A comparative study of plant water extraction methods for isotopic analyses: Scholander-type pressure chamber vs. cryogenic vacuum distillation

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Abstract.

Recent tracer-based studies using stable isotopes of hydrogen and oxygen showed that different methods for extracting water from plant tissues can return different isotopic composition due to the presence of organic compounds, and the extraction of different plant water ~~pools~~domains. One of the most used methods to extract plant water is the cryogenic vacuum distillation (CVD), which tends to extract total plant water. Conversely, the Scholander-type pressure chamber (SPC), which is commonly used by tree physiologists to measure ~~shoot water potential~~ water potential in plant tissues and determine plant water stress, ~~has been rarely applied~~ likely accesses only the more mobile plant water (i.e., xylem and inter-cellular water). However, only few studies reported the application of SPC to extract plant water for isotopic analyses, and therefore, inter-method comparisons between SPC and CVD are urgently needed.

In this work, we analyzed the variability in the isotopic composition of plant water extracted by SPC and CVD, also considering the potential variability in the isotopic signature of the plant ~~tissues~~ water extracted from various tissues by CVD (i.e., leaves, twig without bark, twig with bark, twig close to the trunk of the tree, and wood core)~~and~~ , and from different plant species (i.e., alder, apple, chestnut and beech). The extraction of plant water by SPC is simple, can be carried out ~~*in situ*~~in the field, and it does not require specific laboratory work as in case of CVD. However, the main limitation of SPC is the very small water volume that can be extracted from the lignified ~~shoots~~ twigs during conditions of water deficit, compared to CVD.

Our results indicated that plant water extracted by SPC and CVD were significantly different. The difference in the isotopic composition obtained by the two extraction methods was smaller in the beech samples compared to alder, apple and chestnut samples. The isotopic signature of alder, apple and chestnut plant water extracted by SPC was more enriched in δ^2H and δ^{18}O, respectively, than the samples obtained by CVD (except for the leaf water obtained by CVD). We conclude that plant water extraction by SPC is not an alternative for CVD, as SPC likely extracts ~~only water within the xylem (dead cells)~~mostly the mobile plant water, whereas CVD tends to retrieve all water stored in the sampled tissue, from both living and dead cells. However, studies aiming to quantify the relative contribution of the water sources to transpiration should rely more on the

isotopic composition of xylem water transpiring during the sampling day (which is theoretically sampled by SPC), than the isotopic composition of total plant water (sampled by CVD), which also contains a fraction of water that could be stored in plant tissues for ~~long~~ a longer time.

Keywords: stable isotopes of hydrogen and oxygen; cryogenic vacuum distillation; Scholander-type pressure chamber; plant water; xylem water.

1 Introduction

Stable isotopes in the water molecule (^{2}H and ^{18}O) have been extensively used as environmental tracers in ~~atmospheric and~~ hydrological studies to track water ~~movement, estimate~~ fluxes and estimate water flow pathways, mean residence times, and water storage (e.g., Dansgaard, 1953; Craig, 1961; Klaus and McDonnell, 2013). The development of low-cost and easy-to-use spectroscopic 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 isotopes to investigate ~~the water fluxes~~ water transfer in the soil-plant-atmosphere continuum (Brooks et al., 2010; McDonnell, 2014). An increasing number of ~~new~~ studies has been recently conducted to better understand water dynamics, such as water uptake and evapotranspiration partitioning, in the soil-plant-low atmosphere continuum in different climates and in both 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; Quade et al., 2019; Zhang et al., 2019; Penna et al., 2020) environments. ~~Compared to~~ Despite the rapid increase of the number of studies ~~using a~~ based on the stable isotope approach, only a small fraction of them focused on the comparison of two or more soil or plant water extraction techniques (Sprenger et al., 2015; Orlowski et al., 2016b; Millar et al., 2018; Fischer et al., 2019).

Ecohydrological studies relying on the isotopic signature of plant water require sampling methods that extract water representative of transpiration, and that do not alter the ~~true~~ original isotopic composition of the plant material~~, which~~ . This is still a critical ~~issue~~ aspect because a standardized and shared procedure and method for isotope-based ecohydrological studies has not been ~~developed~~ defined 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)~~ (Sprenger et al., 2015; Volkmann et al., 2016; Marshall et al., 2020), microwave extraction (Munksgaard et al., 2014), cryogenic vacuum distillation (Koeniger et al., 2011; Orlowski et al., 2013, 2016a), centrifugation (Peters and Yakir, 2008), and high-pressure mechanical squeezing (Böttcher et al., 1997). Among these, cryogenic vacuum distillation (abbreviated in CVD thereinafter) is widely applied (Orlowski et al., 2018; Amin et al., 2020). ~~This~~ During CVD, the soil or plant material is heated in a tube under a specified vacuum to evaporate the sample water, that afterwards is frozen and collected in a cryogenic trap (Koeniger et al., 2011; Orlowski et al., 2013). As such, this technique extracts the entire volume of water from plant tissues ~~, which~~ (Millar et al., 2018). This volume may include water with a different age, and stored in dead and living cells for ~~months or years~~days or weeks (Sprenger et al., 2019), so not only water that is ~~being~~ transported at the time of the sampling. This might be a serious limitation in ecohydrological and physiological studies aiming at understanding water sources for plant

transpiration ~~. Different~~ as the isotopic composition of water stored in plant tissues for a long time is possibly different from that of xylem water. Moreover, experimental evidence showed that different techniques might return different isotopic values ~~because of~~ due to intrinsic methodological differences ~~. This is also the case of CVDthat, for examples, provides a large variability of results in~~ (Beyer and Penna, 2021). CVD, in the case of soil water~~extraction (Orlowski et al., 2018). Indeed, the inter-laboratory comparison of the cryogenic water extraction systems carried out by Orlowski et al. (2018) showed that there were~~ , was shown to reveal large differences in the isotopic composition of ~~the extracted soil water among~~ water extracted from soil samples by different laboratories, although ~~the same procedure, soil, and labelling water was used. The~~ strictly consistent procedures were applied (Orlowski et al., 2018). These authors also observed no clear trends in the results~~due to construction systems and applied extraction conditions, but~~ , and differences depended on the ~~interaction~~ interplay of multiple factors, such as soil type and properties, soil water content, system setup, extraction efficiency, extraction system leaks, and each ~~lab~~laboratory's internal accuracy.

Recently, Millar et al. (2018) performed a thorough comparison of ~~different techniques for plant water extraction was presented by Millar et al. (2018), who performed an inter-method comparison of~~ six plant water extraction techniques (i.e., direct ~~vapor~~ vapour equilibration, microwave extraction, two versions of CVD, centrifugation, and high-pressure mechanical squeezing) ~~tested~~ based on four plant portions of spring wheat (*Triticum aestivum*). The authors found marked differences among the measured isotopic compositions of ~~the~~ plant water, with the CVD systems and the high-pressure mechanical squeezing producing waters more depleted in heavy isotopes ~~. Millar et al. (2018)~~ compared to the other techniques. Particularly, Millar et al. (2018) associated the differences in the isotopic compositions of plant water to the ability of each extraction system to access different plant water domains. The authors argued that CVD, microwave extraction, centrifugation, and high-pressure mechanical squeezing could access all plant water domains (i.e., the more mobile xylem water and inter-cellular water, and the less mobile intra-cellular, cell wall, and organelle constrained water), whereas direct vapour equilibration could only extract the mobile xylem and inter-cellular water. Millar et al. (2018) concluded that, in terms of limited co-extraction of organic compounds and speed of sample throughput, the direct ~~vapor~~ vapour equilibration outperformed CVD. ~~Fischer et al. (2019) proposed~~

Fischer et al. (2019) proposed and described various low-tech plant water sampling and extraction techniques, and compared them to the ~~widely-used~~ CVD developed by Koeniger et al. (2011) ~~. These authors developed six simple and low-cost methods to extract plant water and compared them to CVD. They~~ for different plant species. Fischer et al. (2019) found that the new methods ~~extracted plant water consistently and comparably to what was done with CVD.~~ produced consistent and comparable results to those provided by CVD. However, these authors, due to the limited amount of plant material, could not assess the water domains accessed by the different methods for each plant type. Fischer et al. (2019) also showed that other factors, such as appropriate transport and storage of the samples from the field site to the laboratory, fast sample processing, and efficient workflows, significantly influenced the accuracy and precision of the measured isotopic composition.

~~Despite the increasing~~ Comparing different techniques for plant water extraction, understanding which water domain each method accesses, and whether isotopic fractionation occurs during the extraction process are becoming increasingly important, particularly when isotopic differences between plant water and the respective potential water sources used for transpiration are observed. Indeed, Barbeta et al. (2019) found that isotopic fractionation resulting in an unexpected depleted δ^2H of xylem

water complicated the identification and quantification of the water sources used by beech trees in a temperate forest in France. These authors recommended that future research should investigate the physico-chemical fractionation processes occurring in the unsaturated zone, and improve the understanding of the isotopic dynamics of water stored within the plant tissues. If plant water domains have distinct isotopic signatures, new techniques should be developed with the aim of extracting the target plant water domain.

Despite the number of studies ~~focusing on inter-laboratory and inter-method~~ that focused on the comparison of techniques for plant water extraction, previous ~~research did not consider~~ and current research, so far has not considered ecophysiological-based methods that tree physiologists usually adopt to measure leaf water potential and determine plant water stress ~~(e.g., Meiri et al., 1975; Grossiord et al., 2017; Bowling et al., 2017)~~ (e.g., Scholander, 1966; Meiri et al., 1975; Grossiord et al., 2017; Bowling et al., 2017). One of these methods, ~~i.e.~~ namely the Scholander-type pressure chamber (abbreviated in SPC thereinafter) ~~takes advantage of~~ is based on an external pressure to retrieve the mobile water transported within the xylem conduits to measure the ~~shoot~~ plant water potential. ~~Despite that SPC is widely-used~~ Although SPC is widely used in plant water relations studies ~~, it seems that it~~ to measure plant water potential, it is not commonly applied to collect the extracted water for isotopic analyses. ~~So far, we~~ We have found only ~~two studies (i.e., Penna et al., 2013; Geiβler et al., 2019) reporting the isotopic composition of plant water extracted by SPC. Of the two studies, Geiβler et al. (2019)~~ four published studies (Ellsworth and Williams, 2007; Penna et al., 2013; Geiβler et al., 2019; Magh et al., 2020) that used SPC to extract plant water for isotopic analysis. One of them, Geiβler et al. (2019), made a simple comparison between δ^{18}O of water extracted by SPC and CVD in ~~six~~ stem water samples collected from *Acacia mellifera* shrubs~~, and found no~~. Samples were 10 cm long and lignified, and, for SPC extraction, the authors removed leaves, bark, and green tissues to avoid contamination with phloem. Overall, Geiβler et al. (2019) found no significant difference in the isotopic composition of the plant water extracted by the two methods. However, ~~despite this first basic comparison made by Geiβler et al. (2019), no other robust attempts were made to compare SPC with the widely-used CVD extraction technique~~this analysis was based on the comparison of six samples and one plant species only, and more robust comparative tests are missing. Therefore, assessing potential differences in isotope data retrieved by using SPC and the CVD extraction techniques based on a larger number of samples and different plant species is urgently needed.

~~Therefore, in~~ In this study we ~~compare the isotopic composition of plant water extracted by~~ assume that SPC extracts relatively mobile plant water only, in contrast to CVD, which accesses all plant water domains (Millar et al., 2018). Given that the relatively mobile plant water might have a different age and a different isotopic composition compared to the less mobile water domain (Sprenger et al., 2019), we hypothesize that SPC and CVD ~~(considered here as the reference method). Specifically, our research aims~~ return significant differences in the isotopic composition of the extracted plant water. Therefore, our specific objectives are to: *i)* quantify the differences in the isotopic composition of plant water extracted by the two techniques, and *ii)* assess how differences in the isotopic composition are related to plant species or plant tissue type used for CVD.

2 Study sites and sampling

2.1 Ahr/Aurino

Samples from grey alder trees (*Alnus incana*) were taken at two sites in the riparian area of the Ahr/Aurino River in the Eastern Italian Alps (Fig. 1) (Engel et al., in review). The study site is located at about 882 m a.s.l. in the lower valley, where the typical valley form is U-shape. The ~~basin~~ catchment is mostly composed of metamorphic (gneiss, micaschists) and magmatic (tonalite) rocks. The median diameter of sediment in the upper meter of soil in the former floodplain ranges from 0.3 to 0.5 mm (Andreoli et al., 2020). The climate is cold temperate with an average annual air temperature of about 7.7 °C (period 1992-2018) and an average yearly precipitation amount of about 821 mm/yr (period 1972-2018) ~~(Autonomous Province of Bozen-Bolzano)~~(Engel et al., in review). The Ahr/Aurino River regime is nivo-glacial (the glacierized area is about 4 %). Riparian vegetation mainly consists of ~~relatively mature patches~~ mature (at the upstream site in Fig. 1) and juvenile patches (at the downstream site) of grey alder with a thick tall herb (*Rubus caesius*, *Glechoma hederacea* and *Urtica dioica*, *Sambucus nigra* shrubs and the vine *Humulus lupulus*). Gravel mining activities in the 1950s to the 1980s resulted in riverbed incision, and a floodplain being disconnected from its channel (Campana et al., 2014).

The sampling campaign was carried out on 7 June 2017 during a period of ~~prolongated~~ prolonged water deficit. ~~Samples for plant water extraction were~~ Due to logistic issues, and to collect samples when the transpiration fluxes were close to their minimum, plant water was collected after the sunset from four alder trees (two at the downstream and two at the upstream site) in the Ahr/Aurino study area. ~~The selected alder trees were located within few meters to each other to avoid high spatial variability in the isotopic composition of plant water~~Samples for water extraction by SPC and CVD were taken from the same position in the trees.

2.2 Laas/Lasa

Samples from cultivated apple trees (*Malus domestica*, cv. *"Pinova"* grafted on *"M9"* rootstock) were collected in two apple orchards in the Laas/Lasa (Vinschgau/Val Venosta region, South Tyrol; Fig. 1). The orchards are located at about ~~800~~ 860 m a.s.l. on the right and left side of the river Etsch/Adige, with different distance from the river (50 m vs. 450 m, respectively) (Penna et al., 2021). Within each orchard, a plot of about 400 m^2 was selected for sampling. The average annual ~~air temperature is about year 9 °C and the average yearly precipitation amounts about 512 mm~~precipitation recorded at the Laas/~~yr (2009-2018)~~ ~~(~~Lasa weather station (874 m a.s.l., operated by the Hydrographic Office of the Autonomous Province of Bozen-Bolzano) ~~.~~ was approximately 480 mm (period 1989–2012) (Penna et al., 2021). Minimum average temperatures are below 0 °C during winter (from December to February), while maximum average temperatures can reach 24 °C in July (Penna et al., 2021). The soil in both orchards had a silty loam texture.

The sampling campaign was performed on 8 June 2017 during a period of water deficit. All samples were equally taken both at the right and the left field~~,~~. Due to logistic issues and to collect samples when the transpiration fluxes were close to their minimum, samplings were carried out after the sunset. Samples for water extraction by SPC and CVD were taken from the same position in the trees.

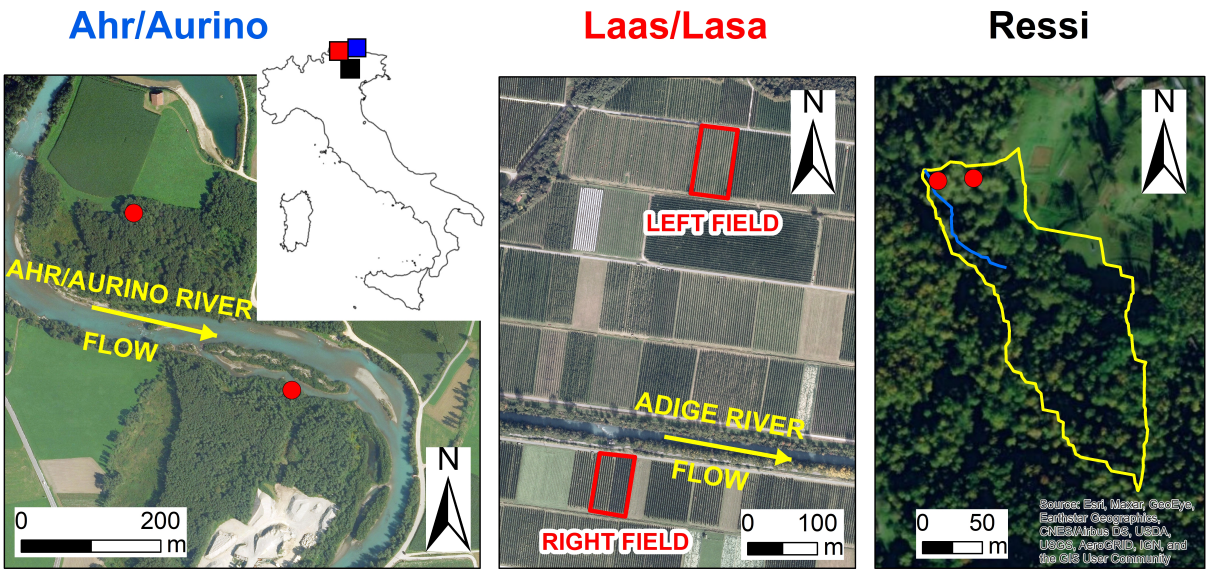

Figure 1. Aerial photos of the study sites, and location in northern Italy (blue: Ahr/Aurino; red: Laas/Lasa; black: Ressi). Red dots in Ahr/Aurino and Ressi indicate the approximate position of the sampled trees. In Ressi, the blue and the yellow solid lines mark the stream network and the catchment divide, respectively. Sources of aerial photos: ©Autonomous Province of Bolzano-South Tyrol (study sites: Ahr/Aurino and Lasa); Esri, "World Imagery" [basemap], scale not given, "World Imagery", August 14, 2020, http://www.arcgis.com/home/item.html?id=10df2279f9684e4a9f6a7f08febac2a9, (September 1, 2020) (study site: Ressi). The maps were made using Esri ArcGIS 10.7.1.

2.3 Ressi

Samples from beech (*Fagus sylvatica*) and chestnut (*Castanea sativa*) trees were collected in the 2.4-ha Ressi catchment in the Italian pre-Alps (Fig. 1) ~~(Penna et al., 2013; Zuecco et al., 2014; Penna et al., 2015; Zuecco et al., 2016)~~ (Zuecco et al., 2014; Penna et al., 2015; Zuecco et al., 2016, 2021). 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 (period 1992-2007) recorded by a weather station approximately 4.5 km from Ressi is 1695 mm/yr (Penna et al., 2015). 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). The top 10 cm of the soil has a sandy clay loam texture; deeper in the profile the soil has a sandy clay texture (Penna et al., 2015; Zuecco et al., 2021).

The sampling campaign was carried out on 5 July 2017 during a period of ~~prolongated~~ prolonged water deficit. Samples for plant water extraction were retrieved at the sunrise from five beech and five chestnut trees at two sites in the lower part of the Ressi catchment. Samples for water extraction by SPC and CVD were taken from the same position in the trees. The sampling design aimed to replicate the sample collection in all the selected trees with the two investigated methodologies (Table 1). However, plant water extraction was not always possible by the SPC method because, in some cases (1 out of 5

Table 1. Sample size~~and median~~, mean sample volume, weighted mean isotopic composition (based on the extracted water volumes used as weights), and weighted mean lc-excess of the samples extracted by Scholander-type pressure chamber (SPC) and cryogenic vacuum distillation (CVD) from different plant tissues (L: leaves; T: twig without bark; TwB: twig with bark; WC: wood core; TcT: twig close to the trunk), and species in the three study sites (Ahr/Aurino, Laas/Lasa and Ressi). Note that SPC samples consisted of lignified twigs with bark and leaves attached to the twig.

Plant species	Sample type	Sample size	~~Median~~ δ^2H ~~Mean~~ volume (μl)	~~Median δ^{18}O~~ Weighted mean δ^2H (‰)	Weighted mean δ^{18}O (‰)	Weighted mean lc-excess (‰)
Alder (Ahr/Aurino)	SPC	4	~~-37.8~~ 117	~~-4.95~~ -38.0	-5.08	-2.7
	CVD-L	4	~~-7.6~~ 1025	~~8.99~~ -9.1	8.64	-74.2
	CVD-T	4	~~-53.7~~ 575	~~-6.51~~ -54.9	-6.80	-7.0
	CVD-TwB	4	~~-80.7~~ 550	~~-7.37~~ -77.2	-7.62	-23.4
	CVD-WC	4	~~-49.4~~ 125	~~-6.86~~ -48.1	-6.79	-0.3
Apple (Laas/Lasa)	SPC	8	~~-63.8~~ 104	~~-8.91~~ -64.0	-9.02	1.1
	CVD-L	8	~~-12.9~~ 1910	~~11.92~~ -13.8	11.26	-103.3
	CVD-T	5	~~-75.7~~ 610	~~-10.10~~ -76.1	-9.84	-4.8
	CVD-TwB	8	~~-80.6~~ 711	~~-9.91~~ -81.0	-9.90	-9.2
	CVD-WC	5	~~-85.7~~ 254	-84.9	-10.52	-8.5
Chestnut (Ressi)	SPC	4	~~-14.2~~ 183	~~-3.55~~ -13.6	-3.20	-2.4
	CVD-L	4	~~8.8~~ 1600	~~5.76~~ 11.1	5.45	-47.9
	CVD-T	4	~~-30.6~~ 975	~~-5.69~~ -29.3	-5.41	-0.1
	CVD-TwB	2	~~-26.9~~ 1050	~~-4.48~~ -27.5	-4.67	-4.3
	CVD-TcT	4	~~-35.4~~ 1350	~~-5.74~~ -32.6	-5.69	-1.1
Beech (Ressi)	SPC	3	~~-24.8~~ 180	~~-5.75~~ -28.7	-5.47	0.9
	CVD-L	3 (1 for δ^2H)	1267	7.5	~~4.87~~ 7.58	-83.9
	CVD-T	3 (2 for δ^2H)	~~-22.2~~ 1133	~~-5.65~~ -26.0	-5.66	5.2
	CVD-TwB	3	~~-32.8~~ 600	~~-5.74~~ -32.2	-5.67	-0.9
	CVD-TcT	3	~~-33.1~~ 1267	~~-5.64~~ -28.9	-5.46	0.7

chestnut samples, and 2 out of 5 beech samples), the extracted plant water volume was not always enough for isotopic analysis. In addition, we discarded some plant water samples extracted by CVD, affected by bad injections, and for which we could not perform a second run of isotopic analyses due to the small water volume. Therefore, in this study we reported only the isotopic data relative to the plant water extracted by both methods (i.e., SPC and CVD) from the same trees (Table 1).

3 Materials and methods

3.1 Extraction of plant water: the SPC method

The SPC is an instrument normally used by tree physiologists to measure shoot water potential (e.g., Meiri et al., 1975; Donovan et al., 2003; Grossiord et al., 2017)water potential in plant tissues (e.g., Scholander, 1966; Meiri et al., 1975; Donovan et al., 2003; Grossiord et al., 2017). Typically, SPC is used to determine plant water potential (Cochard et al., 2001) and, being a proxy of the tissue water content, it can signal the occurrence of water deficit. The basic working principle is the use of an external pressure to retrieve the water within the xylem conduits (Scholander et al., 1965; Turner, 1981; Castro Neto et al., 2004) (Fig. 2). In this study, we used the SPC to force water out of the xylem tissuestwigs, and collect water samples for isotopic analyses. The sampling material consisted of lignified twigs, with a diameter ranging between 3 and 6 mm. In agreement with Penna et al. (2013, 2021), we kept the bark and the leaves attached to the twig.

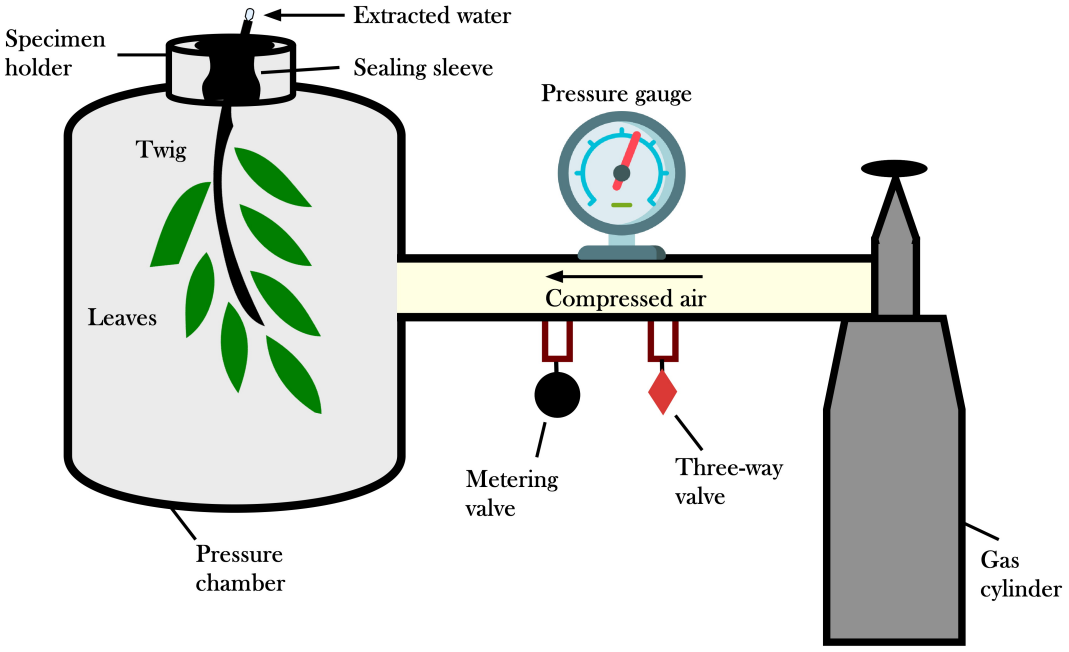

Figure 2. Sketch showing the technical setup of the Scholander-type pressure chamber (SPC).

The SPC contains a cutting board used to prepare the sample, i. e. one-year old suberized shoots. sampled twigs. The set up consisted of one or more leaves sealed inside the chamber, while the cut end of the suberized shoot lignified twig was exposed to the atmosphere (Fig. 2). After connecting the SPC to the gas tank, a three-way control valve was turned to pressure and the

metering valve was slowly opened to begin to pressurize the unit. A pressure equal to ~~shoot~~ the water potential was applied until water sprang from the cut end of the ~~one-year old shoot~~twig. For the SPC plant water extractions, we used a Pump-Up Chamber with a 2.0 MPa gauge (PMS Instrument Company, Oregon, USA) in Ahr/Aurino and Laas/Lasa, and a SAPS II portable plant water status console (model 3115) with a 4.0 MPa gauge (Soilmoisture Equipment Corp., California, USA) in Ressi. The ~~xylem~~ plant water was collected ~~directly (or by using pipettes)~~ in 2 ml glass vials ~~, (~~which were immediately capped~~.~~) by using pipettes or with the help of gravity (SPC was put on its side).

To extract water from the plant tissues, we had to apply a pressure of about 0.5 MPa in Laas/Lasa, 1.0-1.5 MPa in Ahr/Aurino, and 3.0 MPa in Ressi; the different ~~shoot~~ plant water potentials indicate that the sampled vegetation in Ressi suffered higher water deficit conditions than the sampled plants in Ahr/Aurino and Laas/Lasa. The ~~water~~ lower water deficit in Laas/Lasa than in the other two sites can be explained by the irrigation of the apple orchards during dry periods. The water extraction by the SPC method ended when we collected all the water flowing out from the ~~shoots.~~ twigs. The duration of the extraction was different among the samples (due to the different water deficit conditions), but we kept it as short as possible (less than 10 min) to minimize the evaporation. Note that the sampled volume was smaller than ~~2 ml~~ 200 μl during the sampling campaigns carried out for this study (Table 1). All the samples were stored in a fridge at 4 °C until the isotopic analyses.

3.2 Extraction of plant water: the CVD method

To extract plant water by CVD, we collected samples from different plant tissues, along a branch, in 12 ml glass Exetainer® vials (Labco Ltd., UK). After cutting the twigs from the trees, we removed all the leaves and other green tissues close to the leaves. Some of these leaves were collected in the vials for the extraction by CVD (i.e., CVD-L samples). CVD-L samples were used to determine the isotopic composition of the fractionated leaf water. The twig samples were lignified and approximately 85 mm long and 3-6 mm thick. For some of the twig samples, we kept the bark (i.e., twig with bark samples, abbreviated in CVD-TwB), whereas for others, bark was peeled using a knife (i.e., twig without bark samples, abbreviated in CVD-T; Table 1). ~~Samples with and without bark were used to test whether the plant water extracted by the SPC method had an isotopic composition more similar to~~ CVD-extracted ~~bulk~~ total plant water (i.e., CVD-TwB) ~~or to~~ and total plant water deprived of ~~phloem tissues~~ the phloem tissue (i.e., CVD-T) were used to assess the differences in the isotopic composition with the plant water extracted by the SPC method. In Ahr/Aurino and Laas/Lasa study sites, the diameters at the breast height of the alder and apple trees allowed for the collection of wood core samples (abbreviated in CVD-WC), that were retrieved by an increment borer (phloem tissue was removed, and the heartwood was not collected during the samplings). In Ressi, instead of wood cores (the sampling was not possible because of the small tree diameters and the location in a private land), we collected additional twig samples that were located close to the trunk (abbreviated in CVD-TcT). For these samples, we removed the bark by a knife. CVD-TcT samples were supposed to have older tissues and more dead cells than the twigs collected closer to the leaves (i.e., CVD-T and CVD-TwB). However, we need to consider that CVD applied to lignified samples could still extract water stored in living xylem parenchyma cells, and the total ray and axial parenchyma tissue fractions can be 21.1 $\pm$ 7.9 % (average $\pm$ standard deviation) in temperate angiosperm trees (Morris et al., 2016).

The plant water volume of CVD samples was larger than volume of SPC samples (Table 1), with a minimum of 100 μl (three CVD-WC samples from alder trees) and a maximum of 2690 μl (a CVD-L sample from an apple tree). All the samples for CVD were stored in a fridge at 4 °C until the extraction and the consequent isotopic analyses.

The CVD was performed in the laboratory of the Faculty of Science and Technology of the Free University of Bozen-Bolzano (Italy) (Fig. 3). The CVD system was developed based on the method of Koeniger et al. (2011). The system consists of independent extraction-collection units, where the capped sample vial was connected to a second empty vial (hereafter collection vial) using a 1.56 mm diameter stainless steel capillary tube. After the preparation of the extraction-collection unit, the samples were frozen by immersing the sample vials in liquid nitrogen (approximately at –196 °C) to prevent loss of water ~~vapor~~ vapour 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 (Fig. 3). At the same time, during the extraction process, the bottom of the collection vials was immersed 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~~ vapour 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 liquid nitrogen cold trap, defrosted at room temperature under perfect sealed conditions and stored in a refrigerator after labelling, and tightly wrapped with Parafilm® 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 before and after water extraction, and after the oven-drying at 100 °C for 24 hours to determine the extraction efficiency. We obtained an average extraction efficiency of 98.6 % (n = 65), whereas the median was 100 %.

3.3 Isotopic analysis

Isotopic analyses were performed by isotope-ratio mass spectrometry (IRMS) at the Faculty of Science and Technology of the Free University of Bozen-Bolzano. All water samples were analyzed using an IRMS (Delta V Advantage Conflo IV, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), coupled with a Thermo Scientific Gas Bench II to determine δ^{18}O.

For δ^{18}O, water samples 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 hours, the headspace ~~vapor~~ vapour phase was injected 8 times.

δ^2H was determined by direct injection of sample into the IRMS, through Thermo Scientific High Temperature Conversion Elemental Analyzer (TC/EA, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), equipped with an autosampler (Thermo Scientific AI/AS 3000).

The samples were calibrated with standards relative to the Vienna Standard Mean Ocean Water. The standard deviation of the isotopic measurements performed by the IRMS was 2.5 ‰ and 0.10 ‰ for δ^2H and δ^{18}O, respectively.

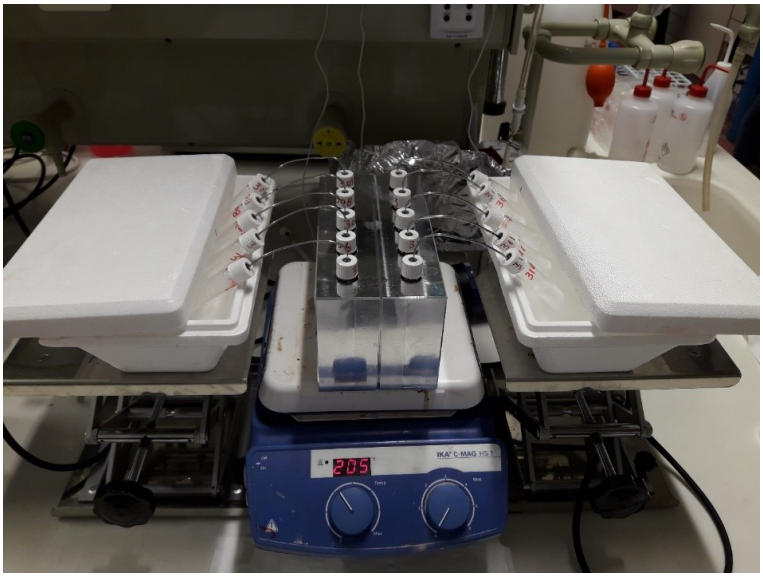

Figure 3. The cryogenic vacuum distillation (CVD) system at the Faculty of Science and Technology of the Free University of Bozen-Bolzano, based on the method developed by Koeniger et al. (2011).

3.4 Data analysis

The isotopic values of the samples were grouped based on the extraction method (i.e., SPC and CVD) and plant species (i.e., alder, apple, chestnut, and beech trees). In addition, samples extracted by CVD were grouped based on the collected plant tissue (i.e., leaves (CVD-L), twig with bark (CVD-TwB), twig without bark (CVD-T), twig close to the trunk of the tree (CVD-TcT) and wood core (CVD-WC)). In total, we considered 24 groups of samples for data analyses.

The samples were plotted in the dual-isotope space, together with the Local Meteoric Water Lines (~~LMWL~~LMWLs) of the three study areas, obtained by the ordinary least squares regression (Marchina et al., 2020), to identify potential evaporated samples. For each sample, we also computed the ~~deuterium excess (d-excess) (?),~~ line-conditioned excess (lc-excess) (Landwehr and Coplen, 2004), which considers the deviation from the LMWL, as follows:

$$d-excesslc-excess = \delta^2 H - 8H - a \times \delta^{18} OO - b \quad (1)$$

~~We report the d-excess values, which take into account deviation from the Global Meteoric Water Line, instead of the line-conditioned excess (Landwehr and Coplen, 2004), which accounts the deviation from the LMWL, to compare samples from the different study areas~~where a and b are the slope and the intercept of the LMWLs of the three study sites (equations reported in Fig. 4). Negative values of lc-excess mean that the samples experienced isotopic fractionation by evaporation (these samples plot below the LMWL).

Scatter plots between SPC with CVD-T, CVD-TwB, CVD-TcT and CVD-WC samples were used to assess differences (overestimation or underestimation) in the isotopic values. The Friedman repeated measures analysis of variance on ranks,

paired with a multiple comparison test based on the Tukey method, was used to identify ~~possible effects of the extraction method on~~ significant differences (at the 0.05 level) in the isotopic composition and ~~d-excess~~ lc-excess of plant water extracted by the two methods and for the various tissues, collected from alder and apple trees (these tests were not applied to chestnut and beech data because the paired samples were < 4). The Welch two-sample t-test was used to assess whether the differences in the isotopic composition of SPC and CVD-L samples from alder and apple trees were significant (at the 0.05 level).

To evaluate the differences in the isotopic composition between SPC and CVD extracted samples, while accounting for the uncertainty in the isotopic measurements, we computed the Z-scores for each paired sample and isotope (Wassenaar et al., 2012; Orlowski et al., 2016b), as follows:

$$Z - score = \frac{|CVD - SPC|}{SD} \quad (2)$$

where CVD is the δ^2H or δ^{18}O value of the cryogenic extracted samples, SPC is the δ^2H or δ^{18}O value of the SPC samples, and SD is the typical standard deviation of the isotopic measurements ~~performed by the IRMS~~ (in our case, 2.5 ‰ and 0.10 ‰ for δ^2H and δ^{18}O, respectively). For the CVD samples, we distinguished the various plant tissues, i.e. CVD-T, CVD-TwB, CVD-TcT and CVD-WC. Similar to Orlowski et al. (2016b), for Z-score < 2, we considered the difference between the extraction methods acceptable (i.e., the observed difference can be considered equal or lower than the uncertainty in the isotopic measurements), for 2 < Z-score < 5, the difference was considered questionable, whereas for Z-score > 5 the difference was defined as unacceptable.

Scatter plots, the Friedman repeated measures analysis of variance on ranks (Scheff, 2016), and the Z-score analysis were applied only to those groups of samples that were not greatly affected by evaporation (i.e., CVD-L samples were not considered). We applied the Friedman repeated measures analysis of variance on ranks, instead of analysis of variance, because the repeated samples were few and non-normally distributed. The statistical analyses and the plots were prepared using SigmaPlot, Microsoft Excel and R.

4 Results

4.1 Isotopic variability across extraction methods and plant tissues

The isotopic composition of plant water varied considerably across the different plant tissues (Table 1 and Fig. 4). We found that CVD-L samples were more enriched in heavy isotopes than all the other plant tissues samples, and they plotted to the right side of the three LMWLs, highlighting a distinct evaporation signature (Fig. 4). Plant water extracted by SPC, CVD-T, CVD-TwB, CVD-WC and CVD-TcT generally plotted close to the LMWLs, except for three CVD-TwB samples from alder trees that were more depleted in heavy isotopes and plotted on the right side of the LMWL ~~(Fig. 4a)~~, and two samples from beech trees (one CVD-T and one CVD-TcT) that slightly plotted on the left side of the LMWL (Fig. ~~4c~~4). SPC samples were more enriched in heavy isotopes than CVD-T, CVD-TwB and CVD-WC samples collected in Ahr/Aurino ~~(Fig. 4a)~~ and Laas/Lasa ~~(Fig. 4b)~~, whereas the differences between SPC and CVD samples (except for CVD-L) were less marked in Ressi,

for both beech and chestnut trees (Fig. ~~4c~~4). The Welch two-sample t-test, applied only to alder and apple tree samples, showed that there was a significant difference in δ^2H and δ^{18}O of SPC and CVD-L samples ($p < 0.001$ for all four tests).

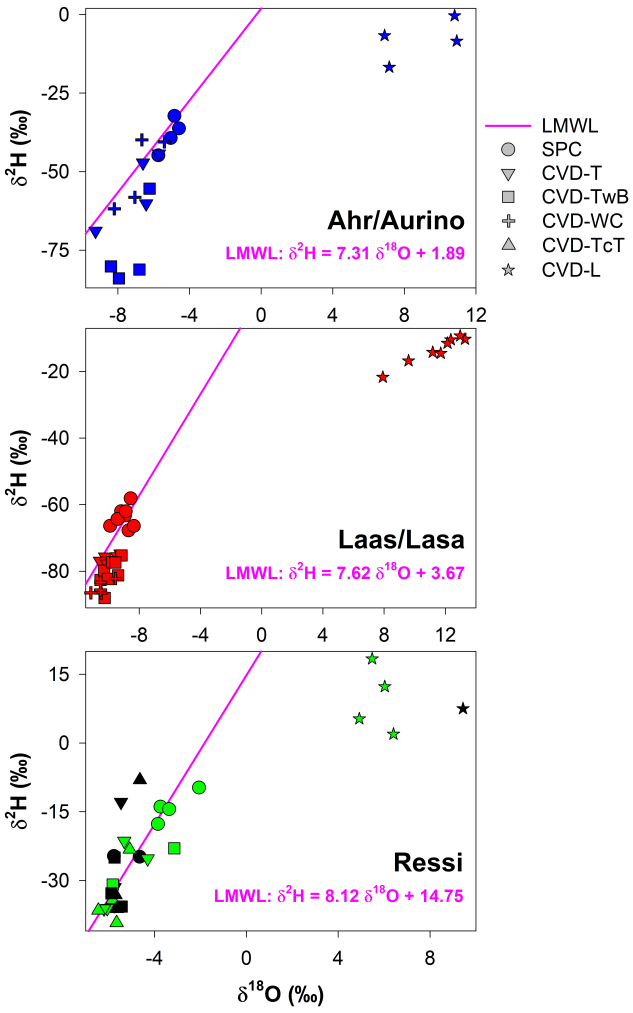

Figure 4. Dual-isotope plot for plant water samples extracted by Scholander-type pressure chamber (SPC), and cryogenic vacuum distillation (CVD) for different plant tissues (CVD-T, CVD-TwB, CVD-WC, CVD-TcT and CVD-L indicate samples extracted by CVD from twig without bark, twig with bark, wood core, twig close to the trunk of the tree and leaves, respectively) and species (alder, apple, chestnut and beech). Local Meteoric Water Lines (~~LMWL~~LMWLs) of the three study sites are also plotted in pink: Ahr/Aurino: $\delta^2H = 7.31 \times \delta^{18}O + 1.89$ (Engel et al., in review), Laas/Lasa: $\delta^2H = 7.62 \times \delta^{18}O + 3.67$ (~~unpublished data~~Penna et al., 2021), Ressi: $\delta^2H = 8.12 \times \delta^{18}O + 14.75$ (Marchina et al., 2020).

The relation between δ^2H and δ^{18}O of plant water extracted by SPC and CVD showed differences among plant tissues and the four species (Fig. 5 and 6). Indeed, we observed that most of the samples did not plot on the 1:1 line, and there were

very large differences between δ^2H of SPC and CVD-TwB, particularly for alder tree samples (the absolute differences varied between 16.1 and 48.9 ‰) and apple tree samples (the absolute differences varied between 12.0 and 21.7 ‰) (Fig. 5b). For alder, apple and chestnut tree samples, we found that δ^2H of SPC was always more positive than δ^2H of CVD samples, except for CVD-L (Fig. 4). The δ^2H of plant water collected from beech trees by CVD-T, CVD-TwB and CVD-TcT was not systematically more enriched or depleted than δ^2H that of SPC samples.

Likewise, we found differences in δ^{18}O values between SPC and CVD samples (Fig. 6). However, compared to δ^2H, more samples plotted closer to the 1:1 line. The differences between SPC with CVD-T and CVD-TwB of beech samples were small (the median of the absolute differences was 0.22 ‰, n = 6), and the samples plotted very close to the 1:1 line (Fig. 6a,b). SPC samples from alder, apple and chestnut trees were less negative in δ^{18}O than CVD samples, but for apple tree samples the differences between SPC and CVD-TwB were relatively small (the median of the absolute differences was 0.57 ‰, n = 8).

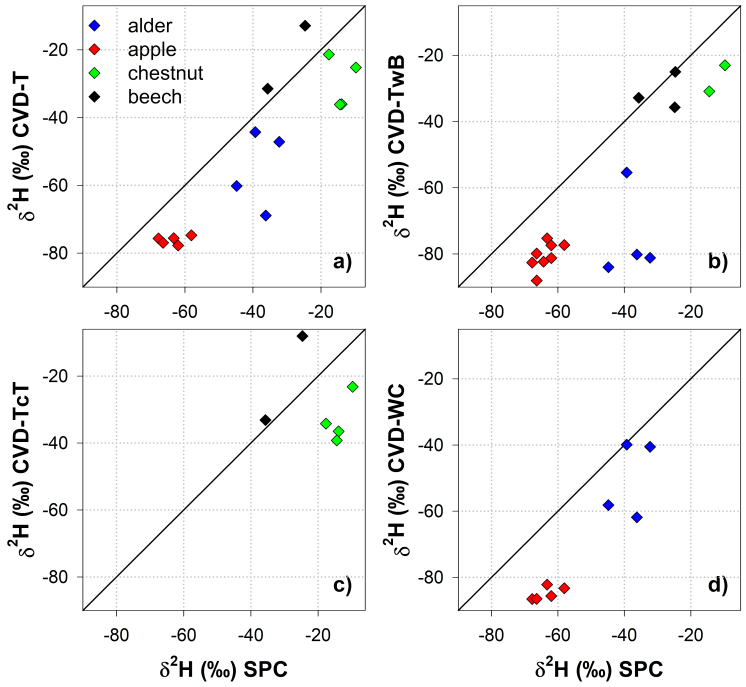

Figure 5. Relation between δ^2H values in plant water extracted by SPC (i.e., Scholander-type pressure chamber) and CVD (i.e., cryogenic vacuum distillation), grouped by plant tissue and species. The solid black lines represent y = x.

4.2 Effect of the extraction method on plant water isotopic composition

The Friedman repeated measures analysis of variance on ranks (applied only on alder and apple isotopic data) showed that there was a significant effect (with $\alpha = 0.05$) of the extraction method and plant tissue on δ^2H and δ^{18}O of plant water (Fig. 7). For alder trees, we found that SPC samples were significantly different in δ^2H and δ^{18}O from CVD-TwB samples ($p < 0.05$,

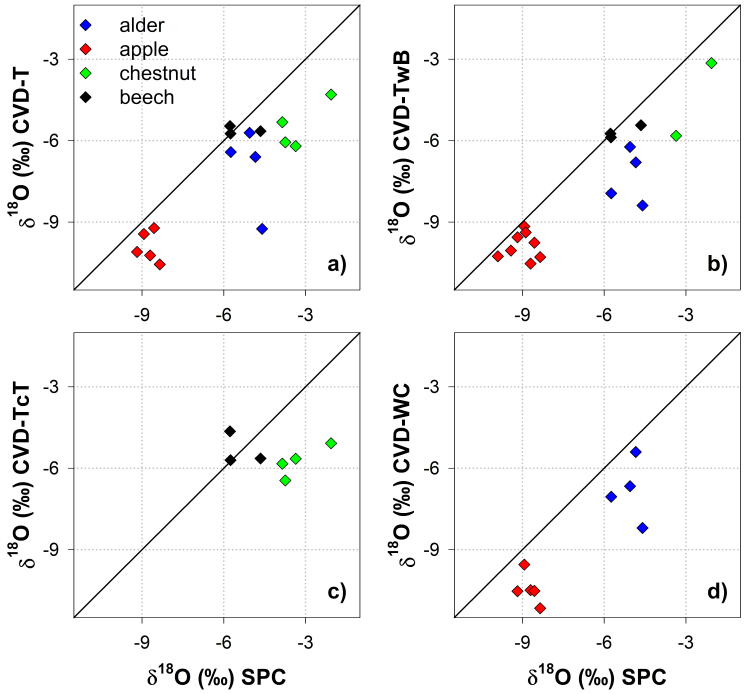

Figure 6. Relation between δ^{18}O in plant water extracted by SPC (i.e., Scholander-type pressure chamber) and CVD (i.e., cryogenic vacuum distillation), grouped by plant tissue and species. The solid black lines represent y = x.

pairs = 4, Tukey test). For apple trees, SPC samples differed in δ^{2}H and δ^{18}O from CVD-WC samples ($p < 0.05$, pairs = 5, Tukey test).

~~When considering d-excess, we~~ We observed that there was not a significant effect of the extraction method on ~~the sample isotopic composition~~ lc-excess ($p > 0.05$, Friedman repeated measures analysis of variance on ranks)~~. d-excess~~ , except for CVD-L samples (weighted mean lc-excess was always very negative; Table 1). lc-excess highly-varied among the various samples~~; values were quite large~~ , even without considering CVD-L samples in the analyses (Fig. 7). Weighted mean lc-excess was quite close to zero for beech tree samples ~~, slightly less positive for chestnut tree samples , and below 8.0 or even negative for~~ (some samples even had positive values), and slightly more negative for chestnut, alder and apple tree samples (Fig. 7 and Table 1). A marked evaporation signature was found only for CVD-TwB samples collected from alder trees. Interestingly, SPC samples from apple trees had a positive weighted mean lc-excess compared to the negative lc-excess of CVD-T, CVD-TwB and CVD-WC samples collected from the same plants (Fig. 7 and Table 1).

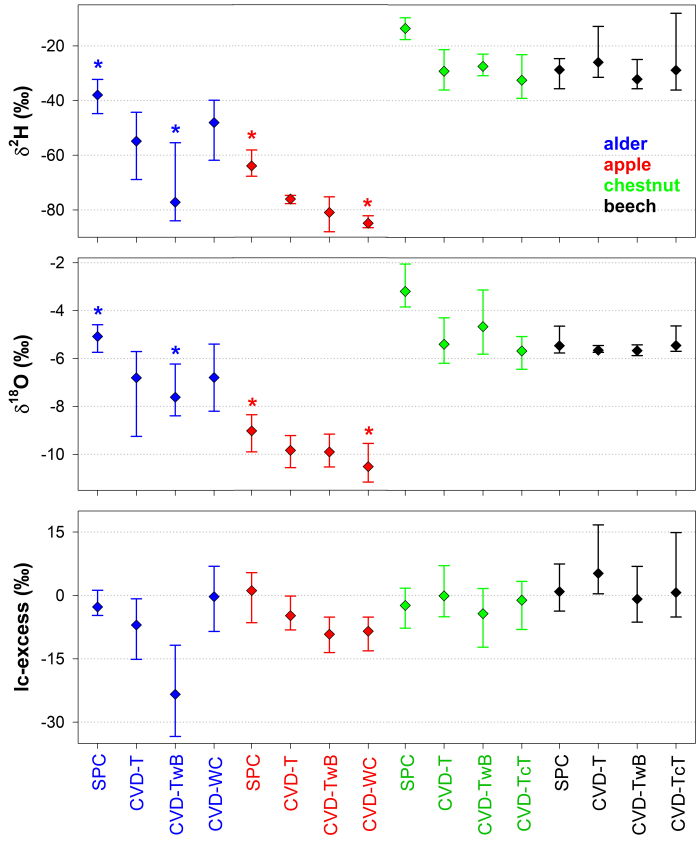

Figure 7. ~~Median~~ Weighted mean isotopic composition and ~~d-excess~~ lc-excess of plant water extracted by SPC (i.e., Scholander-type pressure chamber) and CVD (i.e., cryogenic vacuum distillation), grouped by plant tissue and species. Error bars represent the ~~median absolute deviations~~minimum and the maximum values. Asterisks above the dots indicate significantly different groups ($p < 0.05$, multiple comparison test based on Tukey method, run after the Friedman repeated measures analysis of variance on ranks).

4.3 Are the differences between SPC and CVD larger than the uncertainty in the isotopic measurements?

The Z-score analysis showed that generally the differences between δ^2H and δ^{18}O of SPC and CVD samples were larger than the uncertainty in the isotopic measurements (Fig. 8). Due to the larger uncertainty in δ^2H measurements compared to δ^{18}O (based on the IRMS used in this study), we observed that the computed Z-scores were smaller for δ^2H than for δ^{18}O.

335 For δ^2H, Z-scores varied between 0.1 (computed between SPC and CVD-TwB for samples collected from a beech tree in Ressi) and 19.6 (computed between SPC and CVD-TwB for samples collected from an alder tree in Ahr/Aurino). The median Z-scores for δ^2H were 6.0, 6.5, 6.6 and 7.6 computed between SPC with CVD-T, CVD-TwB, CVD-TcT and CVD-WC, respectively; these median values indicate that more than 50 % of the Z-scores were above the limit for questionable differences (Z-score = 5) between the extraction methods (Fig. ~~8~~8a). For δ^2H, only few Z-scores (about 10 %) were lower than

the upper limit for acceptable differences (Z-score = 2) between the methods. Overall, the smallest differences in δ^2H (and Z-scores) were found between SPC and CVD-T, followed by SPC and CVD-TwB (Fig. ~~8~~8a).

For δ^{18}O, Z-scores varied between 0.1 (computed between SPC and CVD-T for samples collected from a beech tree in Ressi) and 46.6 (computed between SPC and CVD-T for samples collected from an alder tree in Ahr/Aurino). The median Z-scores for δ^{18}O were 12.4, 10.8, 19.8 and 16.1 computed between SPC with CVD-T, CVD-TwB, CVD-TcT and CVD-WC, respectively; these ~~median values~~ results indicate that about 75 % of the Z-scores were above the limit for questionable differences between the extraction methods (Fig. ~~8~~8b). For δ^{18}O, only few Z-scores (less than 10 %) were lower than the upper limit for acceptable differences between the methods. The smallest differences in δ^{18}O (and Z-scores) were observed between SPC and CVD-TwB, followed by SPC and CVD-T (Fig. ~~8~~8b).

5 Discussion

5.1 Advantages and limitations of water extraction by SPC

The SPC has the advantage of extracting plant water that is likely used for transpiration and it is not tightly stored in the plant tissues (Meiri et al., 1975; Grossiord et al., 2017). The water extraction by SPC can be applied directly ~~*in situ*~~ in the field 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 (such as, ~~the handling of~~ handling liquid nitrogen and ~~the transfer of the sample in~~ transferring samples to different vials), as for the CVD system. In addition, water extraction by SPC generally lasts few minutes depending on the ~~shoot~~ plant water potential, whereas the extraction by CVD could last from few minutes (15 min in this study) up to hours (Millar et al., 2018). The easy and fast application (without extensive laboratory work) of the SPC for plant water extraction can be considered comparable to the simple and low-cost methods developed by Fischer et al. (2019).

Despite ~~these advantages , our samplings showed that the~~ the advantages listed above, our sampling approach showed that water extraction by SPC is not always satisfactory in terms of sampling volume and extraction times~~(e. g., in the Ressi catchment~~ . For instance, for some twig samples collected in Ressi during a dry period in July 2017~~at the sunrise). For some shoot samples collected in Ressi~~, we had to apply a 3.0-MPa pressure for the extraction of at least ~~200~~ 60 μl for the isotopic analysis by IRMS, and the whole sample extraction lasted about 10 min. The sampling procedure was also complicated by the extraction of few small water droplets and air bubbles that were difficult to trap into the vials. Conversely, the plant water extraction by CVD was performed for all the samples, and generally obtaining sampling volumes much larger than ~~200~~ 100 μl. Furthermore, plant water extracts obtained by SPC usually were darker (yellowish or even brownish) compared to water extracts by CVD. The dark colour of the SPC plant water extracts suggests a possibly large concentration in organic compounds (Millar et al., 2018), likely due to a partial destruction of plant cell walls. In our case, the sampling volume was not enough to quantify the concentration of organic compounds. Compared to this study, Geiβler et al. (2019) performed a post-processing analysis (by a spectral contamination identifier software) to quantify the spectral contamination of organic compounds, and found that for six stem water samples from *Acacia mellifera* shrubs ~~,~~the relative degree of interference from contaminants in the extracted

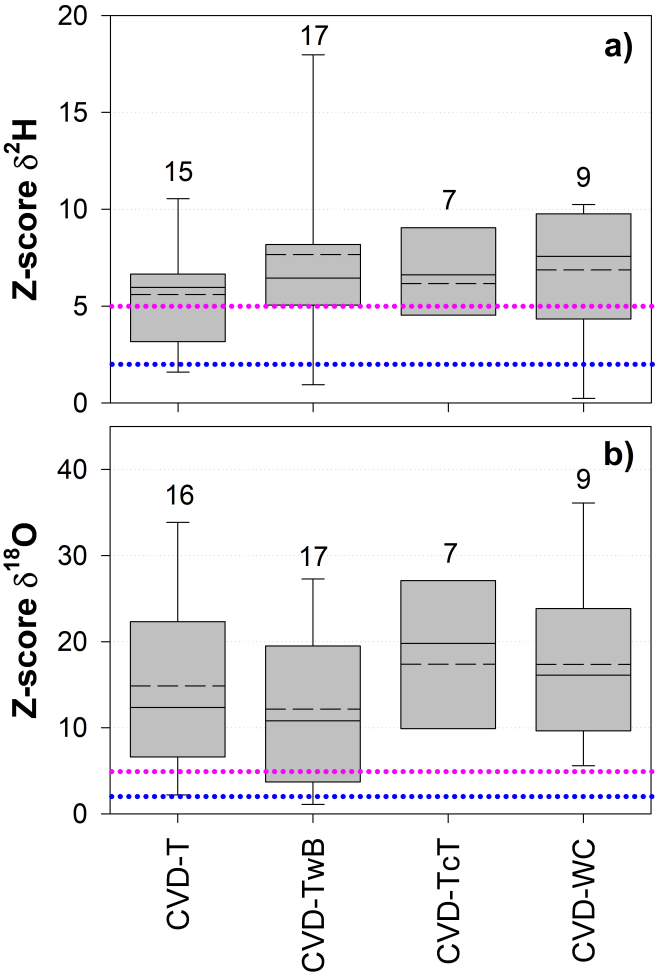

Figure 8. Dimensionless Z-score values for δ^2H (a) and δ^{18}O (b) grouped by sample types (CVD: cryogenic vacuum distillation; T: twig without bark; TwB: twig with bark; TcT: twig close to the trunk; WC: wood core). Samples from the four species were grouped together, and numbers above the boxes represent the sample size. The boxes indicate the 25^{th} and 75^{th} percentile, the whiskers indicate the 10^{th} and 90^{th} percentile, whereas the horizontal solid and dashed lines within the box mark the median and the mean, respectively. The dotted blue and pink lines represent the upper limits for acceptable (Z-score = 2) and questionable (Z-score = 5) differences, respectively, between the SPC (i.e., Scholander-type pressure chamber) and the CVD extracted samples.

water was clearly higher for CVD than SPC ~~extracted plant water~~ (Fig. S1 in Geiβler et al. (2019)). ~~Given that we were not able to quantify the concentration in organic compounds in our samples, and Geiβler et al. (2019) carried out a quantification of the spectral contamination for just six samples and only one plant species, we recommend that future inter-method studies should compare the isotopic composition of plant water extracted by SPC and CVD, as well as the measured concentration of organic compounds.~~

5.2 ~~Does the~~ Do SPC ~~water extraction represent an alternative to the~~ and CVD techniques access different plant water domains?

Our results ~~suggest that SPC can extract plant water in a simple and fast procedure that does not require laboratory work compared to other methods, such as CVD (Fischer et al., 2019; Geiβler et al., 2019). However, some drawbacks, such as the small sampling volume that can be extracted during water deficit conditions, and the isotopic differences found with the plant water extracted by the widely-used CVD, indicate that the SPC and CVD methods cannot be considered as interchangeable plant water extractions.~~

~~Our inter-method comparison~~ showed that the isotopic composition of the ~~samples~~ twig samples (with attached leaves) obtained by SPC, differently from the CVD-L ones, were not affected by fractionation (samples plotted close to the three LMWLs, Fig. 4)~~, indicating~~ . This might suggest that we did not extract significant volumes of ~~plant~~ leaf water, which is ~~subject to water-vapor~~ typically subject to water-vapour exchanges with the low atmosphere ~~. The d-excess~~ (e.g., Cernusak et al., 2016; Benettin et al., 2021). The lc-excess values of plant water extracted by SPC and CVD were generally ~~positive~~ close to zero (except for CVD-L, and CVD-TwB samples collected from alder trees, Fig. 7), indicating ~~no~~ low isotopic fractionation, with ~~values even above 10~~ even positive values for plant water samples extracted from beech and some chestnut trees in Ressi. This observation indicates that either the applied methods did not alter the isotopic composition of plant water by fractionation, if any isotopic fractionation occurred within the plants, or that both methods did not access fractionated water. Despite the similarity in ~~d-excess~~ lc-excess between samples extracted with the two methods (no significant differences were found by the Friedman repeated measures analysis of variance on ranks, $p > 0.05$, Fig. 7), we observed that plant water collected by CVD method from alder, apple, and chestnut trees was always more depleted in heavy isotopes (both δ^2H and δ^{18}O), and in some cases significantly different ~~(i.e., comparison between SPC with CVD-TwB from alder trees and CVD-WC from apple trees, $p < 0.05$, Tukey test) from~~ from plant water samples extracted by SPC (Figs. 5, 6 and 7). ~~These results are similar to those found by Millar et al. (2018) who reported a depletion in heavy isotopes for spring wheat (*Triticum aestivum*) samples derived by CVD compared to other techniques, such as direct vapor equilibration and microwave extraction. The~~ As expected, given the different plant water domain accessed by the two methods, the water extracted by SPC and CVD showed differences in the isotopic composition among plant tissues, larger than the uncertainty in the isotopic measurements, and such differences were considered unacceptable in terms of Z-scores ~~for more than 50 % of the samples (even more than 75 % when considering δ^{18}O,~~ (Fig. 8). ~~These~~ As expected, these results are in contrast with those found by Geiβler et al. (2019), who reported a large variability in δ^{18}O, but no statistical differences for six stem water samples of *Acacia mellifera* extracted

by CVD and SPC. However, we must consider that Geiβler et al. (2019) compared a limited number of samples and a different species, and used samples deprived of the phloem tissue.

In our study, we relate the observed isotopic differences between SPC and CVD samples to various factors, such as the possible effect of organic compounds on the isotopic composition of plant water (although we did not check this effect) ~~or~~ ~~the sampling material~~ and/or the plant water domain accessed by each method (e.g., ~~xylem water extracted at the shoot water potential~~ more mobile plant water extracted by SPC vs. ~~more-tightly bound water stored in plant cells and likely~~ all plant water extracted by CVD). Millar et al. (2018) reported that different methods can extract different water ~~pools~~ domains within the plants, and CVD extracts up to 99 % of the water in a sample, i.e., CVD accesses total plant water. Conversely, SPC ~~only~~ mainly extracts water present in the xylem conduits, and given the much smaller sample volumes we collected by SPC than by CVD (on average $\approx$ ~~200~~ 135 μl for SPC vs. $\approx$ ~~2 ml for CVD~~955 μl for CVD, Table 1), we likely ~~extracted~~ accessed different plant water ~~pools~~ domains when using the two methods, with ~~the former method~~ SPC pulling out more easily ~~mobilized xylem water than water stored in living cells.In addition, we must consider that, while SPC likely extracts only water within the xylem (consisting of dead cells) , CVD tends to retrieve all the~~ mobile water (i.e., xylem and inter-cellular water) than water stored in ~~the sampled tissues (including dead and living cells), which may have very different residence times (Millar et al., 2018)~~ living cells. Water taken up by roots and transported in the xylem conduits can reach ~~very rapidly the leaves~~ the leaves very rapidly and be available for transpiration, whereas the living cells (which are particularly abundant in the leaves and other non-lignified tissues) may store water taken up several days or even ~~months~~ weeks before the sampling date (Sprenger et al., 2019) . Therefore, water taken up by roots in different periods and stored in different tissues might have very different isotopic compositions, ~~and this can mask the method comparison because that water can be retrieved by CVD, but not by SPC~~something that both methods cannot clearly distinguish.

The ability of SPC and CVD to extract different plant water ~~pools~~ domains has implications for studies investigating the water sources exploited by plants for transpiration ~~(e.g., Bowling et al., 2017; Barbeta et al., 2019; Amin et al., 2020)~~ (e.g., Zhao et al., 2016; Bowling et al., 2017; Barbeta et al., 2019; Amin et al., 2020). Indeed, significantly different isotopic compositions in the extracted plant water can complicate the identification of the water sources contributing to transpiration, and can result in different estimations of the contributing water sources (Barbeta et al., 2019). In this view, such studies should rely more on methods extracting apoplastic water from transpiring trees (like SPC) than on methods extracting, in addition to xylem water, also other plant water fractions (stored in living cells) that are likely much older (even decades, as reported in Zhang et al. (2017)) than the actual xylem water~~(Zhang et al., 2017; Millar et al., 2018).~~ . In addition, possible exchanges during transportation among different plant water domains would result in mixing of water having different ages, and likely different isotopic composition (Ellsworth and Williams, 2007; Zhao et al., 2016).

Furthermore, our results show that the differences in the isotopic composition between SPC and CVD vary not only based on the plant tissue used for CVD, but also based on the plant species (~~differences were more marked for alder, apple and chestnut tree than for beech tree samples~~Figs. 5, 6 and 7). Given the isotopic differences among various species and the results obtained by Geiβler et al. (2019), more research is needed to compare multiple extraction methods (SPC should not only be compared to CVD, but as well as to direct ~~vapor~~ vapour equilibration, microwave extraction, centrifugation etc.~~),~~ , that might

access different plant water domains). Future inter-method comparisons should be carried out across various environments and plant species ~~,~~ to investigate the technical and physical factors possibly altering the isotopic composition of plant water during the extraction ~~, and~~and/or potentially different plant water domains accessed by each method, in order to elaborate standard protocols for ecohydrological research relying on the isotopic signature of plant water.

6 ~~Concluding remarks~~

~~Recent studies highlighted problems associated to the reliability of~~

5.1 Limitations of this study

Our results contribute to the pressing need of comparing different plant water extraction ~~methods for isotopic analyses, mainly because different methods do not extract the same water pool in the plant tissues, and some of them can extract large concentrations~~ techniques to understand which plant water domains are accessed by different methods (Penna et al., 2018). Despite the importance of our findings for the isotope ecohydrological community, we acknowledge some limitations in the experimental setup, which may impact the interpretation of the results.

Firstly, our experiment was not designed to test whether plant water extracted by SPC from twigs with or without bark had a significantly different isotopic composition. Contrary to Geiβler et al. (2019), who performed their experiment after we performed ours, we did not remove bark and the leaves attached to the twig. However, we found no direct influence of leaf water isotopic composition on our SPC samples (no deviation from LMWLs, see Fig. 4), and therefore, we could compare plant water extracted by SPC to plant water obtained by CVD-TwB. Nonetheless, our results are not directly comparable to the findings by Geiβler et al. (2019), and future research should aim to test whether SPC is able to extract phloem from twigs with bark and green tissues, and whether there is a significant isotopic difference with SPC samples obtained from twigs without bark.

Secondly, our experimental scheme did not include the quantification of organic compounds, and the water volume obtained for SPC samples was not enough to carry out such analyses. The quantification of organic compounds would have been important to determine whether the destruction of plant cell walls in SPC samples could increase the number of organic contaminants in the extracted water, as well as alter the isotopic composition of the samples, and to quantify and compare the type and amount of organic compounds in SPC and CVD samples. Given that we were not able to quantify the concentration of organic compounds in our samples, and Geiβler et al. (2019) carried out a quantification of the spectral contamination for just six samples and only one plant species, we recommend that future inter-method studies should compare the isotopic composition of plant water extracted by SPC and CVD, as well as the measured concentration of organic compounds.

Finally, based on our experimental setup, we were not able to determine exactly which plant water domain was accessed by SPC. In terms of sample type, our SPC samples were more comparable to CVD-TwB samples because we did not remove bark, and our results confirmed that there was no influence of evaporated leaf water on the SPC samples. Therefore, similarly to CVD-TwB, we cannot exclude that our SPC samples contained phloem. However, the smaller sample volumes we collected by

SPC compared to CVD (Table 1), and the different isotopic composition, particularly shown by the Z-score analysis (Fig. 8), indicate that SPC tended to access more easily the mobile xylem water and inter-cellular water than the less mobile intra-cellular, cell wall, and organelle constrained water (Millar et al., 2018). Despite this, our study did not resolve whether SPC was able to extract all the less mobile plant water (besides likely cell walls), and whether the results were affected by other factors, such as the presence of organic compounds~~, potentially affecting the isotopic analysis.~~. Given that we were not able to determine exactly the plant water domains accessed by SPC, future comparison studies between the SPC and CVD techniques should carefully consider the sample types (both with bark and without bark to assess whether SPC extracts phloem), the quantification of organic compounds, and the extraction of plant water using different external pressures. By applying different external pressures to plants not suffering from high water deficit, and under the assumption that more and less mobile plant water have different isotopic compositions, it may be possible to test whether SPC can extract mobile plant water when a low external pressure is applied, and a mixture of more and less mobile plant water during the application of higher pressures.

6 Concluding remarks

In this work, we analyzed the variability in the isotopic composition of plant water extracted by cryogenic vacuum distillation (CVD) and Scholander-type pressure chamber (SPC), also considering the potential variability in the isotopic signature of the plant ~~tissues~~ water extracted from various tissues by CVD (i.e., leaves, twig without bark, twig with bark, twig close to the trunk of the tree, and wood core)~~and~~ , and from different plant species (i.e., alder, apple, chestnut and beech~~trees~~). The procedure for the extraction of plant water by SPC is simple, can be carried out ~~*in situ*~~in the field, and it does not require specific laboratory work as in case of CVD. However, the main limitation of SPC is the very small water volume that can be extracted from the lignified ~~shoot~~ twig during water deficit conditions~~(and high transpiration moments)~~, compared to CVD. Moreover, our results indicated that the isotopic composition of plant water extracted by SPC and CVD was significantly different. While SPC and most of the CVD samples (except for CVD applied to leaves) did not exhibit an evaporative signature, there was a large isotopic variability among the samples. We found that, for beech tree samples, the difference in both δ^2H and δ^{18}O obtained by the two extraction methods was smaller compared to the difference observed for alder, apple and chestnut tree samples. Specifically, the isotopic composition of alder, apple and chestnut plant water extracted by SPC was more enriched in heavy isotopes compared to samples obtained by CVD applied to twigs or wood cores. Based on these results, we conclude that ~~plant water extraction by~~ SPC accesses only the more mobile part of the plant water fraction that CVD does, and therefore, SPC is not an alternative to CVD ~~, as SPC likely extracts only xylem water (which was theoretically recently taken up by roots and is easily available for transpiration), whereas CVD tends to retrieve total plant water (likely stored both in dead and living cells) from the samples~~in terms of plant water accessed. Therefore, studies aiming to quantify the relative contribution of the water sources to transpiration should rely more on the isotopic composition of xylem water transpiring at the moment of the sampling or during the sampling day (which is theoretically sampled by SPC), than the isotopic composition of total plant water (sampled by CVD), which also contains a fraction of water that could be stored in plant tissues for ~~long~~ a longer time.

Based on our findings, we call for future research investigating the same methods across more plant species, and quantifying the organic compounds in both SPC and CVD samples to determine the effect on the isotopic composition of plant water.

Data availability. Data are available from the corresponding author upon reasonable request.

Author contributions. DP conceptualized the methodological comparison between the two methods. JF, GZ and ME designed the research. JF and ME collected field data in the Ahr/Aurino and Laas/Lasa sites, whereas CM, GZ and AA carried out the field campaigns in the Ressi catchment. JF and AA performed the plant water extraction by the cryogenic vacuum distillation. FS and DZ provided support for the sampling in Laas/Lasa and the laboratory activities. VC and TA provided technical support and comments for the plant water extraction by the Scholander-type pressure chamber. GZ analyzed the data set, and prepared the first draft of the manuscript with contributions from AA, JF, ME and CM. All the authors contributed to the editing of the manuscript. FC, MB and MT funded the research.

Competing interests. The authors declare that they have no conflict of interest.

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