^{18}O$ plant water and soil water materials extracted in Lab 1 vs. Lab2 (Figure 3.9) indicated that both plant and soil water extracts obtained by cryogenic vacuum distillation in Lab 1 found comparable to Lab 2. Root samples and soil water samples at 15-25 cm depth were the only ones plotting away from the 1:1 line as compared to core and twig samples. A little scatter of root water samples and soil water (at 15-25 cm depth) can be seen on the either side of the 1:1 line (Figure 3.9) indicating enrichment in heavy isotopes of few soil water samples extracted by the cryogenic system of Lab 2 in comparison to Lab 1. Slope values (Table 3.3) are close to almost one for both δ^2H and $\delta^{18}O$ of plant and soil water samples and thus, showing the samples extracted in two labs are identical.

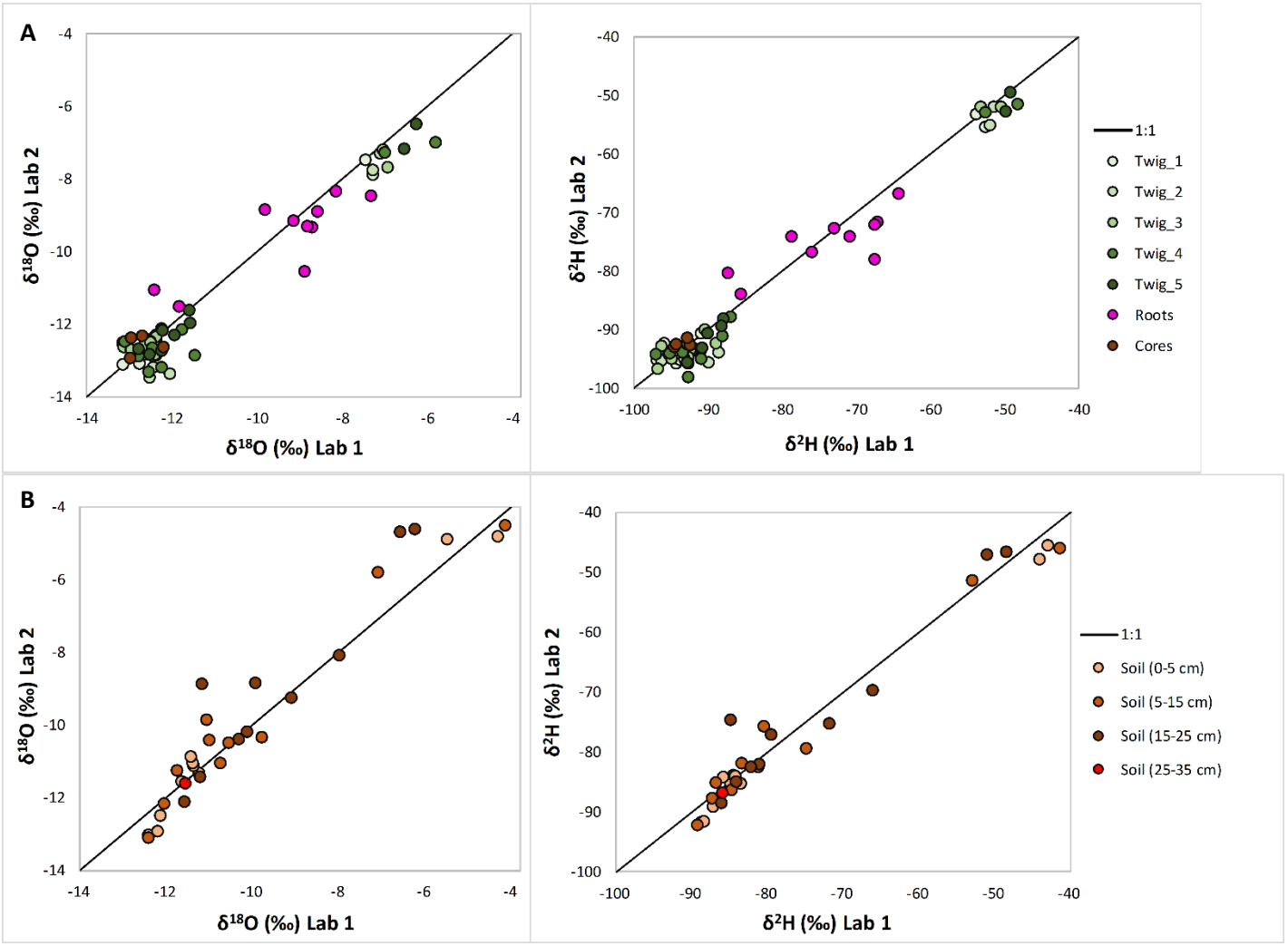

Figure 3.9. Scatterplots of oxygen ($\delta^{18}O$), and deuterium ($\delta^{2}H$) of A) plant water and B) soil water extracted in Lab 1 vs. Lab 2 Indicated in red is the 1:1.

Further, multiple comparison tests were performed to evaluate the differences across different plant tissues types and soil water at different soil depths. Similarity of isotopic composition was found between twig water samples taken at different locations along the branch and between the core water taken at different heights along the trunk (Kruskal-Wallis one-way analysis of variance test, $p>0.05$ for $\delta^{18}O$ and $p>0.001$ for $\delta^{2}H$) during the labelled water experiment. While a statistically significant difference was found between isotopic compositions of soil water at 15-25 cm soil depth (Kruskal-Wallis one-way analysis of variance test, $p<0.001$ and $p>0.001$ for both $\delta^{2}H$ and $\delta^{18}O$). Irrespective of some scatter for the root water samples, no significant differences were observed between the root water extracts between two labs (Mann-Whitney rank sum test, $p>0.10$ for both $\delta^{2}H$ and $\delta^{18}O$). Therefore, the isotopic compositions of plant water extracts of different tissue water (i.e., cores, twigs,

and roots) and soil water extracts at shallow soil depths (at 0-5 cm and 5-15 cm) obtained from the cryogenic extraction techniques developed at Lab 1 are comparable to Lab 2.

Table 3.3. Slopes and intercepts of the linear regressions between $\delta^{18}O$, $\delta^{2}H$ and d-excess of plant tissue water and soil water between Lab 1 and Lab 2. Lab 1 plotted along x-axis while Lab 2 along y-axis.

	Plant water			**Soil water**		
(Lab 1 vs. Lab 2)	**$\delta^{18}O$**	**$\delta^{2}H$**	**d-excess**	**$\delta^{18}O$**	**$\delta^{2}H$**	**d-excess**
Slope	0.92	0.96	0.74	1.08	0.97	1.21
Intercept	-1.15	-4.38	2.40	1.04	-2.44	-3.25
R^2	0.94	0.97	0.62	0.92	0.96	0.70

3.4 Discussion

3.4.1 Isotopic fractionation of plant water

No noteworthy differences were found in $\delta^{18}O$ and $\delta^{2}H$ between different plant tissue, i.e., twig water taken at different locations along the branch and the core samples taken at different heights during tap water as well as labelled water treatments in two olive plants (Figure 3.7 and Figure 3.8). In the past, Washburn and Smith (1934) found that no appreciable isotopic fractionation occurred in three studied species (i.e., cow peas, Indian corn, and Weeping willow) when grown hydroponically, likewise Walker and Richardson (1991) in a study carried out with plants in pots (barely and range of native species) and plants under field conditions (barely, bluebush and Pine) reported no isotopic fractionation. Thorburn et al. (1993) in a follow up study under controlled environmental condition (i.e. in lab and greenhouse set up) have shown that plants did not discriminate towards isotopes. Furthermore, few studies tested fractionation of oxygen and hydrogen isotopes. For instance, Lin and Sternberg (1993) found no isotopic fractionation of stem water and they reported no significant differences in the oxygen isotope of stem waters in coastal wetland plants. Our results are in line with previous studies reporting no isotopic fractionation in plants (naming a few; Dawson et al., 2002; Dawson and Ehleringer, 1993, 1991; Thorburn et al., 1993; Walker and Richardson, 1991; Washburn and Smith, 1934; Wershaw et al., 1966; White et al., 1985; Zimmermann et al., 1967). Though, our study is aligned with the above-mentioned studies reporting "no fractionation of isotopes by plants", however, we did observe some differences in case of root water and soil water at greater than 15 cm soil depths. The only differences exist between twig water and root water and/or that of soil water at >15 cm depth, which contradict with some of the existing studies, as such Ellsworth and Williams (2007) that found no significant differences in the $\delta^{18}O$ isotopic composition of taproot water from its source water (soil water).

Ellsworth and Williams (2007) observed significant differences in the hydrogen isotopic composition between taproot tissue water and soil water source and thus reported $\delta^{2}H$ fractionation of water taken up by plants at soil-root interface (Ellsworth and Williams, 2007). Our results somehow agree with Ellsworth and Williams (2007) for $\delta^{2}H$, but, in addition to hydrogen isotope fractionation, our results also found $\delta^{18}O$ fractionation i.e. significant difference in the oxygen isotope composition between root water and plant tissues water. However, so far, no studies directly compared the $\delta^{18}O$ and $\delta^{2}H$ isotopic values between plant water and water source (i.e., labelled water) under control conditions as in the current work. Differences were highlighted between $\delta^{18}O$ and $\delta^{2}H$ of twig water and soil water at depth of >15 cm and/or root water (Figure 3.7 and Figure 3.8) while the isotopic composition

of mobile soil water and soil water at 0-5 cm depth (cryogenically extracted) was similar to plant water.

No significant differences were observed for both $\delta^{18}O$ and $\delta^{2}H$ between different plant tissue types except roots, which suggests no oxygen and hydrogen isotopic fractionation by plants during its transport from roots to upper part of the branch and distribution to the other plant parts. Our results also differ from what was reported by Lin and Sternberg (1993) that there is significant hydrogen isotope fractionation by some halophyte plant species during uptake through a comparison of $\delta^{2}H$ values between stem water and its source water (Lin and Sternberg, 1993).

It is interesting that there was the fractionation of root water and soil water at depth >15 cm, nevertheless, such differences can be expected. The enrichment of soil water extracted cryogenically is commonly reported for the soils with low water content and tested in several spike experiments (Ingraham and Shadel, 1992; Meißner et al., 2014; Orlowski et al., 2016). Recent work by Vargas et al. (2017) reported preferential uptake of ^{1}H and ^{16}O and leaving the heavy isotopes (^{1}H and ^{18}O) in the remaining soil water pools.

3.4.2 Factors affecting the isotopic composition of plant water

Fractionation in plants can be linked with water ratios of the living cells versus xylem vessels (which are the dead cells), the variations can be due to changes in cell volumes as a part of entire volume of several tissues. In biological systems it is difficult to find a constant offset of the isotope fractionation between reactant and the product.

Cernusak et al. (2005) stated a tendency of *Eucalyptus globulus* for xylem water to be enriched slightly in oxygen isotope in the upper stem as compared to the lower stem, and for branch tips to slightly more enriched than the branch part in the middle and additionally, reported significant isotopic enrichments of phloem water as compared to xylem water (Cernusak et al., 2005). Deuterium enrichment of proximal branches have been observed also by some earlier studies (for instance, Dawson and Ehleringer, 1993). While taking in account the phloem enrichment, there could be some possible effects of phloem water $\delta^{2}H$ and the associated bark enrichment on the xylem water. However, in the current study, we found no significant isotopic differences in the plant water extracted from different parts of the stem. All the branch samples regardless of their central, proximal and extremity positions were still comparable. A few samples did report lower $\delta^{2}H$ values which are in agreement with previous studies (e.g., Ellsworth and Williams, 2007), but this can also relate to sampling and handling errors, therefore, the underlying mechanism still need further investigation.

As said earlier, a possible fractionation between entire plant tissue water and the root water may result in δ^2H depletion of water in the living cells by aquaporins (Mamonov et al., 2007) or even heavy isotope enrichment due to other metabolic processes related fractionation within specific cells (Yakir, 1992). In biological systems it is well known that water in the cells can have different $\delta^{18}O$ and δ^2H isotopic compositions than the water of the neighbouring growth media due to the contribution of O and H to intracellular water by the metabolites (Kreuzer-Martin et al., 2005; Kreuzer et al., 2012). The proportion between metabolic fluxes and water amount inside the living cells depend on the residence time of water within a particular cell type. Then, another assumption is that the flow of xylem water through the dead cells (xylem conduits) is different than the parenchyma (living cells in the sapwood). Leaves produces sugars that are depleted in δ^2H than leaf water while on the other side leaf water have high rates of evaporative enrichment (i.e., in the range of 0 to 100 ‰) compared to xylem water (Cernusak et al., 2005). Some of the sugars can be transported back to the parenchyma cells in sapwood, thus, may result in an exchange of δ^2H during the enzymatic catalysis of sucrose (Roden et al., 2000; Yakir, 1992). One mole of respiratory CO_2 release is linked with an exchange of hydrogen atom of carbon-dioxide with one mole of H_2O hydrogen atom which tells how the cellular metabolism influence the isotopic composition of cell water (Cernusak et al., 2006). This also predicts the behaviour that if the cellular water is much depleted as compared to xylem water due to the metabolism of sugars, therefore, a significant exchange can take place between δ^2H of the cell water and xylem water (Zhao et al., 2016).

Given that, there is a small change in vibrational energy due to the replacement of ^{16}O for ^{18}O in water molecule in comparison to the replacement of ^1H for ^2H, so, fractionation during transport through aquaporins (transmembrane transport) likely more pronounced for hydrogen then oxygen (Ellsworth and Williams, 2007), which is not that significant in our case but still can be observed (Figure 3.7). The fractionation occurs for hydrogen due to lower permeability of $^2H_2{}^{16}O$ than $^1H_2{}^6O$ through aquaporin. Also, under stress conditions aquaporins are regulating leaf and root hydraulics (Rodríguez-Gamir et al., 2019). Additionally, the aquaporins are involved in water movement between xylem and phloem cells (Hacke and Laur, 2016), thus explaining a possibility of fractionation of water isotopic compositions, which can potentially lead to enrichment of stem water as well. However, this is far from reaching on a certain conclusion as the mechanism involved in the possible fractionation and their magnitude is unknown. It also highlights a further investigation of water passage through aquaporin to explain the fractionation occurring during the water movement through aquaporins.

Recent studies, such as the one by Poca et al. (2019) also revealed the effect of fungi on the isotopic fractionation during root water uptake. The arbuscular mycorrhizal fungi (AMF) occupy the apoplastic section of roots of the plants (Poca et al., 2019). Thus, instead of having a direct influence on the isotopic composition of water, AMF may boost fractionation by obstructing movement of water through apoplast. Further, Poca et al. (2019) suggested that the presence of AMF may switch the root water pathways based on environmental situations (e.g., can happen in xerophyte species under drought conditions).

In current study no significant differences were observed between different plant tissue water and the labelled water (Figure 3.4 and Figure 3.5), the plant tissues water in this case were obtained with cryogenic extraction method. In addition, the methodology was checked for reliability through an interlaboratory comparison. The only fractionation observed for root water and deep soil water (>15 cm depths), which can be expected. For instance, heavy isotope enrichment of cryogenically extracted soil water were already reported in literature (Orlowski et al., 2018a; Orlowski et al., 2016a, 2016b) which was associated with soil water content and for the clay rich soils (Meißner et al., 2014; Orlowski et al., 2016b). But our results found to be same for both labs i.e. the root water and soil water heavy isotopic enrichment was detected in both laboratories.

Although we observed some sample handling, preparation and after extraction filtering method differences between the two laboratories involved in the interlaboratory comparison, still we find no significant effect of methodology on the isotopic compositions. The underlined labs resulted into water extracts which were comparable isotopically.

3.5 Conclusions

Deuterium isotope fractionation of stem water with respect to water source has been observed previously in coastal plants, woody xerophytes plant species and other multiple species under both greenhouse and field conditions. However, the location of isotopic discrimination within the plants or from roots to leaves pathways is not yet clear and it is still not certain if the proposed deuterium depletion or enrichment appears in natural conditions. In this work, through a greenhouse experiment utilizing labelled water for the irrigation of two olive plants, we explored the isotopic fractionation in plants. The isotopic compositions were measured in the water samples obtained from twigs, cores materials (located along the entire length of branch and the trunk), roots and the soil water at different depths. We not only investigated the underlying assumption of fractionation in plants, but in addition tested the reliability of cryogenic extraction system developed in two different laboratories.

Our results showed no isotopic fractionation between irrigation water and the whole plant tissue water (i.e., twig water and the core water at different locations), however, we did provide an evidence of isotopic enrichment of root water and soil water at depth >15 cm. The observed isotopic enrichment of soil water at depth and root water might relate to the soil specific extraction time and temperature and may be due to the transmembrane mixing and fractionation processes. We also found that measured isotopic composition were comparable between the two laboratories. Plant tissue water (i.e., core, twig water), shallow soil water (cryogenically extracted) and mobile soil water fall within the range of tap water and labelled water used for irrigation. Furthermore, little differences were observed sometimes between $\delta^{18}O$ and $\delta^{2}H$ isotopes. These results together with other studies dealing with isotopic fractionation (Ellsworth and Williams, 2007; Lin and Sternberg, 1993; Poca et al., 2019; Vargas et al., 2017; Zhao et al., 2016) provide important information on the behaviour of $\delta^{18}O$ and $\delta^{2}H$ in the plant-water related studies.

Finally, our research suggests that such greenhouse water labelling experiments need to be performed with different plant species and especially with plants undergoing stress due to environmental conditions (such as xerophyte and halophyte plant species). Such experiments performed under controlled environment, could also help to unravel the mechanisms and dynamic processes occurring at the soil–plant interface.

Our work further highlights the need to carry out interlaboratory comparisons for other existing extraction methodologies while considering the correct cryogenic extraction method parameters (such

as those related to temperature, extraction timing, vacuum pressure, methodological uncertainty, post extraction filtration of soil and plant water extracts, and extraction efficiencies computations).

SUPPLEMENTARY TABLES AND FIGURES

Table S3.1. Summary statistics (mean, median and standard deviation) for oxygen and hydrogen isotope ($\delta^{18}O$ and $\delta^{2}H$) values and d-excess of the first sampling round (R_1) irrigated with tap water. Shown are cryogenically extracted A) soil and plant water and B) mobile water collected from the suction cups.

A		LAB 1										LAB 2									
		$\delta^{18}O$				$\delta^{2}H$			D-excess			$\delta^{18}O$				$\delta^{2}H$			D-excess		
	Water Source	Sample size	mean	median	SD	mean	median	SD	mean	median	SD	Sample size	mean	median	SD	mean	median	SD	mean	median	SD
OvA	Soil																				
	0-5 cm	2	-4.34	-4.34	0.0	-43.0	-43.0	0.2	-8.28	-8.28	0.6	2	-4.80	-4.80	0.6	-45.6	-45.6	2.5	-7.2	-7.2	2.5
	5-15 cm	2	-4.16	-4.16	2.0	-41.5	-41.5	5.0	-8.18	-8.18	10.6	2	-4.50	-4.50	0.1	-46.1	-46.1	0.1	-10.1	-10.1	0.6
	15-25 cm	2	-6.25	-6.25	0.1	-48.5	-48.5	0.0	1.53	1.53	0.5	1	-4.59	-4.59	-	-46.6	-46.6	-	-9.9	-9.9	-
	Twig	5	-6.76	-6.95	0.7	-50.6	-50.4	2.2	3.44	5.19	3.7	5	-7.28	-7.47	0.5	-51.5	-51.8	1.4	6.7	6.6	3.3
OvB	Soil																				
	0-5 cm	1	-6.04	-6.04	-	-46.6	-46.6	-	1.72	1.72	-	3	-4.88	-4.93	0.1	-47.9	-47.7	0.4	-8.8	-8.9	1.1
	5-15 cm	2	-7.10	-7.10	0.1	-52.9	-52.9	0.3	3.87	3.87	0.2	2	-5.81	-5.81	0.5	-51.4	-51.4	1.1	-5.0	-5.0	2.9
	15-25 cm	2	-6.60	-6.60	0.6	-51.1	-51.1	2.4	1.74	1.74	2.4	2	-4.68	-4.68	0.4	-47.1	-47.1	0.6	-9.7	-9.7	2.9
	Twig	5	-7.00	-7.05	0.3	-52.0	-52.5	1.3	3.95	3.48	1.3	5	-7.37	-7.28	0.3	-53.5	-52.8	1.5	5.4	5.4	1.6

B		MOBILE SOIL WATER									
		$\delta^{18}O$				$\delta^{2}H$			D-excess		
		Sample size	mean	median	SD	mean	median	SD	mean	median	SD
OvA	15 cm	2	-7.0	-7.0	0.6	-49.2	-49.2	2.0	6.8	6.8	2.5

Table S3.2. Summary statistics (mean, median and standard deviation) for oxygen and hydrogen isotope ($\delta^{18}O$ and $\delta^{2}H$) values and d-excess of the sampling rounds (R_2-R_6) irrigated with labelled water. Shown are the cryogenically extracted A) soil and plant water and B) mobile water collected from the suction cups.

A		LAB 1										LAB 2									
		$\delta^{18}O$				$\delta^{2}H$			D-excess			$\delta^{18}O$				$\delta^{2}H$			D-excess		
	Water source	Sample size	mean	median	SD	mean	median	SD	mean	median	SD	Sample size	mean	median	SD	mean	median	SD	mean	median	SD
OvA	Soil																				
	0-5 cm	7	-11.75	-11.78	0.5	-85.6	-84.5	2.6	8.4	8.4	1.9	7	-11.68	-11.60	0.7	-86.0	-85.7	2.6	7.4	7.2	3.0
	5-15 cm	5	-10.84	-10.79	0.6	-81.0	-82.5	3.2	5.7	5.5	2.4	6	-10.28	-10.33	0.3	-79.7	-79.8	2.5	2.5	3.0	1.4
	15-25 cm	4	-9.48	-9.14	0.8	-75.5	-74.4	5.1	0.3	1.1	3.2	6	-9.23	-9.25	1.1	-75.7	-75.2	5.7	-1.8	-2.1	3.2
	Root	4	-8.30	-8.49	0.7	-68.9	-67.6	5.0	-2.4	-2.2	5.7	4	-9.16	-8.88	1.0	-73.3	-74.4	5.1	0.0	-1.1	4.7
	Twig	26	-12.46	-12.47	0.5	-92.2	-92.6	2.5	7.5	7.8	2.2	23	-12.69	-12.76	0.4	-92.5	-93.0	2.2	9.0	8.9	2.0
	Core	12	-12.68	-12.72	0.7	-93.6	-93.9	1.2	7.9	7.2	5.4	10	-12.57	-12.63	0.4	-92.2	-92.5	1.8	8.3	8.4	4.3
OvB	Soil																				
	0-5 cm	12	-10.54	-11.15	1.4	-79.6	-83.0	7.8	4.7	5.8	3.6	8	-11.10	-11.20	0.7	-84.5	-85.3	2.7	4.2	5.0	2.8
	5-15 cm	5	-11.09	-10.98	0.7	-84.2	-84.8	3.4	4.5	4.6	2.9	5	-11.02	-10.88	0.6	-85.1	-83.5	2.9	3.0	3.8	2.5
	15-25 cm	5	-10.28	-10.07	0.5	-81.7	-81.8	2.3	0.5	0.1	2.5	5	-9.39	-9.36	0.8	-78.8	-79.0	3.8	-3.7	-3.7	3.2
	Root	3	-9.04	-8.72	0.7	-72.3	-70.9	5.9	0.1	-0.2	2.4	3	-9.02	-8.89	0.3	-73.2	-74.0	1.4	-1.0	-2.9	3.6
	Twig	17	-12.37	-12.39	0.3	-92.3	-92.7	2.5	6.7	6.2	2.4	16	-12.67	-12.66	0.4	-94.2	-94.5	1.9	7.2	6.9	2.5

B		MOBILE SOIL WATER									
		$\delta^{18}O$				$\delta^{2}H$			D-excess		
		Sample size	mean	median	SD	mean	median	SD	mean	median	SD
OvA	15 cm	22	-12.28	-12.24	0.7	-89.9	-91.1	3.1	8.3	8.5	4.4
	30 cm	1	-11.09	-11.09	-	-79.8	-79.8	-	9.0	9.0	-
OvB	15 cm	2	-12.14	-12.14	0.2	-86.8	-86.8	0.4	10.3	10.3	0.9
	30 cm	6	-12.74	-12.59	0.4	-90.2	-89.9	1.7	11.7	11.2	1.9

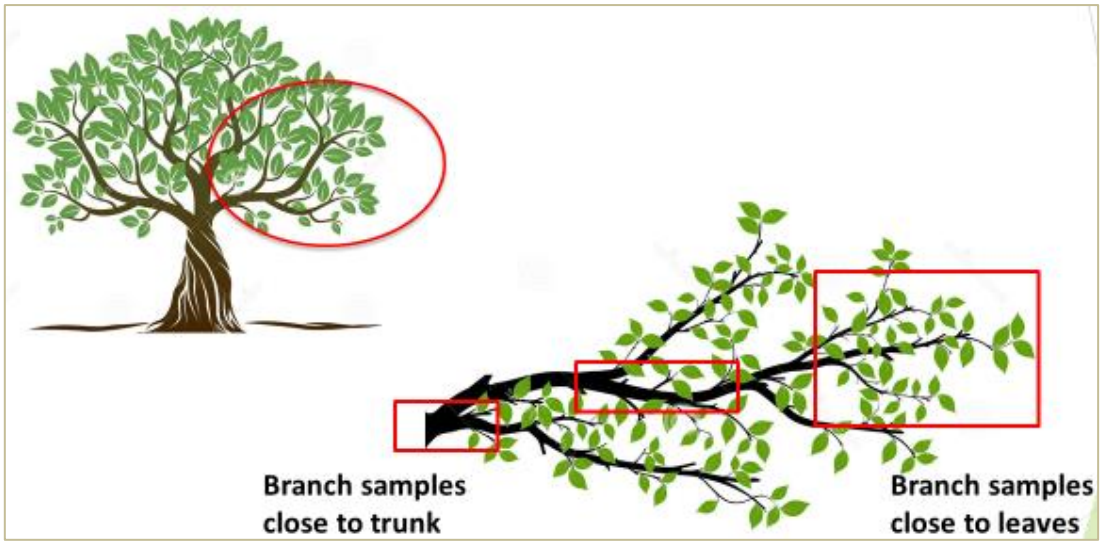

Figure S3.1. Sketch showing how samples were considered at different locations along the entire length of branch.

4 Extracting plant water for isotopic analysis: comparison of Scholander-type pressure chamber and cryogenic vacuum distillation methods[3]

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4.1 Introduction

Stable water isotopes (^{2}H and ^{18}O) are ubiquitous and powerful tools that have been extensively used in atmospheric and hydrological studies to track water movement, estimate mean residence times and water storage (e.g., Craig, 1961; Dansgaard, 1953; Klaus and McDonnell, 2013). The development of cheaper techniques for the collection and isotopic analysis of water samples at a high temporal resolution (e.g., Kerstel et al., 1999; Penna et al., 2010; von Freyberg et al., 2017) stimulated the application of stable water isotopes for investigating the water fluxes in the soil-plant-atmosphere continuum (Brooks et al., 2010; McDonnell, 2014). An increasing number of new studies has been conducted to better understand water dynamics, such as water uptake and evapotranspiration partitioning, in the soil-plant-low atmosphere continuum in different climates and both in natural (e.g., Allen et al., 2019; Dubbert et al., 2019; Liu et al., 2019a; Oerter et al., 2019; Qiu et al., 2019) and managed (agricultural and agroforest, e.g., Liu et al., 2019b; Ma and Song, 2019; Quade et al., 2019; Zhang et al., 2019) environments.

Ecohydrological studies relying on the isotopic signature of plant water require sampling methods that do not alter the true isotopic composition of the plant material, which is still a critical issue because a standardized and shared procedure and method has not been developed yet (Penna et al., 2018). Indeed, there is a variety of different techniques for the extraction of plant water, such as in situ direct vapour equilibration (Sprenger et al., 2015; Volkmann et al., 2016), microwave extraction (Munksgaard et al., 2014), cryogenic vacuum distillation (Koeniger et al., 2011; Orlowski et al., 2013), centrifugation (Peters and Yakir, 2008), and high-pressure mechanical squeezing (Böttcher et al., 1997). Among these, cryogenic distillation is widely applied (Orlowski et al., 2018a), although it presents the main drawback to extract the entire volume of water from plant tissues, which may include water stored in tissues for months or years, so not only water that is being transported at the time of sampling. Different techniques might return different isotopic values because of intrinsic methodological differences. This is also the case of the cryogenic vacuum distillation that, for examples, provides a large variability of results in case of soil water extraction (Orlowski et al., 2018b). Indeed, the inter-laboratory comparison of the cryogenic water extraction systems carried out by (Orlowski et al., 2018b) showed that there were large differences in the isotopic composition of the extracted soil water. They also observed no clear trends in the results due to construction systems and applied extraction conditions, but differences were affected by the interaction of

multiple factors, such as soil type and properties, soil water content, system setup, extraction efficiency, extraction system leaks, and each lab's internal accuracy.

Recently, a thorough comparison of different techniques for plant water extraction was presented by Millar et al. (2018), while Fischer et al. (2017) proposed various low-tech plant water sampling and extraction techniques and compared them to the widely-used cryogenic vacuum distillation developed by Koeniger et al. (2011).

Millar et al. (2018) performed an inter-method comparison of six plant water extraction techniques tested on four plant portions of spring wheat. The six different extraction methods included direct vapor equilibration, microwave extraction, two versions of cryogenic vacuum distillation, centrifugation and high-pressure mechanical squeezing. The authors found that there were marked differences among the measured isotopic compositions of the plant water, with the cryogenic vacuum distillation systems and the high-pressure mechanical squeezing producing waters more depleted in heavy isotopes, depending upon the sample type. They also observed that the various extraction techniques produced different concentrations of co-extracted organic compounds. Millar et al. (2018) concluded that, in terms of limited co-extraction of organic compounds and speed of sample throughput, the direct vapor equilibration outperformed the cryogenic vacuum distillation, but further research is needed to test the different methods on other plant species.

Fischer et al. (2019) developed six simple and low-cost methods for the extraction of plant water and compared them to cryogenic vacuum distillation (as a reference). They found that the new methods extracted plant water consistently and comparably to what was done with the reference technique. Fischer et al. (2019) also showed that other factors, such as appropriate transport, fast sample processing and efficient workflows significantly influenced the accuracy and precision of the measured isotopic composition. The authors concluded that their simple and low-cost methods could be particularly useful for the expansion of isotope-based studies in vegetated remote areas with severe technological limitations or in citizen science.

Despite the increasing number of studies focusing on inter-laboratory and inter-method comparison of techniques for plant water extraction, we found that previous research did not consider ecophysiological-based methods that tree physiologists normally adopt to measure leaf water potential (e.g., Meiri et al., 1975; Grossiord et al., 2016). One of these methods, i.e. the Scholander-type pressure chamber, is theoretically ideal because it samples active xylem

tissue. However, so far, we have found only one study (i.e., Penna et al., 2013) reporting the isotopic composition of plant water extracted by the Scholander-type pressure chamber, but no attempts were made to compare this method with the widely-used cryogenic vacuum distillation.

Therefore, in this study we want to compare the isotopic composition of plant water extracted by the Scholander-type pressure chamber and the cryogenic vacuum distillation (considered here as the reference method). Specifically, our research aims to:

i) evaluate if there are any differences in the isotopic composition of plant water extracted by the two techniques;

ii) determine whether any differences in the isotopic composition could be related to the plant species or the considered sample type used for the cryogenic vacuum distillation.

4.2 Materials and methods

4.2.1 Study site and sampling scheme

In this work, we collected samples for plant water extraction from an experimental catchment, where research in isotopy hydrology has been carried out since August 2012.

Samples from beech and chestnut trees were collected in the 2.4-ha Ressi catchment (45°47′11.79″ N; 11°15′54.12″ E; Figure 4.1) in the Italian pre-Alps (Penna et al., 2015, 2013; Zuecco et al., 2016). The catchment is located at the foothills of the eastern Italian Alps (elevation range: 598-721 m a.s.l.) and is densely vegetated. The climate is humid temperate and the average annual precipitation (1992-2007) recorded by a weather station approximately 4.5 km from Ressi is 1695 mm/yr. Monthly distribution of rainfall is bimodal with peaks in spring and fall. The mean annual temperature is 9.7 °C; on average the minimum monthly temperature is in January (1.2 °C) and the maximum in July (18.7 °C). Previous research in the study area showed that shallow riparian groundwater mainly sustains the baseflow and the riparian zone is much wetter compared to the hillslope (Penna et al., 2015; Zuecco et al., 2016).

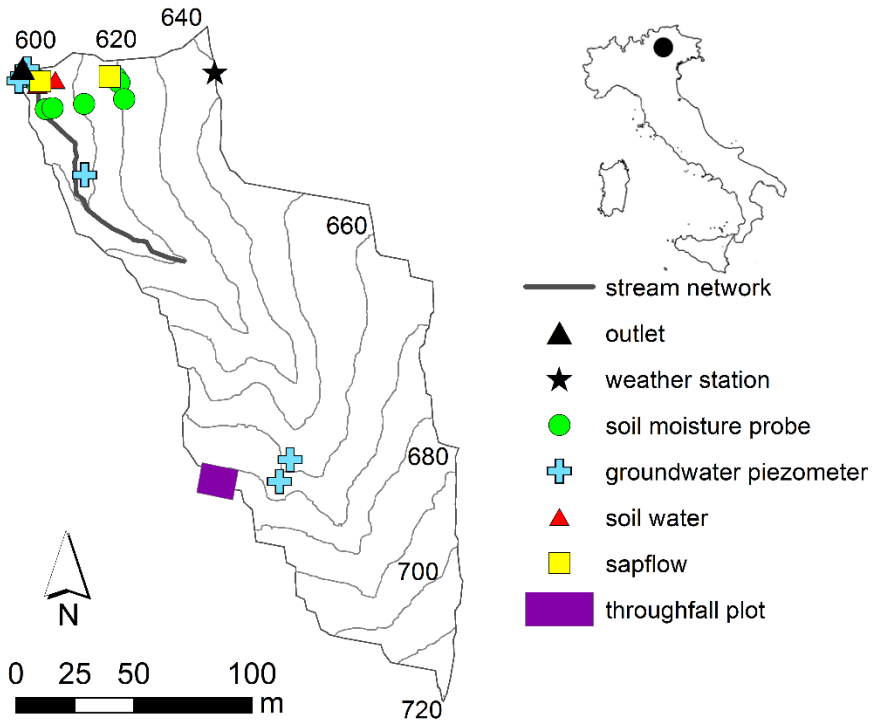

Figure 4.1. Ressi experimental catchment with installed instrumentation, and its location in Italy.

The vegetation is typical of the deciduous forests in the eastern Italian pre-Alps at elevations ranging between 500 and 1000 m a.s.l.. The main tree species in the catchment are beech (*Fagus sylvatica* L.), chestnut (*Castanea sativa* Miller), maple (*Acer campestre* L.) and hazelnut (*Corylus avellana* L.).

The sampling campaign was carried out on 5 July 2017 during a dry period. Samples for plant water extraction were retrieved from four beech and five chestnut trees in the lower part of Ressi catchment. The sampling design aimed to replicate the sample collection in all the selected trees with the two investigated methodologies (Table 4.1). However, plant water extraction and isotopic analyses were not always possible because of the small water volume contained in the plant tissues. Samples extracted by Scholander-type vacuum distillation were stored in 1.2 ml vials. For plant water extraction by cryogenic vacuum distillation, we collected samples from different plant tissues (twigs and leaves), along a branch, in glass Exetainer vials (12 ml, Labco Ltd., Wales, UK.). For some samples (i.e., twig without bark and twig core close to the trunk), bark was peeled using a knife, while for the remaining samples bark was kept (Table 4.1). All the samples were stored in a fridge at 4°C until the isotopic analyses.

Table 4.1. Samples size and median isotopic composition of the samples extracted by Scholander-type pressure chamber and cryogenic vacuum distillation from different plant tissues and species.

Plant water extraction method	**Plant tissue**	**Plant species**	**Sample size**	**Median δ^2H (‰)**	**Median $\delta^{18}O$ (‰)**
Scholander-type pressure chamber	Twig with leaf/leaves	Beech	3	-24.8	-5.75
		Chestnut	4	-14.2	-3.55
Cryogenic vacuum distillation	Leaves	Beech	4	9.7	6.09
		Chestnut	5	5.3	6.03
	Twig without bark	Beech	3	-22.2	-5.65
		Chestnut	4	-30.6	-5.69
	Twig with bark	Beech	3	-32.8	-5.74
		Chestnut	2	-26.9	-4.48
	Twig core close to the trunk	Beech	3	-33.1	-5.64
		Chestnut	4	-35.4	-5.74

4.2.2 Extraction of plant water and isotopic analyses

The Scholander-type pressure chamber (PPWS console: model 3115) (Figure 4.2) is an instrument normally used by tree physiologists to measure leaf water potential (e.g., Grossiord et al., 2017; Meiri et al., 1975). The set up consisted of a leaf sealed inside the chamber, while the cut end of the twig was exposed outside the chamber. Then, pressure (which is equal to leaf water potential) was applied until water exited from the cut end of the twig and it was collected in vials. Due to the dry conditions in the study area, to extract water from the plant tissues, we had to apply water potentials varying between 25 and 35 bar.

The plant water extraction by cryogenic vacuum distillation (Figure 4.3) was performed in a laboratory of the Faculty of Science and Technology of the Free University of Bozen-Bolzano (Italy). The cryogenic vacuum distillation system was developed based on the method of Koeniger et al. (2011). The samples to be extracted were stored directly into Exetainer vials that were later used in vacuum extraction. These vials were caped and connected to a second empty vial (hereafter collection vial) using a 1.56 mm stainless steel capillary tube. Then, the samples were frozen by immerging the sample vials in liquid nitrogen (approximately at –196 °C) to prevent loss of water vapor during evacuation (vials were evacuated to a pressure of 0.95 kPa). The sample vials were then loaded in an aluminum block (with slots for 10 vials) and heated to a temperature of 200 °C (Figure 4.3). At the same time, during the extraction process, the bottom of the collection vials was immerged into the liquid nitrogen trap, which allowed for the evacuation of the sample from the heated vial and its condensation in the collection vial. All the individual plant samples were extracted at a temperature of 200 °C for an extraction time of 15 min per sample. A heat gun (at 300 °C) was used at the end of each extraction round to remove from the steel tube any water vapor trapped in the capillary tube. After the water had been quantitatively transferred from the plant tissue to the collection vial, vials were removed from the cold trap, defrosted in perfect sealed conditions at room temperature and stored in a refrigerator until the isotopic analysis. The exhausted vials were successively recovered in 100 °C oven for 24 hours, while the capillary tubes were cleaned by acetone and then dried. All the plant samples were weighted pre-and-post water extraction, and after the oven-drying to determine the extraction efficiency.

Figure 4.2. SAPSII portable plant water status (PPWS) console (model 3115).

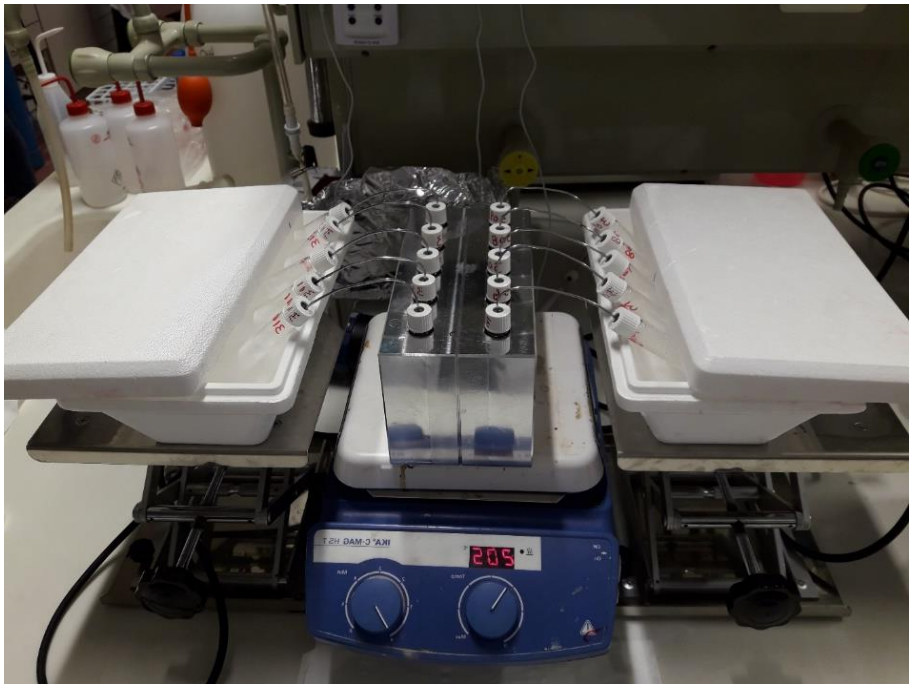

Figure 4.3. The cryogenic vacuum distillation system at the Faculty of Science and Technology of the Free University of Bozen-Bolzano, based on the method developed by *Koeniger et al. (2011).*

Isotopic analyses were performed by isotope-ratio mass spectrometry (IRMS) at Free University of Bozen-Bolzano (Italy). Each plant water sample was pipetted into a 2 mL vial. All water samples were analyzed using a Thermo Scientific IRMS (Delta V Advantage Conflo IV), coupled with a Thermo Scientific Gas Bench II to determine $\delta^{18}O$. Water samples (0.2 ml) were placed in Exetainer vials and the headspace flushed by a 0.3% CO_2-He gas mixture of known isotopic composition. After an equilibration phase of 24 h, the headspace vapor phase was injected 8 times. δ^2H was determined by direct injection on the same IRMS, coupled with a Thermo Scientific High Temperature Conversion Elemental Analyzer (TC/EA), equipped with an autosampler (Thermo Scientific AI/AS 3000).

4.3 Data analyses

Before the statistical analyses, all the isotopic data were grouped based on the extraction method (i.e., Scholander-type pressure chamber and cryogenic vacuum distillation; Figure 4.2 and Figure 4.3), plant tissue (i.e., twig with leaves, leaves, twig without bark, twig with bark and twig core close to the trunk of the tree) and plant species (i.e., chestnut and beech). Since the Scholander-type pressure chamber allows only the water extraction from one specific plant tissue (twig with leaf/leaves), we considered a total of 10 groups. For each sample, we computed the deuterium excess (d-excess; Craig, 1961), as follows:

$$d - excess = \delta^2H - 8\,\delta^{18}O \qquad \text{(Eq. 1)}$$

The isotopic composition of plant water was analyzed by means of descriptive statistics and a dual-isotope plot for a visual inspection of the overall variability in the samples and the identification of potential evaporated samples. Scatter plots between the isotopic composition of the plant water extracted by Scholander-type pressure chamber and cryogenic vacuum distillation were used to assess differences (overestimation or underestimation) in the isotopic values. Factorial analysis of variance (ANOVA), paired with a multiple comparison test based on Bonferroni method, was used to identify possible effects of the extraction method and plant species on the isotopic composition and d-excess of plant water. Scatter plots and factorial

ANOVA were applied only to those groups of samples that were not affected by isotopic fractionation due to likely evaporation processes.

4.4 Results

4.4.1 Isotopic variability across extraction methods and plant tissues

The isotopic composition of plant water varied considerably across the different plant tissues (Table 4.1 and Figure 4.4). As well known, the leaf water is normally enriched in isotopic composition, due to the water vapour exchange occurring at the stomatal level. We found that water extracted from leaves by cryogenic vacuum distillation was much more enriched in heavy isotopes (except for one sample of beech leaves) than all the other plant tissues samples (Figure 4.4). Leaf water also plotted to the right side of Ressi Local Meteoric Water Line (LMWL), highlighting a distinct evaporation signature.

Plant water extracted by Scholander-type pressure chamber and cryogenic vacuum distillation (twig with and without bark, and twig core collected close to the tree trunk) plotted together or close to the LMWL, with three samples from beech trees slightly deviating on the left side of the LMWL (Figure 4.4). The isotopic composition of water from plant tissues of the two plant species plotted together with a similar and large variability in the isotopic composition. For chestnut samples, $\delta^{2}H$ varied between -39.2 – -9.7 ‰ and $\delta^{18}O$ between -6.45 – -2.06 ‰, while for beech samples $\delta^{2}H$ and $\delta^{18}O$ varied between -36.2 – -8.1 ‰ and -5.88 – -4.64 ‰, respectively. Based on the dual-isotope plot, it seems that there was not a clear difference (especially for beech samples) in the isotopic composition of plant water extracted by Scholander-type pressure chamber and cryogenic vacuum distillation. However, we observed that chestnut samples extracted by pressure chamber were slightly more enriched in heavy isotopes than most of the samples obtained by the other extraction method.

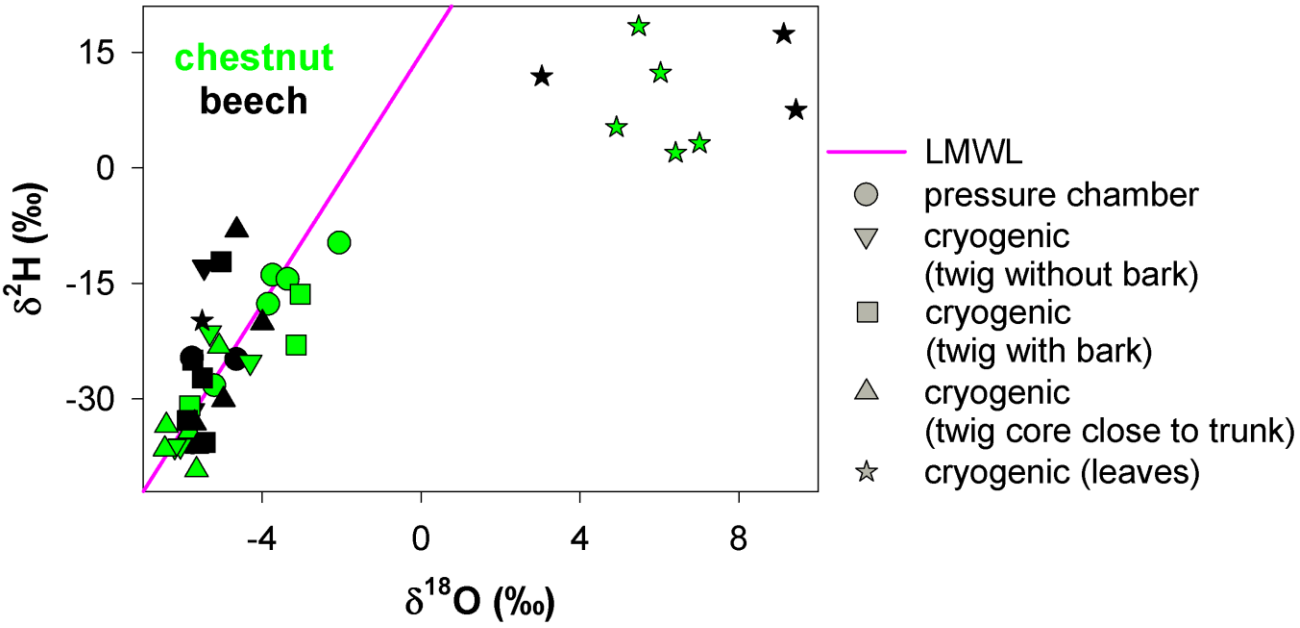

Figure 4.4. Dual-isotope plot for plant water samples extracted by Scholander-type pressure chamber and cryogenic vacuum distillation for different plant tissues and species (chestnut and beech). Ressi Local Meteoric Water Line (LMWL) is also shown ($\delta^2H = 8.12\ \delta^{18}O + 14.76$).

4.4.2 Effect of extraction method and species on plant water isotopic composition

The relation between the compositions in δ^2H and δ^{18}O for plant water extracted by Scholander-type pressure chamber and cryogenic vacuum distillation showed that there were differences between plant tissues and the two species (Figure 4.5). Indeed, we observed that most of the samples did not plot on the 1:1 line, and there were particularly very large differences in δ^2H of samples extracted by the two methods (i.e., samples obtained from twig cores located close to the trunk and twigs without bark of beech trees). Xylem water collected from beech trees by cryogenic vacuum distillation was isotopically very different from δ^2H of waters extracted by Scholander-type pressure chamber, but not systematically more enriched or depleted in heavy isotopes. Conversely, plant water extracted from beech trees by cryogenic vacuum distillation had δ^{18}O values similar to the ones obtained by Scholander-type pressure chamber (Figure 4.5). Plant water collected from chestnut trees by cryogenic vacuum distillation was always more depleted in heavy isotopes (both δ^2H and δ^{18}O) and very different than plant water samples extracted by Scholander-type pressure chamber. This difference probably indicates an overestimation in the isotopic composition of plant water extracted by

the pressure chamber compared to the more widely-used cryogenic vacuum distillation technique.

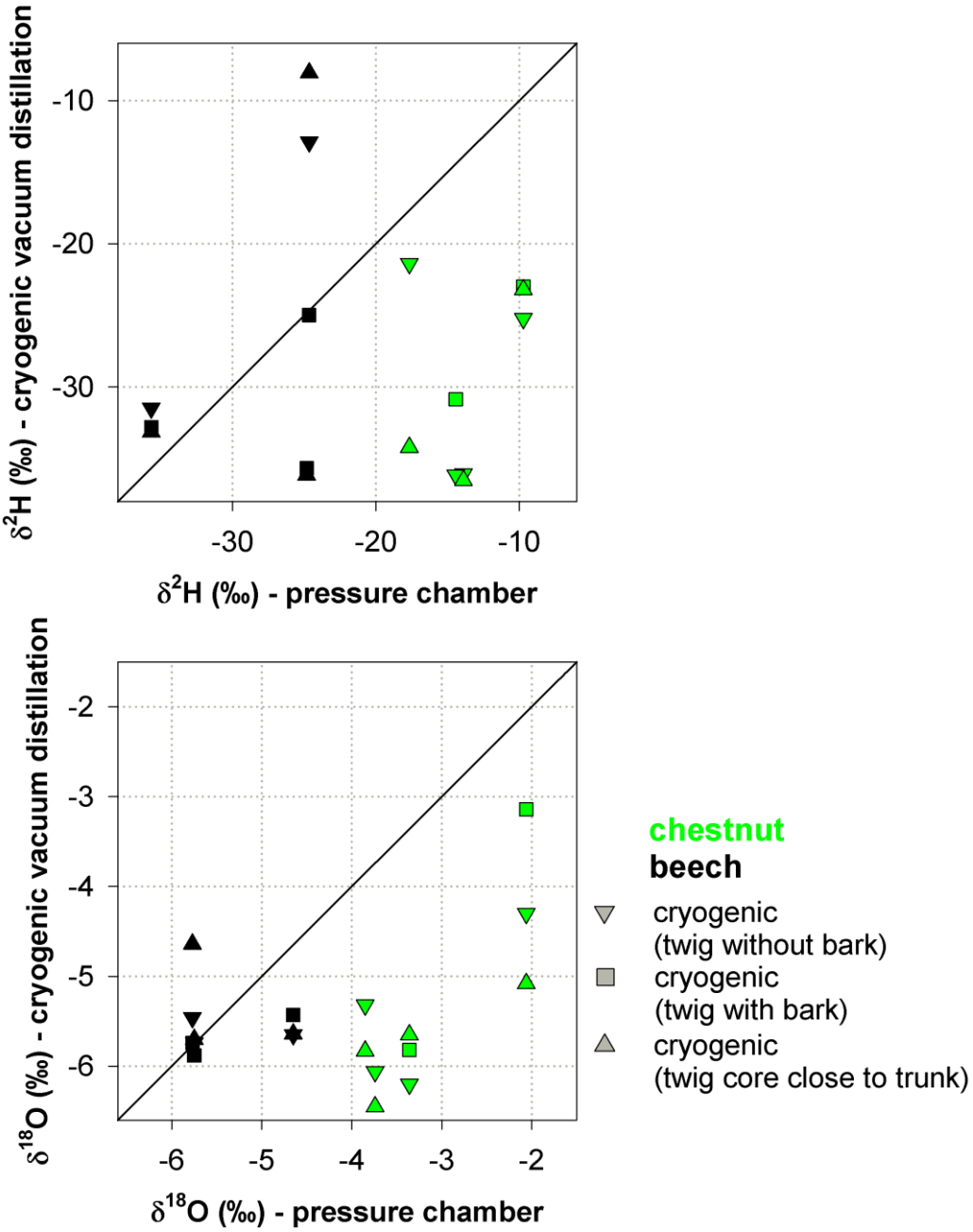

Figure 4.5. Relation between the composition in δ^2H and $\delta^{18}O$ for plant water extracted by Scholander-type pressure chamber and cryogenic vacuum distillation, grouped by plant tissue and species. The solid black lines represent y=x.

The factorial ANOVA showed that there was not a significant effect ($p>0.05$) of the extraction method and the species on δ^2H of plant water (Figure 4.6). For beech trees, average δ^2H of

plant water were quite similar for all the plant tissues and the two extraction methods, while for chestnut trees average δ^2H derived by Scholander-type pressure chamber was more enriched in heavy isotopes, although non-significantly, compared to average δ^2H obtained by cryogenic vacuum distillation. d-excess of plant water was also not affected significantly ($p>0.05$, factorial ANOVA) by the extraction method and the plant species (Figure 4.7). All average d-excess of plant water were positive, with larger values for beech samples than chestnut. Contrary to δ^2H and d-excess, we observed that there were significant effects ($p<0.05$, factorial ANOVA) of extraction methods, plant species and the interaction of the two factors on $\delta^{18}O$ of plant water (Figure 4.6).

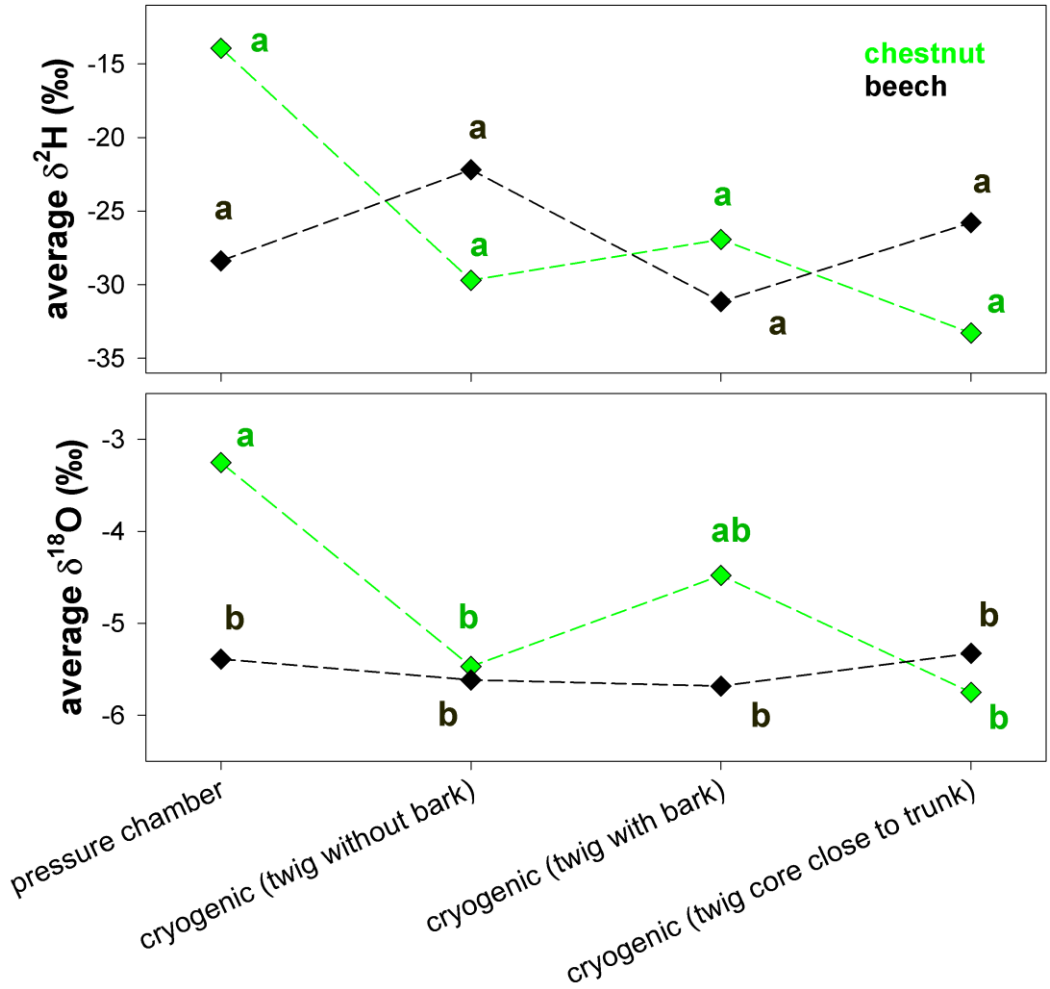

Figure 4.6. Average isotopic composition of plant water extracted by Scholander-type pressure chamber and cryogenic vacuum distillation, grouped by plant tissue and species. Different letters indicate significantly different groups ($p<0.05$, multiple comparison test based on Bonferroni method).

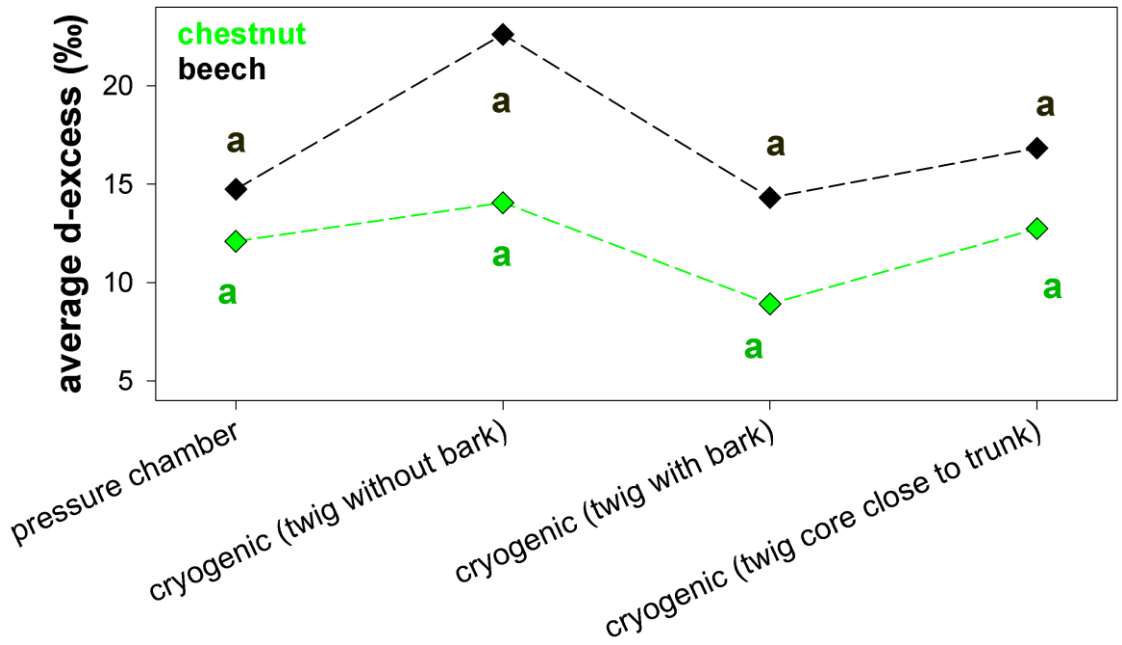

Figure 4.7. Average d-excess of plant water extracted by Scholander-type pressure chamber and cryogenic vacuum distillation, grouped by plant tissue and species. Different letters indicate significantly different groups ($p<0.05$, multiple comparison test based on Bonferroni method).

We identified that the significant difference was due to the enriched $\delta^{18}O$ of chestnut water extracted by Scholander-type pressure chamber ($p<0.05$, multiple comparison test based on Bonferroni method).

4.5 Discussion

The Scholander-type pressure chamber has the advantage of extracting plant water at the water potential level (Meiri et al., 1975; Grossiord et al., 2016), so only water that is likely used for transpiration and it is not tightly stored in the plant tissues. This extraction technique can be applied directly *in situ* or in a laboratory after a proper handling and transport of the vegetation material in sealed bags. The procedure for the extraction of plant water is also simple because it does not require specific laboratory work, like for the distillation system or handling liquid nitrogen during the cryogenic vacuum distillation. In addition, the water extraction by the Scholander-type pressure chamber generally lasts few minutes depending on the leaf water potential. The easy and fast application (without laboratory work) of the Scholander-type pressure chamber for plant water extraction can be considered comparable to the simple and low-cost methods developed by Fischer et al. (2017).

Despite these advantages, our sampling in the Ressi catchment during a dry period (beginning of July 2017) showed that the water extraction by pressure chamber cannot be always applied satisfactorily in terms of sampling volume and extraction times. For some twig samples, we had to apply a 35-bar pressure for the extraction of at least 200 µl for the isotopic analysis by IRMS, and the whole extraction lasted about 10 minutes. For other samples, the sampling procedure was complicated by the extraction of few small water drops and air bubbles that were difficult to trap into the vials. Conversely, the plant water extraction by cryogenic vacuum distillation was performed for all the samples, and generally obtaining sampling volumes larger than 200 µl. Furthermore, plant water extracts obtained by the Scholander-type pressure chamber usually were darker (yellowish, or even brownish) compared to water extracts by cryogenic vacuum distillation. The darker color of the plant water extracts suggest a likely larger concentration in organic compounds (Millar et al., 2018). In our case, the sampling volume was not enough for a quantification of the concentration of organic compounds, but this should be determined in future inter-method comparison studies.

Results of our inter-method comparison showed that the isotopic composition of the samples obtained by the Scholander-type pressure chamber were not affected by fractionation (samples plotting together with the LMWL), indicating that we did not extract significant volumes of leaf water, which is subject to water-vapor exchanges between stomata and the low atmosphere (Figure 4.4). Indeed, the leaf water enrichment is controlled by the Péclet effect (Ellsworth et al., 2013; Kahmen et al., 2008), canopy temperature, air relative humidity and seasonal changes

in water source (Kahmen et al., 2011; Brunello et al., 2019). The microclimate changes around the canopy likely drive the variations in $\delta^{18}O$ and an increase in the isotopic composition is directly proportional to increases in leaf temperature and inversely to relative humidity. Furthermore, d-excess of plant water extracted by the Scholander-type pressure chamber and the cryogenic vacuum distillation (except leaf water) were similar with values close or above 10‰ (Figure 4.7), in agreement with d-excess values observed in the Ressi catchment for rain and stream waters (Penna et al., 2015). Despite the similarity in d-excess between samples extracted with the two methods, we found that plant water collected from chestnut trees by cryogenic vacuum distillation was always more depleted in heavy isotopes (both $\delta^{2}H$ and $\delta^{18}O$) and very different than plant water samples extracted by the Scholander-type pressure chamber (Figure 4.5 and Figure 4.6). These results for chestnut tree samples are similar to those found by Millar et al. (2018). Indeed, they reported a depletion in heavy isotopes for samples derived by cryogenic vacuum distillation compared to other techniques, such as direct vapor equilibration and microwave extraction. The water extracted by the Scholander-type pressure chamber and cryogenic vacuum distillation showed large differences in the isotopic composition among plant tissues and between the two studies species. We relate the observed isotopic differences to various factors, such as the possible effect of organic compounds on the isotopic composition of plant water (Millar et al., 2018) or the different origin of the plant water extracts (e.g., derived from different water pools available for beech and chestnut trees) or the sampling material (e.g., xylem water extracted at the leaf water potential vs. more-tightly bound water stored in plant cells).

The isotopic variability of plant water across various species can depend structure characteristics of the root systems and the water availability in an environment. For instance, the tree species isotopic variability in $\delta^{18}O$ in cellulose of tree ring was observed by Marshall and Monserud (2006) in Ponderosa pine and Douglas-fir. They related their findings to the dimorphic rooting system of Ponderosa pine, which has a feeder root system that extract shallow soil water and a taproot able to take up water from deep soil layers (Ehleringer and Dawson, 1992b), compared to Douglas-fir, which relies only on a deep lateral root system. These rooting systems cause the divergence in the plant use of soil water and thus a variability can be observed in the isotopic compositions of the extracted plant water. Similarly, Richter et al. (2008) has documented tree species marked variations of $\delta^{18}O$ in wood cellulose. Some other studies reported species abilities to access different soil water pools due to different

rooting depths, e.g. variations in $\delta^{18}O$ of source water among co-occurring species (Dawson et al., 2002).

Furthermore, we observed different results when considering the two isotopes, i.e., there were larger differences between the methods and the samples found for $\delta^{2}H$ compared to $\delta^{18}O$. It has been previously noted that this isotopic variability in plant water can be linked with water ratios of the living cells versus dead vessel cells, and to changes in cell volumes as a part of entire volume of several tissues. Oxygen and hydrogen isotope enrichment of xylem water has also been reported by various studies. A slight enrichment of $\delta^{18}O$ in the xylem water upper stem than the lower stem was reported by Cernusak et al. (2005). Similarly, a $\delta^{2}H$ enrichment has also been observed by Dawson and Ehleringer (1993). While considering the phloem enrichment, there could be possible effects of phloem and bark enrichment on the xylem water (e.g., Ellsworth and Williams, 2007). It is well known from the biological systems that different $\delta^{18}O$ and $\delta^{2}H$ isotopic signature may result due to the surrounding growth media and due to contributions of metabolic oxygen and hydrogen to the intracellular water (Kreuzer-Martin et al., 2005; Kreuzer et al., 2012). The proportion of metabolic water and the intracellular water (within the living cells) depend on the residence of water within a specific cell type. Another assumption is the movement of leaf water (enriched in $\delta^{2}H$; Cernusak et al., 2005) back to the xylem vessels and thus this mixing can take place with the xylem water stored in leaves (Cernusak et al., 2006; Zhao et al., 2016). In addition, the enrichment of heavy or lighter isotopes depend on the vibrational energy change that occur during the replacement of ^{16}O for ^{18}O and ^{1}H for ^{2}H in the water molecule, and is more pronounced for hydrogen than oxygen (Ellsworth and Williams, 2007).

Concluding, our results suggest that Scholander-type pressure chamber can extract plant water in a simple and fast procedure that does not require laboratory work (Fischer et al., 2019). However, some drawbacks, such as the small sampling volume that can be extracted during dry conditions, and the isotopic differences found with the plant water extracted by the widely-used cryogenic vacuum distillation, indicate that the two methods used in this study are not interchangeable. Furthermore, we agree with Millar et al. (2018), and other inter-comparison studies carried out for soil water (Orlowski et al., 2018b; Sprenger et al., 2015), that more research is needed to compare multiple extraction methods across various environments and plant species, in order to investigate the technical and physical factors altering the isotopic

composition of plant water during the extraction, and elaborate standard protocols for ecohydrological research relying on the isotopic signature of soil and plant waters.

4.6 Concluding remarks

In this work we analyzed the variability in the isotopic composition of plant water extracted by the two methods, also considering the potential variability in the isotopic signature of the plant tissues (i.e., leaves, twig without bark, twig with bark and twig core close to the trunk of the tree) and plant species (i.e., beech and chestnut). Our results indicated that plant water extracted by Scholander-type pressure chamber and cryogenic vacuum distillation applied to twigs did not have an evaporative signature compared to water extracted from leaves, but there was a large isotopic variability among the samples. We found that, for beech samples, the difference in $\delta^{18}O$ obtained by the two extraction methods was smaller compared to the difference observed for chestnut samples. Specifically, the isotopic composition of chestnut plant water extracted by Scholander-type pressure chamber highlighted a marked enrichment in heavy isotopes compared to samples obtained by cryogenic vacuum distillation.

Furthermore, we observed different results when considering the two isotopes, with larger differences between the methods and the samples found for $\delta^{2}H$ than for $\delta^{18}O$. Based on these results, we recommend that plant water extraction by Scholander-type pressure chamber should not be considered as an alternative for cryogenic vacuum distillation. However, we call for future research comparing the same methods across more plant species and investigating the reasons behind the large differences in $\delta^{2}H$ signature of plant water extracted by the two methods.

5 CONCLUSIONS

This thesis presented an integrated perspective towards the quantification of plant water pools via a stable water isotope tracing approach. The investigation was carried out from a global scale assessment of plant water sources and the main drivers controlling the isotopic compositions of the plant and soil waters, and then moved towards the examination of the underlying assumptions and plant water extraction methodologies, which may limit the application of stable water isotopes in ecohydrological studies.

In this work, for the first time, soil water depth distributions for plant uptake were quantified for the study sites belonging to four climate zones (i.e., tropical, arid, temperate and cold zone) across the world. The former meta-analyses provided the relative contributions of groundwater and soil water, but did not assess the soil water depth contributions to plants. This global scale analysis has quantified the depths contribution of the soil water source to plant transpiration and in addition accounted for the controls of climate, and plant functional group on the isotopic composition of xylem water. This analysis was performed on a global isotopic dataset comprising both $\delta^{18}O$ and $\delta^{2}H$ of soil water and xylem water extracted from 65 papers (77 study sites) published between 1990 and 2017. Overall, a wide overlap was found between the isotopic composition of soil water and xylem water in all the climates suggesting soil water as main plant water source. Climate resulted as the main driver of the isotopic composition of soil water and, in turn, the xylem water, while the other controlling factors for instance plant type likely had less influence on the isotopic signatures of soil and xylem waters.

Further, a new direct inference approach was developed to provide the proportions of soil water based on the evaluation of the graphical overlap of xylem water with soil water at different depths. Results suggest that xylem water reflected mostly the isotopic signature of soil water at shallower soil depths (for instance a median overlap of xylem water of up to 100± 0% (for MCD and MVE methods) was found with 0-10 cm depth in the cold zone and an overlap of 84±16% for MCD (85±15% for MVE) with 10-30 cm in the temperate zone) compared to the deeper soil layers, the only exception being there was the arid climates where plants seems to be exploiting water in the deep soil profiles (e.g., a median overlap of xylem water of 96±4% for MCD, 88±4% for MVE at 30-50 cm depth in arid zone), thus indicating soil water as a major contributor to plant transpiration. Moreover, the new graphical inference method that we developed to quantify water sources contribution is easy to apply, it is an information-based

method, and therefore, it has a great prospective to be tested and applied at other study sites around the globe. However, future work should aim to test this inference approach on specific datasets while comparing the results with existing methods (e.g., mixing models based on Bayesian framework).

Additionally, an effort was made to investigate the underlined assumption and the methodological aspect that remained unclear in plant-water research based on stable water isotopic analysis. Isotopic fractionation of plant water relative to water source has been reported by some studies under both field and greenhouse conditions for different plant species. Here, this thesis attempted to test the isotopic variability in plant water from the roots to the upper part of the branches under a greenhouse setup using labelled water, and it tested the reliability of the cryogenic vacuum distillation technique through an interlaboratory comparison. Results showed that no significant differences were found in the isotopic composition of plant water at different positions along the branch within a tree. Plant water, and shallow soil waters fall within the isotopic composition of labelled water source. However, deep soil water (>15 cm soil depth) and root water seems to be deviating (i.e., heavy isotopes enrichment), hence, indicating that the other processes could occur at soil-root interface. This observed isotopic enrichment of soil water may relate to the soil specific extraction times or temperatures (as indicated by previous work on the water extraction methodologies for soils). Further, no significant differences were observed for an interlaboratory comparison of the cryogenic extraction technique (with same instrumental and parameters set up). Our results together with the other works testing the assumptions behind the use of stable water isotopes, provide important insights on the behavior of $\delta^{2}H$ and $\delta^{18}O$ in plant-water research. In short, such experiments under controlled environmental conditions need to be carried out for different plant species with a special focus on the plants undergoing stress when the environment is not in their favour. Such experiment could also help to solve the puzzle of complex physiological and biogeochemical processes taking place at the soil-root interface.

Recent studies mainly used cryogenic vacuum distillation as extraction method of plant water or compared it to other laboratory and *in situ* extraction approaches. However, no study compared the cryogenically extracted plant water with that obtained via Scholander-type pressure chamber. Therefore, an analysis of the isotopic variability of plant water extracted by the two methods was carried while taking in account the possible isotopic signature variability of different plant tissues (i.e., leaves, twig with and without bark, twig close to trunk and core

samples) and the plant species (chestnut and beech). The isotopic compositions obtained with the above two extraction methods showed significant differences between the methods and large variability found among the isotopic signatures of various sample types. The observed differences in $\delta^{18}O$ for the two extraction methods were smaller for beech plant samples in comparison to chestnut. Pressure chamber water samples highlighted a marked heavy isotope enrichment in comparison to cryogenic extraction method. Besides this, different results were obtained with $\delta^{18}O$ and $\delta^{2}H$. The results of this comparison between cryogenic vacuum distillation and the Scholander-type pressure chamber highlights the need of extending it to different plant species and setting up more comparisons among the existing plant water extraction methods.

Concluding, this thesis work suggests that further ecohydrological research needs to be performed in the climatic zones (especially tropical and arid zones) where few studies have been published so far. Collection of xylem water samples and the corresponding soil water samples (at different soil depths) is needed so that they can be linked in space and time. Future research should also focus on testing the basic underlying assumptions behind the application of water stable isotopes under controlled experimental conditions, which may help to bypass issues posed by research in field areas where boundary conditions are unknown or some key water sources cannot be sampled. In addition, more detailed methodological comparisons are urgently required to be performed for various plant water extraction methodologies by taking into account different extraction method parameters (such as extraction time, temperature, pressure and/or set up) while giving significant consideration to the methodological uncertainties.

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7 List of publications

7.1 Scientific papers

Amin, A., Zuecco, G., Geris, J., Schwendenmann, L., McDonnell, J.J., Borga, M., Penna, D., 2019. Depth distribution of soil water sourced by plants at the global scale: a new direct inference approach. Ecohydrology (accepted for publication in Ecohydrology).

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7.2 Book chapters

Zuecco G., Penna D., Marchina C., Gelmini Y., **Amin A.**, Borga M., 2019. La risposta idrologica in piccoli bacini montani: il caso studio del bacino del Rio Ressi (Prealpi Venete) - Hydrological response of small mountain catchments: the case of the Rio Ressi catchment (Italian pre-Alps). *In AA.VV.: Attualità dell'idraulica agraria e delle sistemazioni idraulico-forestali. Scritti in onore di Giovanni Tournon. Quaderni di Idronomia Montana*, 36, 135-143, a cura di S. Ferraris e M. Previati. EdiBios, 2019. ISBN: 978-88-97181-68-2

7.3 Abstracts

Amin, A., Penna, D., Zuecco, G., Borga, M., 2017. Soil water and xylem water isotope composition across different climate zones. International workshop on "Isotope-based studies of water partitioning and plant-soil interactions in forested and agricultural environments". San Casciano in Val di Pesa (FI), Italy, 27-29 September 2017.

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Zuecco G., Penna D., Marchina C., Gelmini Y., **Amin A.**, Borga M., 2018. La risposta idrologica in piccoli bacini montani: il caso studio del bacino del Rio Ressi (Prealpi Venete). Convegno in memoria del professor Giovanni Tournon, AIIA2018. Turin, Italy, 21-22 June 2018.

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Author contributions

I performed the literature search, compiled the databases, developed R scripts, did the data analyses and contributed to the manuscript write-up of the three underlined papers. I'm responsible for the organization of Greenhouse experiment, field and the indoor data collections, the subsequent laboratory analyses (laboratories in Italy and Canada) and the presentation of the results.

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