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Isotopic fractionation from deep roots to tall shoots: A forensic analysis of xylem water isotope composition in mature tropical savanna trees

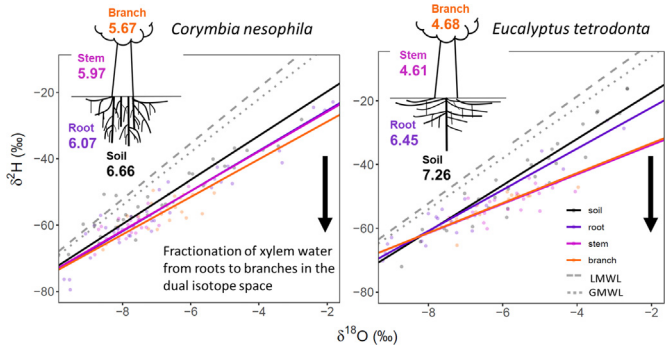
Adriana M. Vega-Grau [a,b,*], Jeffrey McDonnell [c,d], Susanne Schmidt [a,e], Mark Annandale [b], John Herbohn [a,b]

[a] University of Queensland, School of Agriculture and Food Sciences, Brisbane, Queensland 4075, Australia
[b] Tropical Forests and People Research Centre, University of the Sunshine Coast, Queensland 4556, Australia
[c] Global Institute for Water Security, School of Environment and Sustainability, University of Saskatchewan, Saskatoon, Canada
[d] School of Geosciences, University of Birmingham, Birmingham, UK
[e] Centre for Horticultural Science, Queensland Alliance for Agriculture and Food Innovation, St Lucia, Queensland 4067, Australia

HIGHLIGHTS

- Isotopic fractionation of xylem water may affect plant water source identification.
- We analysed xylem δ^2H and $\delta^{18}O$ from roots to branches in mature trees in a savanna.
- Fractionation increased from below- to aboveground xylem in the dual isotope space
- Root structure assessment helped clarify aboveground interpretation of water use.
- Future studies should consider xylem water fractionation and include plant traits.

GRAPHICAL ABSTRACT



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ABSTRACT

Studies of plant water sources generally assume that xylem water integrates the isotopic composition (δ^2H and $\delta^{18}O$) of water sources and does not fractionate during uptake or transport along the transpiration pathway. However, woody xerophytes, halophytes, and trees in mesic environments can show isotopic fractionation from source waters. Isotopic fractionation and variation in isotope composition can affect the interpretation of tree water sources, but most studies to date have been greenhouse experiments. Here we present a field-based forensic analysis of xylem water isotope composition for 12 *Eucalyptus tetrodonta* and *Corymbia nesophila* trees. We used a 25-tonne excavator to access materials from the trees' maximum rooting depth of 3 m to their highest canopies at 38 m. Substantial within-tree variation occurred in δ^2H (−91.1‰ to −35.7‰ *E. tetrodonta*; −88.8‰ to −24.5‰ *C. nesophila*) and $\delta^{18}O$ (−12.3‰ to −5.0‰ *E. tetrodonta*; −10.9‰ to −0.3‰ *C. nesophila*), with different root-to-branch isotope patterns in each species. Soil water δ^2H and $\delta^{18}O$ dual isotope slopes (7.26 *E. tetrodonta*, 6.66 *C. nesophila*) were closest to the Local Meteoric Water Line (8.4). The dual isotope slopes of the trees decreased progressively from roots (6.45 *E. tetrodonta*, 6.07 *C. nesophila*), to stems (4.61 *E. tetrodonta*, 5.97 *C. nesophila*) and branches (4.68 *E. tetrodonta*, 5.67 *C. nesophila*), indicative of fractionation along the xylem stream. Roots of both species were more enriched in ^2H and ^{18}O than soil water at all sampled depths. Bayesian mixing model analysis showed that estimated proportions of water sourced from different depths reflected the contrasting root systems of these species. Our study adds evidence of isotopic fractionation

* Corresponding author at: University of Queensland, School of Agriculture and Food Sciences, Brisbane, Queensland 4075, Australia.
E-mail addresses: a.vegagrau@uq.net.au (A.M. Vega-Grau), jeffrey.mcdonnell@usask.ca (J. McDonnell), susanne.schmidt@uq.edu.au (S. Schmidt), mannanda@usc.edu.au (M. Annandale), jherbohn@usc.edu.au (J. Herbohn).

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from water uptake and along the transpiration stream in mature trees in monsoonal environments, affecting the interpretation of water sources. We discuss the findings with view of interpreting aboveground xylem water isotopic composition, incorporating knowledge of root systems.

1. Introduction

The stable isotopes of hydrogen ($^2H/^1H$) and oxygen ($^{18}O/^{16}O$) are widely used to quantify plant water sources (Brooks et al., 2010; Ehleringer and Dawson, 1992; Evaristo et al., 2015, 2019; Goldsmith et al., 2012; Ogle et al., 2004), providing insights into rooting depths and water uptake patterns. Traditionally, xylem water is assumed to integrate the isotopic composition (δ^2H and $\delta^{18}O$) of the water sources that roots access (Dawson and Ehleringer, 1993, 1991; Ehleringer and Dawson, 1992). It is expected that no fractionation (i.e. deviation from the source signal) occurs with root water uptake (Dawson and Ehleringer, 1991, 1993; Zimmermann et al., 1967) or with transport along the transpiration pathway (Martín-Gómez et al., 2017), so that the isotopic composition of a plant's xylem water should mirror that of its water sources (Zhao et al., 2016).

Generally, studies of plant water sources using stable isotopes have assumed such "steady-state conditions" in trees (Penna et al., 2018). However, this has been challenged with evidence of xylem water fractionation associated with root water uptake in xerophytes and halophytes (Ellsworth and Williams, 2007) and, more broadly, species growing in saline environments (Lin and Sternberg, 1993). A watering experiment in a botanical garden detected $^2H/^1H$ fractionation in trees, confirming that isotopic fractionation can occur in mesic environments (Evaristo et al., 2017). $^2H/^1H$ fractionation between xylem and soil water outside arid or saline environments was also demonstrated in *Fagus sylvatica* with xylem water being more depleted in ^2H than soil water in a temperate riparian forest (Barbeta et al., 2019). In a further drought experiment with *F. sylvatica* saplings grown in pots, Barbeta et al. (2020) found offsets of xylem-soil $^2H/^1H$ which depended on changes in soil water content and plant water status. Furthermore, recent studies detected $^2H/^1H$ fractionation, with xylem water not corresponding to sources in the dual isotope space (Barbeta et al., 2019; Brooks et al., 2010; Evaristo et al., 2017; Geris et al., 2015; Oerter and Bowen, 2017, 2019).

It has been argued that the mechanism associated with hydrogen isotopic fractionation has almost no effect on oxygen, so that no $^{18}O/^{16}O$ fractionation is expected to occur with root water uptake (Ellsworth and Williams, 2007; Rothfuss and Javaux, 2017). If it does occur, $\delta^{18}O$ fractionation with root water uptake should be too small to be detected in most species (Rothfuss and Javaux, 2017). Challenging this argument is the observed xylem water depletion in ^2H and ^{18}O in the presence of arbuscular mycorrhizal fungi, which, due to their ubiquitousness, isotopic fractionation with root water uptake could be common (Poca et al., 2019). Recent work by (Poca et al., 2019) showed discrimination of up to −24.6‰ in δ^2H and − 2.9‰ in $\delta^{18}O$ in potted seedlings of *Acacia caven*, a tree species of the seasonally dry South American Chaco. Similarly, root water uptake in potted avocado plants fractionated both δ^2H and $\delta^{18}O$, depending on soil type and water content (Vargas et al., 2017). Further, a vapour equilibration experiment with a potted pine tree showed that stem water $\delta^{18}O$ differed by up to 4‰ from source water (Marshall et al., 2020), although the authors did not attribute the offset to possible $\delta^{18}O$ fractionation with water uptake.

In addition to isotopic fractionation during water uptake, other plant processes can change xylem water δ^2H and $\delta^{18}O$ signatures, while spatiotemporal variations in water flow can add uncertainty in the isotopic composition of xylem water (von Freyberg et al., 2020). Fractionation can occur as a result of xylem water stagnation after dry periods in deciduous plants (Ellsworth and Sternberg, 2015). Xylem water can become enriched in suberized twigs on short time scales due to limited sap flow rates (Martín-Gómez et al., 2017). Isotopic enrichment can occur during long xylem water residence times or over long distances along a tree due to feedback from leaves through phloem-xylem exchange, so that isotope signals differ between the upper and lower stem and within the crown (Cernusak et al., 2005). Uneven exposure to radiation can cause transpiration differences within the crown (Burgess and Dawson, 2008) and result in further isotopic variation. Water stored in plant tissues may also affect the sampled xylem water (Barbeta et al., 2020; Berry et al., 2017; Penna et al., 2018). If sap flow rates and the use of stored water differ along and across the tree (Čermák et al., 2007; Meinzer et al., 2004), then the isotopic composition of xylem water may reflect these variations. Lastly, variation in carbon fixation rates in the tree crown may cause enrichment in ^{18}O of wood (Cernusak et al., 2005). Collectively, these processes can lead to fractionation in xylem water isotopic composition.

Together, these findings raise questions about the adequacy of tree source water apportionment using water isotope signatures, as uptake and fractionation along the xylem stream may affect the identification of tree water sources and interpretation of tree water use. How reliable are the estimates of proportions of water sources used by plants has also been recently questioned by Beyer and Penna (2021). So how does all this express itself isotopically in the plant? It appears that for some species, isotopic fractionation of xylem water can indeed occur with root water uptake or in association to other plant processes e.g. (Ellsworth and Williams, 2007; Martín-Gómez et al., 2017). Most research to date, however, has been conducted in potted plants and this limits interpretation of plants in natural ecosystems. The required destructive sampling of entire trees is often impossible due to logistics and cost. Yet only systematic analysis of the water isotopic composition from roots to crown, combined with analysis of natural water environment in the rooting zone, permits whole-picture, holistic assessment of xylem water isotopic variation and fractionation in mature trees.

Here we address the question of whether the isotopic signatures in xylem-sampled water accurately reflect the depths of root water uptake. We took advantage of an undisturbed ecosystem prior to clearing for mining operations. Destructive sampling was facilitated by the availability of a hydraulic excavator, which enabled tree extraction and soil excavation down to maximum rooting depth. With this unusual opportunity for forensic investigation, we analysed xylem water isotopic composition along the below-to-aboveground length (~40 m) of twelve mature *Eucalyptus tetrodonta* and *Corymbia nesophila* trees in a northern Australian tropical savanna. We tested the null hypothesis that the source-to-xylem water isotopic composition relationship remains unchanged along the transpiration stream. Specific research questions were (i) Does the isotopic composition of xylem water vary along the tree and its parts? (ii) Do isotope profiles differ between tree species? (iii) Is isotopic variation explained by plant traits including tree height, size, root depth and root structure, and (iv) What are the implications of possible xylem water isotopic variation and fractionation for interpreting tree water sources?

2. Methods

2.1. Study site

Sampling was carried out on an undisturbed area within a bauxite mining lease (Green Coast Resources) near Weipa on the Western Cape York Peninsula in north Queensland, in a flat, open savanna that is dominated by *E. tetrodonta* and *C. nesophila* (Fig. 1). The region has a

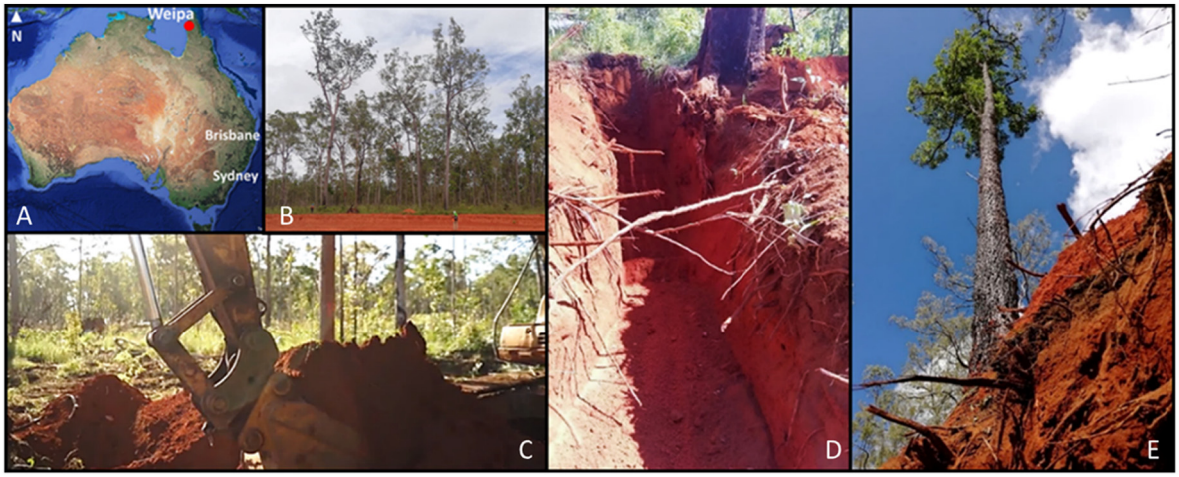

Fig. 1. Site location in Weipa, Cape York Peninsula, North Queensland, Australia (A), and open woodland savanna on site (B). Using a hydraulic excavator (C), trenches were dug, sampling soil and roots down to maximum rooting depth (D), after which the tree was pushed over and xylem was sampled all the way through to branches.

tropical monsoon climate, with a mean annual rainfall of 1900 mm (BOM, 2020), 95% of which falls during the wet season between November–March (Taylor et al., 2008). Humidity ranges from 75% in the wet season to 37% in the dry season (Taylor et al., 2008), and annual mean temperatures are 21–34 °C (BOM, 2021). The site has thin topsoil layer (~0.5 m) followed by bauxitic red kandosol, composed of bauxite, clay, organics and quartz, and beneath this layer lies up to 10 m of loose or cemented bauxite, which is a round-grained rock with a high aluminium content (Taylor et al., 2008). This is followed by a cemented or mottled iron oxide and kaolinite layer (≥2 m thickness) (Eggleton et al., 2008), lying over a 10–20 m zone of up to 90% kaolinite. The water table can vary by up to 10 m across seasons, reaching to the bauxite layer during the wet season (Taylor et al., 2008).

We sampled six trees each of *E. tetrodonta* and *C. nesophila* randomly located within a ~ 1.5 ha area. Potential trees were pre-selected in this area, with view of the diameter-at-breast-height (DBH) distribution (all trees > 10 cm DBH in the plot were surveyed in a separate study). The DBH of sampled trees ranged from 165 to 733 mm and the height ranged from 15 to 38 m. Only healthy, single-stemmed trees were included. Sampling was conducted over 10 days (May 1–10, 2019).

2.2. Sample collection

Soil and plant samples were collected into 12 mL double-wadded Exetainer glass vials (Labco Limited, UK). Groundwater samples were put into 20 mL glass vials which were immediately sealed by wrapping in parafilm. Groundwater samples were collected one day before tree sampling started (April 30, 2019) and on May 1, 2019, after a moderate rain event in the afternoon from an open water table monitoring pit ~1 km from the tree sampling site, where the water table was approximately 2 m below ground level. Samples were kept refrigerated before sending for lab analysis.

Using a 25-tonne excavator, a trench was excavated next to each tree down to maximum root depth (confirmed by digging further as needed) immediately before sampling. Directly within each tree's vertical root zone, within an area of approximately 1 m around the root crown and main roots under each tree, soil was sampled at (0.05, 0.10, 0.20, 0.30, 0.50, 1.0, 1.5, 2.0, 2.5 and 3.0 m) depths down to maximum rooting depth, scraping off the first 3 cm of exposed soil in the trench wall to avoid evaporation effects. Where sample depths were not exactly the fixed depths (e.g., maximum root depth occurring between depths), sample heights were rounded to the nearest fixed sampling height. This procedure was also followed for roots, which were also approximated to the nearest fixed soil sampling height when they could not be sampled at the exact fixed heights. Whenever possible, soil was sampled from ~5 cm around the root tips of sampled roots.

Xylem was collected using a cordless drill with a 25 mm Forstner bit, discarding bark and phloem and quickly sealing wood shavings in a vial. For thin roots and branches or twigs, bark and phloem were removed with a knife. Samples were collected at ~1 cm from the inner bark to collect new xylem and as, although variable, higher sap rates commonly occur in the outer sapwood (Gartner and Meinzer, 2005; Nadezhdina et al., 2007). To avoid evaporation, parallel holes were drilled if more material was needed. Root samples were collected from main, coarse, and fine roots, noting diameter and sampling depth. Because of contrasting rooting morphologies, in *E. tetrodonta*, we use the terms 'main' root for the single taproot and 'coarse' for lateral roots. In *C. nesophila*, 'main' roots denote the main sinker roots (trees having 3–5) and 'coarse' are lateral or secondary sinker roots. Main root samples were collected at a mid-depth and at maximum root depth. For both species, 'fine' roots are roots ≤ 2 mm in diameter. Small roots ~2–5 mm in diameter were also sampled and were included within the 'fine' root category for all analyses.

Each tree was cut at 0.5 m from ground level and toppled with the excavator immediately before sampling. Following the aspect of belowground sampling, xylem was sampled at the DBH level and every 5 m from the ground (0 m) through the stem and main branch. For the purposes of this study, we distinguish between stem samples (i.e. along the bole between ground level (0 m) and before the first main branch division) and branch samples. It is important to note that in plant water studies, xylem samples, generally collected at the DBH level or branch/twig level, are commonly considered stem samples. The direction of xylem sampling along the stem was randomised by starting either at ground or crown level to avoid possible effects of fractionation that could occur after the tree was cut. Additional samples were collected at 0.20 m from ground level for three trees per species. Twig samples (approx. 1 cm diameter, 4–5 cm length) were collected from outer branches off the main branch. Full soil and plant sampling took approximately 1.5–3 h per tree. After isotope sampling, DBH and total tree height were measured, and the root crown was retrieved from the soil to assess rooting morphology. Maximum rooting depth was measured during root isotope sampling.

Water was extracted from samples using the cryogenic vacuum extraction method (Koeniger et al., 2011) (rejection threshold of 96% for extraction efficiency), with 15 min runs at 200 °C and ~ 0.8 mbar (80 Pa). These conditions overcome problems associated with clay minerals in the recovery of the soil water isotopic signal (Gaj et al., 2017). Satisfactory isotopic signal recovery rates were confirmed through previous analysis of stockpiled soil from the site. Groundwater and soil-

extracted water were analysed using liquid water Off-Axis Integrated Cavity Output Spectroscopy (Los Gatos Research OA-ICOS CA, USA), with accuracy of ≤±1.0‰ for δ^2H and ±0.2‰ for $\delta^{18}O$. Due to possible interference from co-extracted wood organic compounds (Millar et al., 2018), plant extracted water was analysed using Isotope Ratio Mass Spectrometry (Elementar Isoprime IRMS), with accuracy of ±2.0‰ for δ^2H and ±0.2‰ for $\delta^{18}O$. To minimize memory effects in laser spectroscopy, nine injections are run per sample, discarding the first three as a control for between-sample memory. In addition, the machine cavity is flushed with dry air between samples. In mass spectroscopy, the memory control procedure is different for $\delta^{18}O$ and δ^2H. For $\delta^{18}O$, obtained through CO^2-H_2O equilibration (Epstein and Mayeda, 1953) and connected to the machine to through a micromass multiflow device, a time lag between measurements allows the gas to flush. For δ^2H, obtained through hydrogen reduction (Morrison et al., 2001) and injected into the machine with a syringe, two injections are used, and the first one is discarded. To ensure the quality and comparability of measurements made through mass spectrometry and laser spectroscopy at the McDonnell Hillslope Hydrology Lab at the University of Saskatchewan, Canada, where the analyses were conducted, the same control waters ('standards') of isotopically known composition are run in both machines (OA-ICOS and IRMS) for every batch of samples. As determined by a recent (March 2021) sample run, the difference between measurements for the standard control waters run through both machines was 0.15‰ for δ^2H and 0.29‰ for $\delta^{18}O$. This is comparable to the laser spectroscopy error and lower than the mass spectrometry error. To correct for machine drifts over the sample runs, the standard control waters are run immediately before a sample and then every few (~5) samples. This ensures that in case any machine drift occurs, the effect is minimal and removes the need of post-analysis drift corrections. Values are reported as parts per thousand (‰) according to Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation (VSMOW-SLAP) scales, with standard δ notation: $\delta^{18}O = [(^{18}O/^{16}O_{sample} - {}^{18}O/^{16}O_{standard})/{}^{18}O/^{16}O_{standard}] \times 1000$.

2.3. Data analysis

The Global Meteoric Water Line (GMWL) is the regression between δ^2H and $\delta^{18}O$ in rainwater on a global scale, while the Local Meteoric Water Line (LMWL) reflects local isotope signatures. Previous studies have shown that Weipa groundwater isotopic composition integrates the rainfall values spanning the Cairns LMWL (Leblanc et al., 2015). Thus, the Cairns LMWL was used in our study (slope 8.4, intercept −15.0), obtained through RMA regression as suggested by Crawford et al. (2014) and Marchina et al. (2020), using the "lmodel2" package (Legendre, 2018) and the rainwater isotope dataset from Munksgaard et al. (2019). Lc-excess ($lc\text{-}excess = \delta^2H - a \times \delta^{18}O - b$), which quantifies the offset, or fractionation, of soil and plant waters from the LMWL (Landwehr and Coplen, 2006) was used to compare isotopic variation and rainwater offset along the tree. Greater fractionation and offset from the LMWL is indicated by lower or more negative the lc-excess values.

All analyses were conducted in R version 3.6.3 (R Core Team, 2020), using packages "ggplot2" (Wickham, 2016), "ggpubr" (Kassambara, 2020a) and "rstatix" (Kassambara, 2020b). Levene's test for homogeneity of variances and quantile-quantile plots for normality showed unequal variances and approximately normal distribution of plant data. We thus used Welch's *t*-test with Benjamini-Hochberg *P*-value adjustment for multiple comparisons to compare isotopic composition and lc-excess per tree part and species. We used linear regressions to assess isotopic composition and lc-excess correlation with sampling height per species, to assess trait effects on aboveground isotopic composition and lc-excess, and relationships between traits. As soil was not normally distributed, we used Kruskal-Wallis followed by the Dunn test to assess differences in δ^2H and $\delta^{18}O$ between soil depths and groundwater. At the species level, plant samples were compared to soil pooled per depths, while individual tree samples were compared to soil water from each tree's root zone. In addition, in the dual isotope space, we also compared the slopes of the regression of δ^2H and $\delta^{18}O$ per tree part, per species, obtained through reduced major axis (RMA) regression method (Crawford et al., 2014; Marchina et al., 2020). Outliers with high leverage (as per Cook's distance) in the regression model were excluded from this analysis, while extreme outliers were removed from the dataset for all analyses.

We used the R package 'MixSIAR', which uses Bayesian mixing models with Markov Chain Monte Carlo sampling to estimate the probability distributions of the proportions of sources contributing to the mixture (Moore and Semmens, 2008; Stock and Semmens, 2016) i.e., xylem water, to assess source water use per species and individual. Sampled soil depths were aggregated into four sources- 'surface' (0.05–0.10 m), 'shallow' (0.20–0.30 m), 'mid' (0.5 m) and 'deep' (≥1 m plus groundwater) based on adjacent, isotopically similar depths and visual assessment in the dual isotope space. Weak correlations between sources in MixSIAR confirmed them to be well separated, i.e., distinguishable in the model (Supp. Fig. 3). Models were run using 'root', 'lower stem' (pooled 0.2 and 1.3 m stem samples), 'upper stem' (pooled >1.3 m stem samples) and 'branch + twig' samples separately as mixtures. 'Species' and 'tree' were set as random factors to assess both species and individual variations, and error structure was set to 'process only'. Discrimination factors were set to zero and models were set to "extreme" (3,000,000 iterations, discarding the first 500,000).

3. Results

3.1. Does xylem water δ^2H and $\delta^{18}O$ vary between tree parts and along the height of the tree?

There were no significant differences in δ^2H between tree parts and between plant and soil for either species. However, patterns of variation in both δ^2H and $\delta^{18}O$ with respect to soil and from roots to branches varied between species (Fig. 2, Table 1). In both species, roots had a much higher variance in δ^2H and $\delta^{18}O$ than aboveground xylem, probably resulting from stem xylem mixing of root water isotopic composition from different depths. While average δ^2H and $\delta^{18}O$ in *E. tetrodonta* roots did not differ from soil water signatures, *C. nesophila* roots were more enriched than soil in both ^2H and ^{18}O, differing significantly in $\delta^{18}O$ ($p = 0.025$) and lc-excess ($p = 0.031$) (Supp. Table 1), suggesting fractionation at the root level. In both species, stem xylem water was more enriched than soil in ^2H and ^{18}O, significantly differing in $\delta^{18}O$ ($p = 0.013$) and lc-excess ($p < 0.001$) in *E. tetrodonta*. In this species, the stem was also more enriched than roots with significantly different $\delta^{18}O$ and lc-excess ($p < 0.001$ for both), suggesting that fractionation occurs at the stem level. *E. tetrodonta* branches were more depleted in ^2H and ^{18}O than stem, but more enriched than roots, significantly differing in $\delta^{18}O$ from roots ($p = 0.009$) and in lc-excess (offset from the LMWL) from roots ($p = 0.001$) and soil ($p = 0.003$). In contrast, *C. nesophila* branches were more enriched than stem, differing significantly in $\delta^{18}O$ ($p = 0.011$), but close to root values ($p = 0.809$), while differing significantly from soil in $\delta^{18}O$ ($p = 0.011$) and lc-excess ($p = 0.005$). No significant differences were present in lc-excess between tree parts in *C. nesophila*. While not discussed here, we additionally calculated the magnitude of isotopic difference (Δ^2H and $\Delta^{18}O$) between plant and soil samples, shown in Supp. Table 4.

When considering DBH and twig samples separately from stem and branch samples, *C. nesophila* branch $\delta^{18}O$ remained significantly different from soil ($p = 0.032$), however, there were no differences in $\delta^{18}O$ or in lc-excess among tree parts, suggesting that twigs are responsible for much of the variation in *C. nesophila* branches (Supp. Tables 2 and 3). *E. tetrodonta* stem, DBH and branch $\delta^{18}O$ were significantly different from roots ($p = 0.002$, $p = 0.018$ and $p = 0.002$ respectively) and soil ($p = 0.033$, $p = 0.036$ and $p = 0.036$, respectively), while roots remained significantly different from stem in lc-excess ($p < 0.001$).

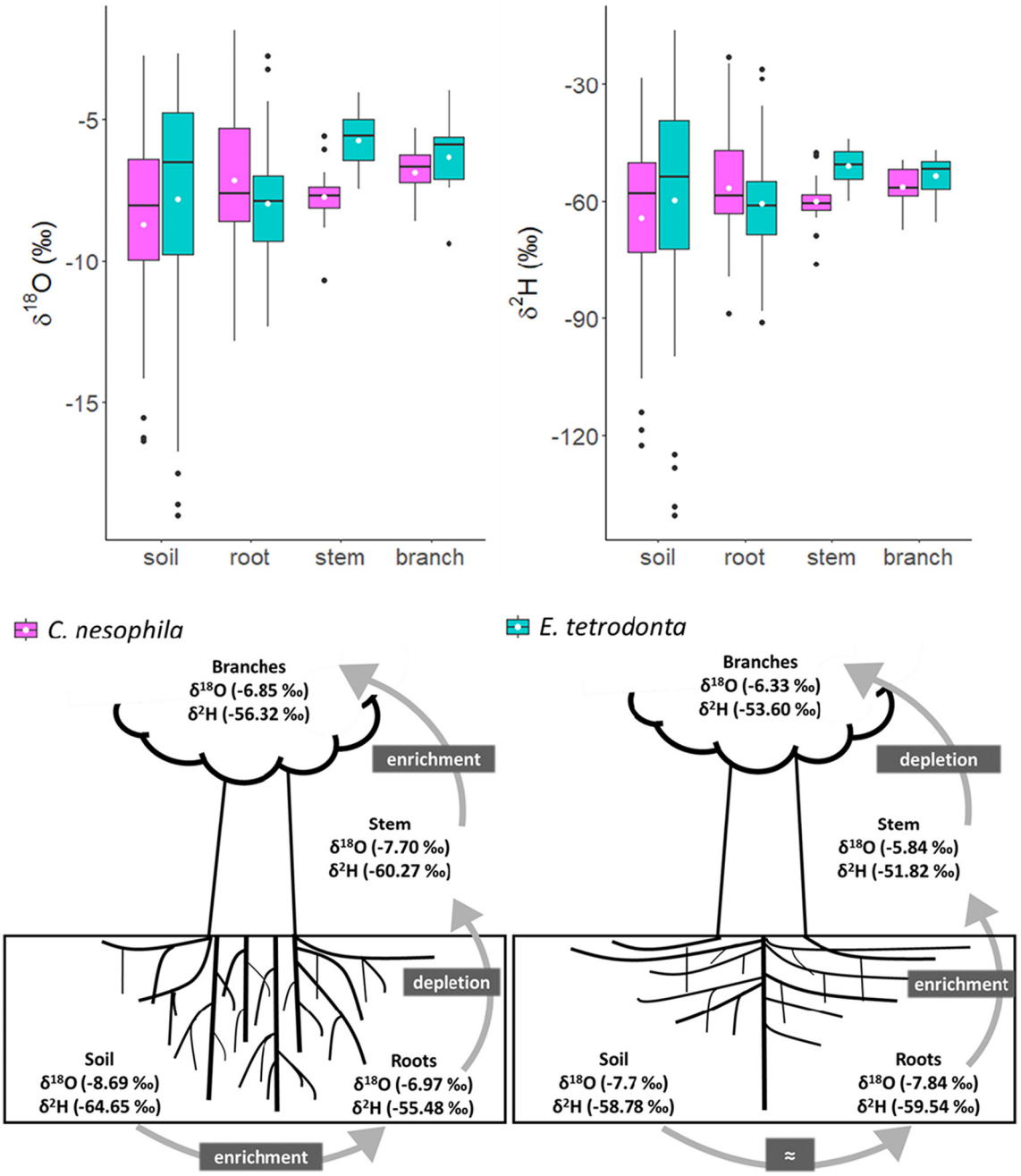

Fig. 2. Top: boxplots of δ^2H and $\delta^{18}O$ differences per tree part per species. Medians are shown in black horizontal lines and means are shown in white dots. Bottom: diagram of mean $\delta^{18}O$ and δ^2H variation from soil to roots, stem and branches per species. Species show contrasting mean xylem water isotopic variation patterns along the tree and in overall enrichment or depletion relative to soil water. Diagrams show a simplified, but not exact, representation of each species' rooting morphology for clearer visualisation, as root densities were not quantified.

When pooling aboveground (stem and branch) xylem samples, water isotopic composition was weakly, but not significantly, correlated with sampling height in *C. nesophila* ($R = 0.24$, $p = 0.118$ for δ^2H and $R = 0.31$, $p = 0.037$ for $\delta^{18}O$), and not correlated in *E. tetrodonta* (R = -0.07, $p = 0.654$ for δ^2H and $R = -0.07$, $p = 0.657$ for $\delta^{18}O$) (Supp. Table 5). Similarly, aboveground lc-excess was weakly negatively correlated to sampling height in *C. nesophila* (R = -0.21, $p = 0.172$) but not correlated in *E. tetrodonta* ($R = 0.03$, $p = 0.828$). This suggests that while isotopic composition and lc-excess markedly differs between roots and aboveground xylem in *E. tetrodonta*, in shoot xylem water *C. nesophila*, isotopic composition and lc-excess vary with sampling height.

3.1.1. How does xylem δ^2H and $\delta^{18}O$ vary between species and individuals?

Between species, stem xylem differed significantly ($p < 0.001$ for both δ^2H and $\delta^{18}O$), suggesting species were using different water sources. This was reflected in the dual isotope space with *C. nesophila* using more depleted water than *E. tetrodonta* (Fig. 3). However, root isotopic composition was similar between species ($p = 0.279$ for δ^2H and $p = 0.136$ for $\delta^{18}O$), suggesting that both species were tapping the same water sources. Branch δ^2H and $\delta^{18}O$ were also similar ($p = 0.101$ for and δ^2H and $p = 0.123$ for $\delta^{18}O$) between species. When considering DBH and twig samples separately, species differed significantly in stem ($p < 0.001$ for δ^2H and $\delta^{18}O$), DBH ($p < 0.001$ for δ^2H and $p =$

Table 1
δ^2H, $\delta^{18}O$ and lc-excess and water line slopes per soil and tree part, per species. Slopes of the RMA regression of δ^2H and $\delta^{18}O$ in the dual isotope space are reported along with their respective 95% confidence interval (C.I.). Data points which were outliers in the regression model were excluded, reducing the sample size.

	Sample	N	δ^2H (‰)			$\delta^{18}O$ (‰)			lc-excess (‰)			δ^2H and $\delta^{18}O$ regression slopes			
			Range	Mean (SD)	CI (95%)	Range	Mean (SD)	CI (95%)	Range	Mean (SD)	CI (95%)	N	r	Slope (95% C.I.)	p-value
C. nesophila	soil	45	(−122.68, −28.17)	−64.65 (23.87)	7.172	(−16.35, -2.75)	−8.69 (3.56)	1.069	(−25.51, 3.24)	−9.69 (5.17)	1.55	44	0.98	6.66 (6.28, 7.06)	0.001
	root	43	(−88.76, −10.92)	−55.48 (16.76)	5.159	(−12.82, -0.33)	−6.97 (2.72)	0.837	(−23.26, −2.44)	−12.40 (4.55)	1.4	41	0.96	5.96 (5.59, 6.58)	0.001
	stem	24	(−76.03, −47.47)	−60.27 (5.82)	2.457	(−10.68, −5.58)	−7.70 (0.98)	0.412	(−21.39, −5.92)	−12.11 (3.61)	1.53	23	0.73	5.97 (4.74, 7.55)	0.001
	branch	21	(−67.4, −49.49)	−56.33 (4.97)	2.261	(−8.57, −5.31)	−6.85 (0.88)	0.402	(−22.47, −5.49)	−14.02 (4.29)	1.95	21	0.89	5.67 (3.49, 9.28)	0.002
E. tetrodonta	soil	46	(−140.55, −16.07)	−58.78 (30.31)	9.001	(−18.99, −0.76)	−7.7 (4.25)	1.263	(−26.28, 7.78)	−10.64 (6.30)	1.87	44	0.99	7.26 (6.83, 7.70)	0.001
	root	33	(−91.1, −24.8)	−59.54 (15.57)	5.52	(−12.33, −2.74)	−7.84 (2.33)	0.826	(−18.74, −3.82)	−10.44 (4.18)	1.48	32	0.97	6.45 (5.83, 7.15)	0.001
	stem	29	(−73.71, −44.26)	−51.82 (5.91)	2.247	(−8.99, −4.05)	−5.84 (1.08)	0.411	(−25.47, −7.68)	−16.49 (4.25)	1.62	28	0.89	4.61 (3.11, 6.81)	0.001
	branch	19	(−65.3, −47.07)	−53.60 (5.26)	2.534	(−9.37, −3.97)	−6.33 (1.16)	0.56	(−24.64, −5.58)	−14.89 (4.06)	1.96	19	0.72	4.68 (3.64, 6.14)	0.001

0.007 for $\delta^{18}O$) and branches ($p = 0.036$ for δ^2H and $p = 0.019$ for $\delta^{18}O$) but not in twigs ($p < 0.804$ for δ^2H and $p = 0.488$ for $\delta^{18}O$).

At the individual tree level, there were no clear patterns of isotopic variation, however, lc-excess generally decreased with sampling height (not shown). In aboveground xylem water, δ^2H in individual trees varied up to 29.4‰ in *E. tetrodonta* and up to 28.5‰ in *C. nesophila*, while $\delta^{18}O$ in both species varied up to 5.4‰ (Table 1). Including roots, δ^2H variation in individual trees extended up to 55% in *E. tetrodonta* and up to 64.3‰ in *C. nesophila*, while $\delta^{18}O$ varied up to 7.3‰ in *E. tetrodonta* and up to 10.6‰ in *C. nesophila*.

3.1.2. How do roots, per type and depth, differ in their water isotopic composition?

Water isotopic composition and lc-excess were not significantly different between root types (Supp. Table 6). However, in both species there was a depletion tendency from fine to main roots (Fig. 4). Correspondingly, values were progressively depleted with diameter increase, with a moderate correlation in *E. tetrodonta* (R = -0.51, $p = 0.063$ for δ^2H and R = -0.56, $p = 0.037$ for $\delta^{18}O$) and a weak correlation in *C. nesophila* (R = -0.31, $p = 0.15$ for δ^2H and R = -0.37, $p = 0.086$ for $\delta^{18}O$). Variance in isotopic composition decreased with root diameter increase for both species, consistent with fine roots plotting along the length of the LMWL in the dual isotope space (Fig. 4). Main and coarse roots plotted more clustered around the lower half of the LMWL, corresponding to deeper sampling heights. Differences in rooting morphologies between species may have resulted in *C. nesophila* main and coarse roots plotting with some overlap in the dual isotope space, while *E. tetrodonta* main roots reflected slightly more depleted values than coarse roots. In both species, roots approximately reflected the observed soil pattern per depth, with the deepest roots (≥1 m) more depleted, plotting above the lower portion of the LMWL and more enriched, shallower roots (≤0.50 m) mostly below the upper portion of the LMWL. However, in both species, root isotopic composition was not correlated with height ($R = 0.17$, $p = 0.56$ for δ^2H and $R = 0.022$,

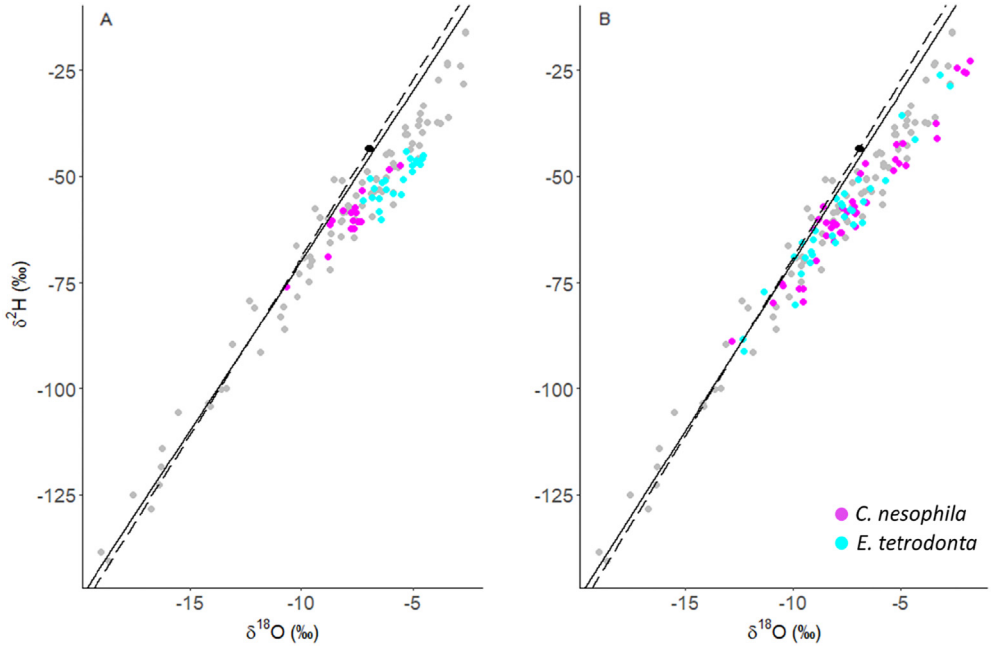

Fig. 3. Dual isotope space for stem (A) and root (B) samples per species. Stem reflects different water sources between species, while roots show similar water isotopic composition. Soil is shown in grey and groundwater in black. Dashed line is Cairns LMWL and full line is GMWL.

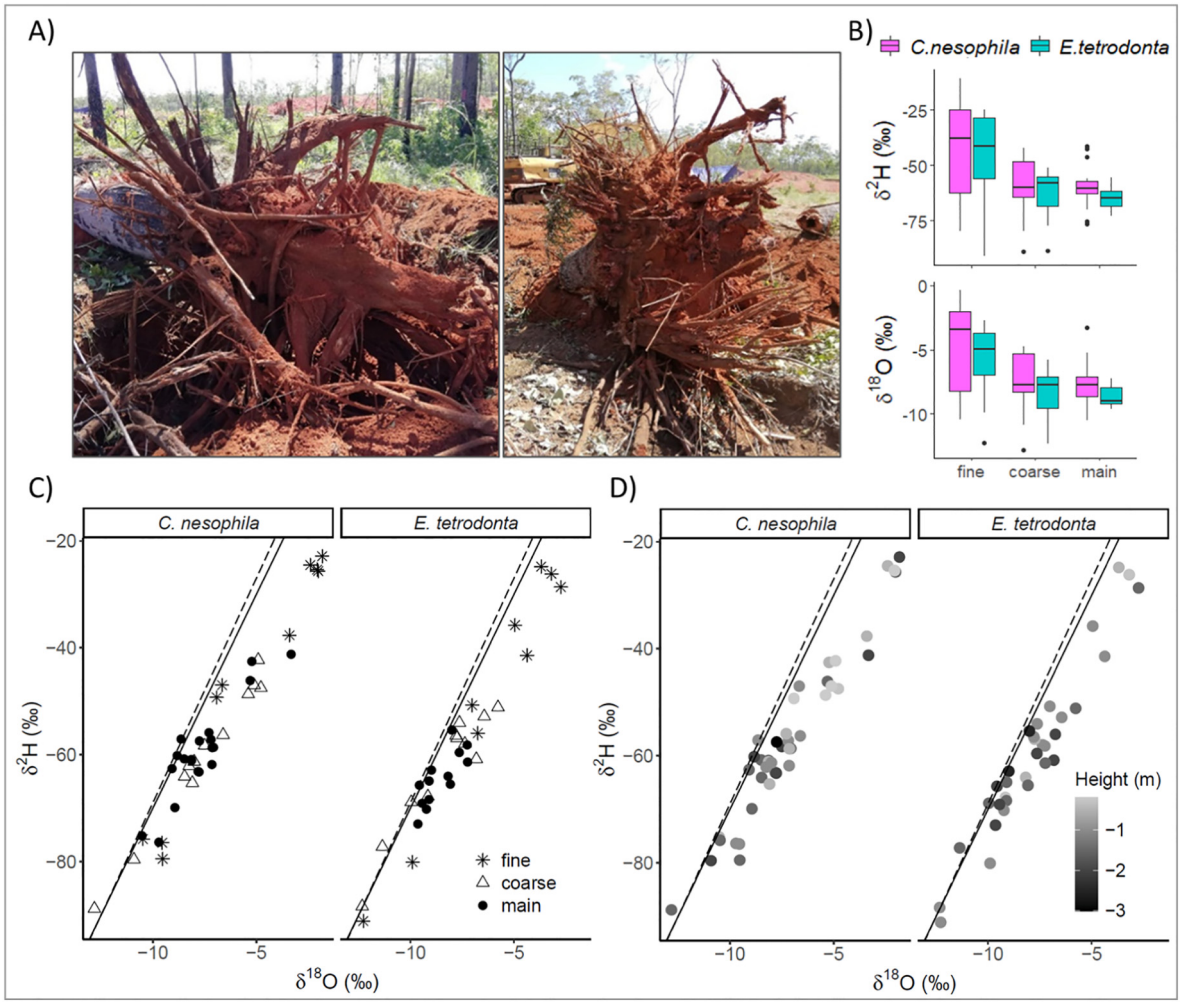

Fig. 4. A: Contrasting rooting morphologies between species (A), *E. tetrodonta* (left) and *C. nesophila* (right). Boxplot per root type (B), dual isotope space with roots per type (C) and per height (D), per species. In the boxplot, fine roots show the highest variance for both species. This is reflected in the dual isotope space, where fine roots plot a wide range along the length of the LMWL, while coarse and main roots are more clustered. In (C), *E. tetrodonta* main roots appear more depleted than coarse, in contrast to *C. nesophila*; this may be related to rooting morphological differences. The dashed line shows the LMWL, and the full line shows the GMWL.

$p = 0.94$ for $\delta^{18}O$ in *E. tetrodonta* and R = -0.019, $p = 0.93$ for $\delta^{2}H$ and R = -0.056, $p = 0.80$ for $\delta^{18}O$ in *C. nesophila*).

At the individual tree level in both species, however, when plotting $\delta^{2}H$, $\delta^{18}O$ and lc-excess along soil profile depths, individual roots plotted with some displacement or "gap" relative to soil sampled at the same height (Supp. Fig. 2). Compared to soil at the same depth, individual roots had either lower or higher $\delta^{2}H$ and $\delta^{18}O$ values, and generally lower lc-excess, suggesting fractionation from soil isotopic composition. At the species level, however, roots were enriched in both ^{2}H and ^{18}O with respect to soil across sampling depths (Fig. 5). This pattern was stronger for *C. nesophila* than for *E. tetrodonta* and is reflected with *C. nesophila* roots having significantly different mean isotopic composition from soil water, which this was not case for *E. tetrodonta*. The species-level trend was also clearer for lc-excess and was more pronounced in *C. nesophila* than in *E. tetrodonta*.

3.1.3. Flattening dual isotope slopes from below- to aboveground samples

Intriguingly, in the dual isotope space, water line slopes became flatter from roots to branches (Fig. 6, Table 1), implying increasing offset from rainwater signal and fractionation of xylem water along the tree. In both species, roots were closest to the soil water line (slope 6.66 and 7.26 in *C. nesophila* and in *E. tetrodonta*, respectively) and the LMWL (slope 8.40), with the root water line in *C. nesophila* having a slightly flatter slope (6.07) than in *E. tetrodonta* (6.45). Flatter slopes were observed with the stem water lines (5.97 in *C. nesophila* and 4.61 in *E. tetrodonta*), and with the branch water lines (5.67 in *C. nesophila* and 4.68 in *E. tetrodonta*).

3.2. Is aboveground isotopic variation explained by plant traits?

The maximum root depth at the studied site was 3 m, with roots sometimes stunted or turning upwards at the mottled or cemented zones, due to the low permeability of these clay-rich layers (Eggleton et al., 2008). While the maximum root depth varied between trees but not species, each species had a characteristic rooting structure (Fig. 4). *E. tetrodonta* had a deep taproot with laterals from shallow to mid depths, while *C. nesophila* had multiple main sinker roots reaching near maximum root depth with branching sinker clusters. Maximum root depth was weakly correlated to tree size DBH) ($R = 0.36$, $p = 0.012$) and total tree height ($R = 0.47$, $p = 0.001$) in *E. tetrodonta*, and not correlated to tree size (R = -0.02, $p = 0.194$) or tree height (R = -0.058, $p = 0.704$) in *C. nesophila*.

Interestingly, the effects of tree size, height and maximum root depth on aboveground isotopic composition were opposite between species. In *C. nesophila*, aboveground isotopic composition was weakly negatively correlated to tree height (R = -0.31, $p = 0.040$ for $\delta^{2}H$ and

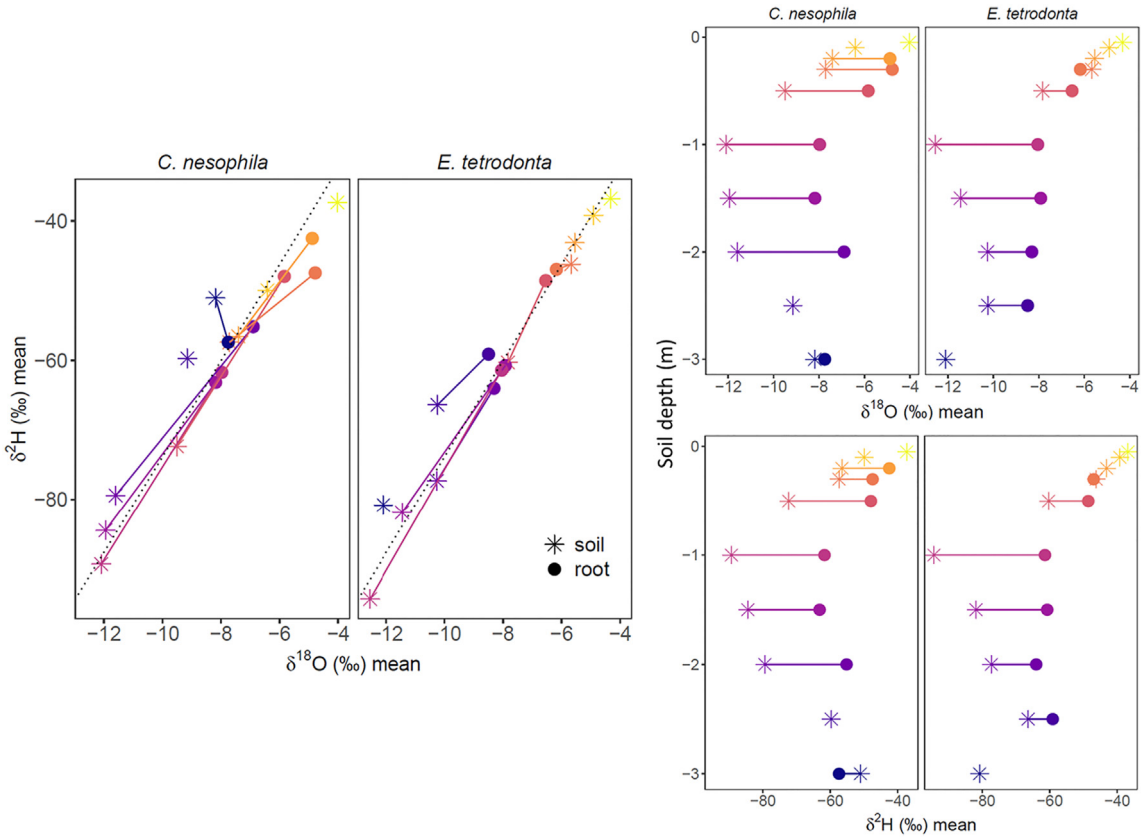

Fig. 5. Mean δ^2H and $\delta^{18}O$ in roots and soil per profile depths. On the right, offset between mean root and soil isotopic composition is shown per depths. On the left, how root-soil offsets translate into the dual isotope space, with root samples plotting along the LMWL while being fractionated with respect to soil at the same depths. While soil and roots were sampled at fixed depths for comparison purposes, it should be noted that where samples could not be collected at the exact fixed depth, they were included in the closest approximate sampling depth.

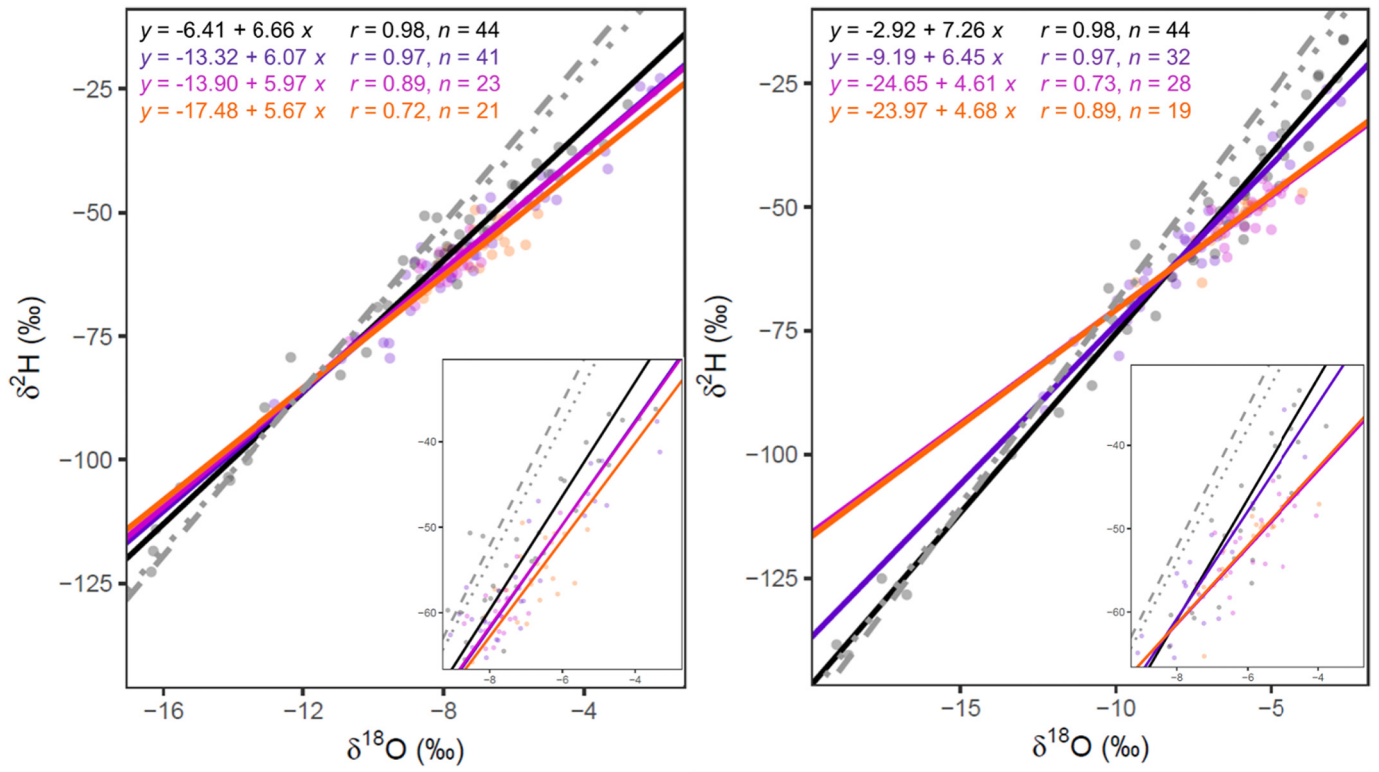

Fig. 6. Dual isotope plot with water lines of root, stem and branch samples per species, including soil. Insets are zoom-ins for detail. Equations show decreasing trendline slopes from roots to branches, indicating progressive offset from the LMWL from belowground to aboveground xylem. The grey dashed and dotted lines show the LMWL and the GMWL, respectively.

R = -0.27, $p = 0.074$ for $\delta^{18}O$), maximum root depth ($R = -0.42$, $p = 0.004$ for $\delta^{2}H$ and R = -0.19, $p = 0.224$ for $\delta^{18}O$), and size (R = -0.23, $p = 0.133$ for $\delta^{2}H$ and R = -0.17, $p = 0.265$ for $\delta^{18}O$) (Supp. Table 5). In contrast, in *E. tetrodonta* it was weakly positively correlated to tree height ($R = 0.17$, $p = 0.251$ for $\delta^{2}H$ and $R = 0.34$, $p = 0.017$ for $\delta^{18}O$), maximum root depth (R = 0.3, $p = 0.036$ for $\delta^{2}H$ and $R = 0.14$, $p = 0.332$ for $\delta^{18}O$), and size ($R = 0.08$, $p = 0.587$ for $\delta^{2}H$ and R = 0.3, $p = 0.037$ for $\delta^{18}O$).

3.3. Bayesian mixing models for soil water source proportions

All models showed that variation in estimated source contributions to xylem water was better explained by species than by individual trees (Supp. Table 7). This was clearest in the lower stem model, with 0.755 species median and 0.207 tree median, and in the upper stem model (0.877 species median and 0.275 tree median), followed by the root model (4.538 species median and 2.575 tree median). The branch + twig model showed less difference, with 0.284 species median and 0.150 tree median.

The lower stem model showed *E. tetrodonta* using mostly surface soil water (47%), with source contributions decreasing with depth (24.6% shallow, 17.3% mid and 7.7% deep) (Fig. 7, Supp. Table 8). The same model showed *C. nesophila* using roughly equal proportions from surface to mid depths (29.4% surface, 24.8% shallow, 28.5% mid), and less use of deep soil water (14.9%). These results are coherent with the observed species' rooting morphologies, where *E. tetrodonta* had lateral roots in the upper half of the profile while *C. nesophila* roots covered soil depths more evenly. Proportions estimated by the upper stem model also followed this trend, with 51.5% surface, 24.3% shallow, 16.2% mid and 6.2% deep for *E. tetrodonta* and 27.7% surface, 23.5% shallow, 30.1% mid and 17% deep for *C. nesophila*. Interestingly, for both species, the branch + twig model showed decreasing proportions from surface to deep soil water sources (41.9% surface, 25.9% shallow, 21.8% mid and 9.1% deep for E. *tetrodonta*; and 37.4% surface, 26.2% shallow, 24.8% mid and 10.8% deep for *C. nesophila*). Because *C. nesophila* branches were more enriched than stem (Fig. 2), the branch + twig model shows this species using greater surface water proportions than the lower stem model. In contrast, for *E. tetrodonta*, greater surface

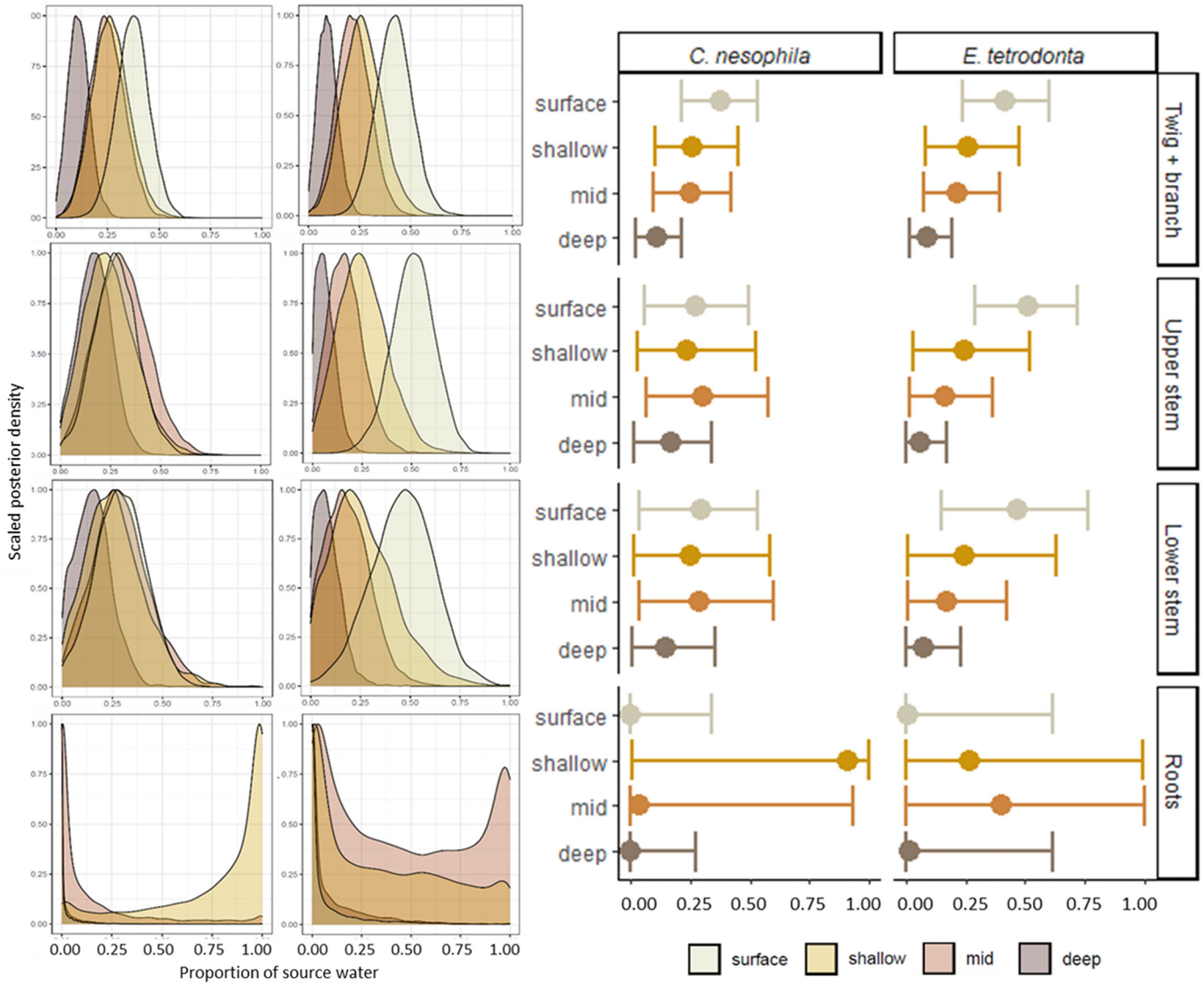

Fig. 7. MixSIAR Bayesian mixing model results for source ('surface', 'shallow', 'mid', 'deep') proportions used by species. Shown from top to bottom are the twig + branch, upper stem, lower stem and root models. On the left are the full probability distributions, or possible solutions (Phillips and Gregg, 2003), of the estimated proportions of each source contributing to xylem per model, with the left column showing *C. nesophila* models and the right column *E. tetrodonta* models). The plot on the right summarizes the medians (dots) and 95% credible intervals (error bars) of the estimated proportions.

water use was estimated by the lower and upper stem models than by the branch + twig model, consistent with the enriched stem water found in this species. The root model showed very different source proportions per species, with mostly shallow and mid soil water sources used by *E. tetrodonta* (1.2% surface, 26.8% shallow, 39.8% mid and 1.7% deep) and almost only shallow soil water use by *C. nesophila* (0.1% surface, 91.2% shallow, 3.3% mid and 0.2% deep). However, for both species, the root model estimations had a high level of uncertainty (Fig. 7), as shown by the wide 95% credible intervals and posterior distribution plots (Phillips and Gregg, 2003). This large uncertainty, particularly for *E. tetrodonta,* which showed a bimodal distribution for the 'mid' source estimate, and this renders the root models inconclusive. The high uncertainty in the estimates can probably be attributed to the large variability and range in root water isotope values as compared to source values (Supp. Fig. 4), because the probability distributions are influenced by the geometry of the of the mixing space in the dual isotope plot (Phillips and Gregg, 2003). In fact, uncertainty decreased from roots to branches, as did the isotopic ranges, with the branch model showing the estimates with the smallest uncertainty.

4. Discussion

Sampling entire mature trees including their root systems to maximum rooting depth is often impossible. Thus, only partial accounts of the actual xylem water isotopic composition are typically obtained, including from suberized twigs (Dawson and Ehleringer (1993) or stem core samples at DBH (Goldsmith et al., 2012; Meinzer et al., 1999). As a consequence, the water sources used by trees are mostly observed only in part (von Freyberg et al., 2020) and most field-based tree water sources have been unable to account for potential isotopic fractionation and variation along the tree transpiration path. Nevertheless, isotopic fractionation of xylem water can have considerable impact on the interpretation of plant water sources (Evaristo et al., 2015), and if ignored, may hinder their identification (Barbeta et al., 2019).

Here, we destructively sampled mature trees from the canopy to the maximum rooting depth to quantify the isotopic variation of xylem water along the transpiration stream. We found substantial variation in isotopic composition of xylem water in individual trees and contrasting species-level patterns of fractionation between tree parts. In both species, we found that root xylem water was enriched in ^{2}H and ^{18}O with respect to soil water across all depths. We also found increasing deviation of xylem water from the LWML from below- to aboveground xylem samples. This fractionation of xylem water from roots to branches challenges the standard assumption where generally, sap water isotopic composition in xylem tissue is assumed to remain unchanged from that of its sources. To our knowledge, this progressive and systematic, directional decrease in dual isotope slope from belowground to aboveground xylem water has not been observed previously. Our findings indicate that the isotopic composition of xylem water along the transpiration stream may differ from that of source waters, and that sampling from different locations in the tree can therefore affect the identification of its water sources, contradicting standing assumptions.

4.1. Differences in δ^2H and $\delta^{18}O$ of xylem water from root, to stem, to branch

Statistically significant differences in isotopic composition between soil and plant samples and between tree parts were only found for $\delta^{18}O$. These differences diverged between species, which suggested fractionation at the root level in *C. nesophila* and between roots and stem in *E. tetrodonta*. Our findings contrast with those of Ellsworth and Williams (2007) who showed that the desert shrub *Prosopis velutina*, had root water $\delta^{18}O$ that was intermediate between soil and leaf values and where stem $\delta^{2}H$ values fell between roots and leaves That roots were enriched with respect to soil across depths also contrasts with previous findings of roots being more depleted than soil (Ellsworth and Williams, 2007), or where root water uptake caused isotopic enrichment in surrounding soil (Vargas et al., 2017). That we did not find significant differences between tree parts for $\delta^{2}H$ in either species is consistent with those of desert tree *Populus euphratica* (Zhao et al., 2016). While cryogenic vacuum extraction, as we have used, is standard practice in plant water studies, it removes all water in a sample, including intra-cellular water that does not form part of the transpiration stream (Millar et al., 2018; Penna et al., 2018). However, while we cannot rule out possible effects associated to cryogenic extraction (Orlowski et al., 2018a, 2018b), it is unlikely the differences observed in $\delta^{18}O$ among tree parts were due to extraction methods since we used the same extraction method on all samples and the isotopic trends, variations and patterns were consistent in each species. In our view, cryogenic extraction cannot explain the fractionation from roots to branches observed in the dual isotope space. Furthermore, Zhao et al. (2016) used the cryogenic method (with different parameters) for tissue water extraction and did not detect differences in $\delta^{18}O$ among tissues.

The lack of significant differences in $\delta^{2}H$ between tree parts in either species and between soil and plant samples, but with greater $\delta^{18}O$ variation than $\delta^{2}H$ along the tree is noteworthy. This is because xylem water fractionation from soil in plant water source studies had been, until recently, only found —for the most part —for $\delta^{2}H$ (Ellsworth and Williams, 2007; Lin and Sternberg, 1993). Recent exceptions include $\delta^{18}O$ fractionation in association with arbuscular mycorrhizal fungi (Poca et al., 2019) or with root water uptake in potted trees (Vargas et al., 2017), and offsets in $\delta^{18}O$ between soil and plant water (Barbeta et al., 2020). To a lesser extent, De Deurwaerder et al. (2020) also detected spatial and temporal variation in xylem $\delta^{18}O$, while Ellsworth and Williams (2007) found some sapwood and stem segments more enriched in ^{18}O than soil water.

Although it cannot be directly compared, the magnitude of isotopic separation between soil and xylem in our study ($\Delta^{2}H = -22.1‰$ to 27.8‰ and $\Delta^{18}O = 3.5‰$ to $-3.8‰$) (Supp. Table 4) would be comparable to the $\Delta^{2}H = -24.6‰$ and $\Delta^{18}O = -2.9‰$ found by Poca et al. (2019) and greater than the $\Delta^{2}H = 9‰$ found by Ellsworth and Williams (2007). Within individual trees in both our species, the variation in aboveground xylem $\delta^{2}H$ and $\delta^{18}O$ alone, greatly exceeded the ~3% variation observed along *P. velutina* ($-141‰$ in taproot, $-138‰$ in stem) by Ellsworth and Williams (2007) and the diurnal variation (13.1‰) in stem in individual trees found by De Deurwaerder et al. (2020). Without considering $\delta^{2}H$ and $\delta^{18}O$ in roots, the variation in aboveground xylem water in our study (up to 29.4‰ in $\delta^{2}H$ and 5.4‰ in $\delta^{18}O$) is possibly the largest yet observed.

Our results suggest that xylem water integration of the isotopic composition of a tree's water sources may not be so straightforward. While twig samples were collected from the top part of the main branch sampled, due to the selection of only fully suberized twigs, half of the twig samples for *C. nesophila* were collected from positions lower than the highest branch sample. Notwithstanding, this does not explain why twigs had more depleted isotopic composition than branches in *C. nesophila*, or why branches were more enriched than the stem, or the higher variation in branch and twig lc-excess with respect to stem in this species. Possible explanations include variation in carbon fixation rates (Cernusak et al., 2005), feedback from leaves through phloem (Cernusak et al., 2005; Ellsworth and Williams, 2007), and differences within the crown in transpiration and radiation exposure, which could result in varying water isotopic composition in the crown (Burgess and Dawson, 2008). It is also possible that the large difference in *C. nesophila* twigs and branches, the higher variance in stem lc-excess as compared to roots and branches in *E. tetrodonta*, and the isotopic variations between tree parts in both species were caused by differences in the amount and use of stored water between the stem and crown of a tree (Čermák et al., 2007) or compartmentalisation in storage water pools (Barbeta et al., 2019). These variations may also have been due to previous water sources being reflected along the transpiration path. However, since both species are evergreen, we would have expected

similar isotopic patterns to appear along the transpiration path, which was not what we observed. That *E. tetrodonta* root water was significantly different from stem water could have been due to the extensive lateral root spread in this species not being completely represented in our sampling.

Although not always significant, the considerable variation in xylem water isotopic composition along the tree affected our Bayesian mixing model estimations. While the root model may have reflected recent root activity, considering that both species' roots were enriched with respect to soil, results may have been biased towards more enriched sources. Similarly, enrichment in *E. tetrodonta* stem water - may have biased the lower and upper stem model results towards greater surface water use. While the root and stem models showed different source proportions reflected by species, the branch + twig model showed little difference between species. If lower stem samples reflected more recent water uptake than branch-level samples, it is possible that differences observed in the stem between species were 'dampened' by fractionation at crown level, e.g. through feedback from leaves (Cernusak et al., 2005) or transpiration differences within the crown (Burgess and Dawson, 2008). This is consistent with the observation that the variability that was explained by species decreased from 75.5% to 58.2% to 28.9% in the lower stem, upper stem and branch + twig models, respectively.

4.2. Links between plant traits and aboveground xylem water isotopic composition

In an ecosystem, variation in rooting depths enables use of different water stores in the profile (Matheny et al., 2017), and stem-sampled water is often used to identify rooting depths. Our species differed strongly in stem water isotopic composition, reflecting their different sources. Notwithstanding, their similarities in maximum rooting depths and in root water isotopic composition indicated similar source access. Contrasting rooting morphologies between the two species may have been responsible for the different sources reflected in aboveground water composition. While not specifically quantified, on visual inspection *C. nesophila* appeared to have higher small root and fine root densities distributed evenly through the soil profile, while *E. tetrodonta* appeared to have much lower fine root densities and a dimorphic root system with a distinct deep taproot and shallow to mid-depth lateral roots. These same differences may have directly affected where in the soil profile water was sourced and consequently, what water was reflected in the stem. They may also help explain the opposite trends observed for the influence of DBH, total tree height and maximum rooting depth on aboveground isotopic composition between species. In *C. nesophila*, a weakly negative correlation suggested that for increasing tree size, height and rooting depth, more depleted (deeper) sources were used, although maximum rooting depths were not correlated to tree size or height in this species. In contrast, the weak positive correlation for *E. tetrodonta* suggested that more enriched (shallower) sources were used with increasing tree size and height (with maximum root depth weakly correlated to tree size and height). We hypothesize that more horizontally extensive shallow roots in large *E. tetrodonta* trees could possibly result in greater proportions of shallow soil water sources used, which coincides with aboveground xylem in this species reflecting more enriched sources than *C. nesophila*. That water isotopic composition in *E. tetrodonta* roots was significantly different from that in stem could also have been due to lateral root spread in this species not being completely represented in our sampling. Importantly, this would imply that rooting depth is not necessarily represented by aboveground xylem water sampling, and that aboveground xylem isotopic differences between species may result from contrasting rooting morphologies. This seemed to be supported by the Bayesian mixing model using xylem samples from the lower stem, which appeared to reflect each species' rooting morphology, suggesting that rooting morphologies should be considered in the interpretation of water sources (Figs. 4 and 7).

4.3. On the importance of belowground isotopic assessment

Xylem water isotopic composition will vary within a plant's rooting system as different roots source water from depths with varying isotopic composition (Rothfuss and Javaux, 2017). Isotopic composition may also vary along the length of a single root as water is incorporated from different source locations via branching roots. Thus, some degree of offset from sampled soil water could be expected in roots. It is important to note that our sources represented the full soil profile down to each tree's maximum rooting depth, i.e., 'all possible sources'. However, in the dual isotope space, both species' roots reflected only a portion of the full range of sampled soil sources, even while soil was sampled down to each individual tree's maximum rooting depth. This would suggest that regardless of rooting depth, neither species was using the full range of available water sources, if available sources are soil profile depths which roots can access. Plotting roots and soil per sampling height revealed that roots were more enriched in both ^{2}H and ^{18}O than soil water at the same heights. This explains why in the dual isotope space, roots reflected only the upper portion of the full soil sources value range. In other words, this resulted from root enrichment with respect to soil, and not by limited access to the available soil water sources. In fact, when tracing mean root isotopic values to soil at the corresponding depth in the dual isotope space, there was considerable distance between values: roots shifted along the LMWL with respect to soil water. This is important since in the dual isotope space, plant samples below the soil mixing space are assumed to indicate H fractionation (Evaristo et al., 2017). However, H as well as O fractionation may be masked if samples still plot along the LMWL and within the soil mixing space. Furthermore, this enrichment in roots was not just reflected as restricted use of water sources in the dual isotope space, but may have also influenced the Bayesian model results for aboveground mixtures, which showed both species using greater proportions of surface (0.05–0.10 m) soil water. Oerter and Bowen (2017) were surprised to find that xylem water from a ~ 5 m tree reflected very shallow (0–0.05 m) soil water in the dual isotope space. Our trees reached 40 m in height and 3 m in rooting depths; it is possible that the mostly surface soil water use estimated by the models is linked to the observed root enrichment.

Many studies have been unable to match some xylem isotope values with those of the sampled water sources (Barbeta et al., 2019; Brooks et al., 2010; Evaristo et al., 2017; Geris et al., 2015; Oerter and Bowen, 2017, 2019), with stem xylem samples plotting outside the soil mixing area in the dual isotope space (Evaristo et al., 2017). This offset from soil water sources and the possibility of a more generalized occurrence of xylem water isotopic fractionation have only recently been addressed by a limited number of papers (e.g., Barbeta et al., 2019, 2020; Penna et al., 2018). Possible explanations include the two water worlds hypothesis (McDonnell, 2014), where trees use less mobile soil water than that which recharges groundwater and streams (Berry et al., 2017; Brooks et al., 2010; Evaristo et al., 2015), separation in both space and time of tree and soil water fluxes (Evaristo et al., 2019), diurnal variation in root water uptake and sap flow (De Deurwaerder et al., 2020), incomplete sampling of all potential soil water sources (Penna et al., 2018) and issues related to extraction methodologies (Gaj et al., 2017; Orlowski et al., 2018, 2016; Penna et al., 2018). Several factors shown by our study may contribute to these offsets observed in some studies. First, our data showed that xylem water samples from roots, stem and branches followed different trend lines in the dual isotope space, showing progressive fractionation from source values along the transpiration stream. This could be related to differences in stem water storage amounts and use along the tree (Čermák et al., 2007) which could affect water isotopic composition as it is transported along the stem. In addition, different fractionation processes may be occuring per species, given the significant differences between roots and stem in *E. tetrodonta* and between soil and roots in *C. nesophila*. Second, that roots were enriched with respect to soil in both ^{2}H and ^{18}O

across depths would be reflected downstream along the tree as an offset from sampled sources. Together, these findings suggest that fractionation may occur in association with root water uptake and possibly as water is transported along the transpiration path. It is likely that a combination of these factors is responsible for plant samples plotting below the soil mixing space in some studies. Benettin et al. (2018) said that "isotope samples need to be understood as combining the effects of source variation, mixing, and fractionation", which, with view of our data, may also include effects of root-soil offsets and of possible fractionating plant physiological processes.

Considering the dual isotope space, Benettin et al. (2018) explain that flatter slopes and samples farther from the LWML result from greater evaporation variability, while steeper slopes and samples closer to the LMWL result from greater variability in sources (Benettin et al., 2018). In our study, divergence of water lines from the LMWL increased from roots to branches. It is possible that flatter stem slopes reflected smaller 'source' variability than roots, i.e. a decrease in water isotopic variation from below to aboveground, as xylem mixes water sourced across depths by roots- which had a steep slope close to the soil water line. It is also possible that the flatter stem and branch slopes also resulted from larger evaporation variability in the crown due to varying radiation exposure (Burgess and Dawson, 2008) and feedback from enriched leaves (Cernusak et al., 2005).

4.4. Implications for future work linking xylem water to soil water sources using stable isotopes

Some studies have used ^{18}O over ^{2}H to trace plant water sources, as there is no evidence for ^{18}O fractionation in plants (Ellsworth and Sternberg, 2015). However, the significant $\delta^{18}O$ variation we found along the tree challenges this assumption. Importantly, we found that, per species, both $\delta^{2}H$ and $\delta^{18}O$ in xylem followed the same trend from roots to branches, implying the fractionating processes that may be occurring are affecting both isotopes of water and not just H as has been previously found (Barbeta et al., 2019; Dawson and Ehleringer, 1993; Ellsworth and Williams, 2007; Evaristo et al., 2017).

Stem xylem water isotopic composition is commonly used to estimate maximum and effective rooting depths, i.e. water uptake depths, as it should integrate the signals of sources that roots have accessed. However, our study suggests that care should be taken in its interpretation. Our data showed that aboveground xylem isotopic composition does not necessarily correspond to that of soil water at maximum root depths and more importantly, may not reflect the full range of soil water sources accessed by trees. In the dual isotope plot (Fig. 3), there appeared to be a relatively limited use of soil water, where both species' roots matched only a portion of the sampled soil in the dual isotope space despite sampling soil to maximum rooting depth. However, this resulted from individual roots not reflecting soil water isotopic composition at the same profile depths, being displaced from soil in individual trees and enriched at the species level. Furthermore, this offset in both $\delta^{2}H$ and $\delta^{18}O$ at the root-level was masked by samples plotting along the LMWL and within the soil mixing space (Fig. 5).

While both species were able to access the same soil water profiles, evidenced by rooting depths and root water isotopic composition, aboveground xylem suggested different use of sources. Together with direct observations of rooting morphology, Bayesian mixing models helped understand water source partitioning between these two species which share access to the same soil water sources. The models showed that despite similar rooting depths and root water isotopic composition, species differed in source proportions used, i.e., that different use of sources, and not necessarily different access to sources was reflected in stem xylem. As argued by Chitra-Tarak et al. (2018), mature tree rooting depths do not necessarily represent actual water uptake depths. Due to the isotopic variation found along the tree, models using samples taken from stem and branch sections resulted in greatly different estimates of where water was being sourced within the soil profile. The estimates based on the isotopic composition of samples collected in the lower parts of the stem (which should best reflect recent water uptake) appeared to reflect the rooting morphology of each species, while differences between species became dampened at crown level in the branch + twig model. However, it is important to note that the enrichment at root level in *C. nesophila*, the enrichment at stem level in *E. tetrodonta* and the root-soil offsets in both species may have biased model estimations, limiting the direct identification of water source proportions. Our findings suggest that rooting morphologies and other traits should be incorporated in the study of plant water sources and have important implications for the accurate identification of root water uptake depths and the understanding and interpretation of tree water use, which is especially important in the context of climate change. Future plant water apportionment studies should thus consider not just the water sources used by trees —and not just different access to sources —but also how these sources are used, which may depend on water use strategies and that traits vary between individuals and species.

5. Conclusions

We have capitalized on a rare opportunity for whole-tree sampling to provide a full-picture view of water isotopic composition from roots and shoots and along the xylem transpiration stream of 12 mature *Eucalyptus tetrodonta* and *Corymbia nesophila* trees in their natural tropical savanna environment in northern Australia. Our results revealed root enrichment with respect to soil in both ^{2}H and ^{18}O and substantial water isotopic variation along individual trees with different patterns between species, greatly affecting the interpretation of water uptake depths. Sampled tissue in the trees showed an overall directional flattening of the slope of the dual isotope values relative to the LMWL from roots to branches, suggesting progressive, directional isotopic fractionation in xylem water along the tree water stream. Belowground assessments of rooting morphology and findings of root-soil offsets across depths shed light on the interpretation of aboveground xylem isotopic composition. A fractionated signal may be carried along the transpiration path, possibly caused by both below- and aboveground processes, which may further vary by species. These findings are especially important for studies using qualitative assessments of water uptake depth in the dual isotope space and may be related to the isotopic offsets observed between xylem and soil water in recent studies, where xylem water samples plotted below the LMWL and outside the soil mixing space. Further research should explore if the recent observations of soil-xylem offsets by others result from a combination of other plant processes along the transpiration stream. Future plant water source studies working with Myrtaceae and other tree species in monsoonal environments should also consider some fractionation in stem-sampled water.

CRediT authorship contribution statement

Adriana M. Vega-Grau: Investigation, Writing – original draft, Conceptualization, Methodology, Formal analysis, Data curation. **Jeffrey McDonnell:** Conceptualization, Writing – review & editing, Methodology. **Susanne Schmidt:** Conceptualization, Writing – review & editing, Methodology. **Mark Annandale:** Project administration, Resources, Funding acquisition. **John Herbohn:** Supervision, Conceptualization, Methodology, Writing – review & editing, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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