Leaf $\delta^{18}O$ of remaining trees is affected by thinning intensity in a semiarid pine forest

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ABSTRACT

Silvicultural thinning usually improves the water status of remaining trees in water-limited forests. We evaluated the usefulness of a dual stable isotope approach ($\delta^{13}C$, $\delta^{18}O$) for comparing the physiological performance of remaining trees between forest stands subjected to two different thinning intensities (moderate versus heavy) in a 60-year-old Pinus halepensis Mill. plantation in semiarid southeastern Spain. We measured bulk leaf $\delta^{13}C$ and $\delta^{18}O$, foliar elemental concentrations, stem water content, stem water $\delta^{18}O$ ($\delta^{18}O_{stem\ water}$), tree ring widths and leaf gas exchange rates to assess the influence of forest stand density on tree performance. Remaining trees in low-density stands (heavily thinned) showed lower leaf $\delta^{18}O$, and higher stomatal conductance ($g_s$), photosynthetic rate and radial growth than those in moderate-density stands (moderately thinned). By contrast, leaf $\delta^{13}C$, intrinsic water-use efficiency, foliar elemental concentrations and $\delta^{18}O_{stem\ water}$ were unaffected by stand density. Lower foliar $\delta^{18}O$ in heavily thinned stands reflected higher $g_s$ of remaining trees due to decreased inter-tree competition for water, whereas higher photosynthetic rate was largely attributable to reduced stomatal limitation to CO₂ uptake. The dual isotope approach provided insight into the early (12 months) effects of stand density manipulation on the physiological performance of remaining trees.

Key-words: Pinus halepensis; competition; drought stress; dual isotope approach; oxygen isotopic composition; stomatal conductance.

INTRODUCTION

Pinus halepensis Mill. is a drought-adapted species that has been extensively used in afforestation programmes in semiarid areas of the Mediterranean basin during the last 100 years (Maestre & Cortina 2004). In semiarid southeastern (SE) Spain, afforestation with P. halepensis has been primarily aimed at preventing catastrophic floods, reducing soil erosion rates and combating desertification. In many instances, afforestation of degraded soils with P. halepensis has led to the establishment of high-density plantations with slow-growing, even-aged trees which are prone to fire and pest attacks (Maestre & Cortina 2004). Silvicultural thinning is a suitable and widely used forest management alternative in this context, as it can enhance resource (light, nutrients, water) uptake and growth in remaining trees (Brèda, Granier & Aussenac 1995; Kolb et al. 2007). Thinning in drought-prone forests is primarily aimed at increasing water availability for remaining trees through reduced canopy interception of rainfall and decreased inter-tree competition for soil water (Brèda et al. 1995; McDowell et al. 2006; Martín-Benito et al. 2010). In semiarid conifer forests, enhanced soil water availability is to a large extent responsible for increased growth of remaining trees after thinning (Klein et al. 2005; McDowell et al. 2006). Several studies have shown that thinning stimulates P. halepensis growth by reducing inter-tree competition for growth-limiting soil resources (water and nutrients; Ne‘eman, Lahav & Izhaki 1995; González-Ochoa, López-Serrano & De Las Heras 2004; Moya et al. 2008). However, diameter growth responses to thinning can take several years to become noticeable in semiarid P. halepensis forests due to very slow tree radial growth. It thus becomes difficult to evaluate the effectiveness of stand density manipulation for improving the performance of remaining trees in these low-productivity forests. Stable isotope measurements in bulk leaf material could help overcome this limitation.

Stable isotope techniques are a powerful research tool in plant ecophysiological studies, as isotopic signatures provide time-integrated information on plant resource uptake and plant responses to the changing abiotic environments (Dawson et al. 2002). In C₃ plants, carbon stable isotope composition ($\delta^{13}C$) is inversely and linearly correlated with $c_i/c_a$, the ratio of intercellular to atmospheric CO₂ concentrations in leaves (Farquhar, Ehleringer & Hubick 1989). This ratio reflects the relative magnitudes of net photosynthetic rate ($A$) and stomatal conductance ($g_s$), and thus, $\delta^{13}C$ is a good indicator of plant intrinsic water-use efficiency ($WUE$), which is given by the ratio $A/g_s$ (Farquhar et al. 1989; Dawson et al. 2002; Klein...
The oxygen stable isotope composition ($\delta^{18}O$) of plant organic material is strongly influenced by the isotopic signature of soil water, which may change with depth of water uptake due to evaporative isotopic enrichment of soil water near the surface (Dawson et al. 2002; Barbour 2007). Plant $\delta^{18}O$ is also inversely related to $e_i/e_o$, the ratio of atmospheric to leaf intercellular water vapour pressure, and thus is strongly affected by changes in $g_s$ (Barbour 2007; Farquhar, Cernusak & Barnes 2007). Since plant $\delta^{18}O$ is related to $g_s$ but is unaffected by $A$, it can help separate the independent effects of $A$ and $g_s$ on $\delta^{13}C$ (Barbour 2007): simultaneous measurement of $\delta^{18}O$ and $\delta^{13}C$ in plant tissues allows discrimination between biochemical and stomatal limitations to photosynthesis (Scheidegger et al. 2000; Dawson et al. 2002; Querejeta et al. 2006; 2007, 2008; Grams et al. 2007).

$\delta^{13}C$ measurements in plant material (leaves and/or tree growth-rings) have been often used to assess the effects of stand density manipulation on the ecophysiological performance of remaining trees, with different results (Warren, McGrath & Adams 2001; McDowell et al. 2003; Sala et al. 2005). Reduced competition for water and increased soil water availability after thinning may lead to reduced $WUE$, due to differential enhancement of $g_s$, over $A$ in remaining trees (Meinzer, Goldstein & Grantz 1993; McDowell et al. 2003; Skov, Kolb & Wallin 2004; Ferrio et al. 2005). However, forest thinning can also increase foliar nutrient concentrations (Lopez-Serrano et al. 2005) and incident light levels in remaining trees, thus enhancing their $A$ and $WUE$, (Warren et al. 2001). Increased soil water availability, on the one hand, and enhanced nutrient and/or light availability, on the other, can exert effects of opposite sign on the $WUE$, of remaining trees after silvicultural thinning. Unsurprisingly, different studies have found widely different responses of $WUE$, and $\delta^{13}C$ to forest thinning. McDowell et al. (2003) reported depletion of $\delta^{13}C$ values in Pinus ponderosa after forest thinning, due to a greater increase in $g_s$ than in $A$ that led to reduced $WUE$, in remaining trees. By contrast, Warren et al. (2001) and Powers, Pregitzer & Palik (2008) reported foliar $\delta^{13}C$ enrichment in various Pinus species after thinning, which they attributed to differential enhancement of $A$ over $g_s$ due to increased foliar nutrient concentrations and greater canopy light interception in remaining trees. Other studies have reported no change in the $\delta^{13}C$ values of remaining pine trees after thinning (Wallin et al. 2004; Sala et al. 2005; McDowell et al. 2006), allegedly due to roughly parallel increases in both $g_s$ and $A$, without significant change in $WUE$.

Simultaneous measurement of $\delta^{18}O$ and $\delta^{13}C$ in bulk leaf material could help elucidate which physiological processes ($A$, $g_s$, $WUE$) are most strongly affected by stand density manipulation in each particular situation. Huang et al. (2008) and Brooks & Coulombe (2009) recently used this dual isotope approach to investigate the effects of various forest management practices (weed control, fertilizer addition) on tree physiological status. However, to the best of our knowledge, only Powers et al. (2008) have used both foliar $\delta^{18}O$ and $\delta^{13}C$ to evaluate the effects of silvicultural thinning on the performance of remaining trees. Powers et al. (2008) found increased $\delta^{13}C$ and no significant change in $\delta^{18}O$ in response to thinning, which they interpreted as evidence of enhanced $A$ (and to a lesser extent, $g_s$) in remaining pine trees.

Several recent studies have successfully used $\delta^{18}O$ and $\delta^{13}C$ measurements to investigate the intensity of interspecific or intraspecific competition for soil resources among plants (Grams et al. 2007; Huang et al. 2008; Ramírez, Querejeta & Bellot 2009). The main goal of the present study was to evaluate the usefulness of the dual stable isotope approach for early (~1 year) detection of differences in tree physiological status between forest stands subjected to different thinning levels in a semiarid environment. We hypothesized that heavy thinning in a 60-year-old P. halepensis plantation would reduce inter-tree competition for water and would improve the water status of remaining trees to a greater extent than moderate thinning. We predicted that remaining trees in low-density stands (subjected to heavy thinning) would show higher $g_s$ and $A$, and more depleted foliar $\delta^{18}O$, than those in moderate-density stands (subjected to moderate thinning). Furthermore, we expected to find no differences in $WUE$, or foliar $\delta^{13}C$ between treatments due to tightly coupled changes in $g_s$ and $A$. We also expected that trees in heavily thinned stands would show higher radial growth rates than those in moderately thinned stands.

**MATERIALS AND METHODS**

**Study site**

The study was conducted in a 60-year-old *P. halepensis* Mill. plantation (‘Los Cuadros’, 38°05′N, 1°06′W) located near the city of Murcia in SE Spain. Elevation in the experimental area ranges from 140 to 170 m above sea level. The climate is semiarid Mediterranean, with mean annual precipitation of 280 mm and average annual temperature of 18.2 °C (data from the Spanish Agencia Estatal de Meteorología, Ministerio de Medio Ambiente, y Medio Rural y Marino). Potential annual evapotranspiration, calculated by the Thornthwaite method (e.g. Dunne & Leopold 1978), is 932 mm. Soils in the area are mostly haplic calcisols, with some lithic leptosols (Alias et al. 1998). The *P. halepensis* plantation was established on abandoned agricultural lands in the 1940s and 1950s, with an initial planting density of 1400 trees ha$^{-1}$. Tree growth is very poor, with heights ranging from 4 to 8 m and basal diameters ranging from 7 to 20 cm (2003 data).

The year 2005 was extremely dry in the experimental area, with a total annual precipitation of only 158.8 mm. Years 2004 and 2007 were moderately wet (324.7 and 363.6 mm annual precipitation, respectively) but had very different rain distributions; whereas 2004 had a rainy spring, 2007 had a dry spring and above-average rainfall during late summer and early fall (Fig. 1).
Thinning intensity influences tree water status

Each plot (six trees were collected from six randomly selected trees in 2007 for isotopic and elemental analyses. In November core area to avoid boundary or edge effects. Thinning treatments: heavy thinning, with approximately 50% reduction of initial basal area, and a final tree basal area of 6.6 m² ha⁻¹ left standing; or moderate thinning, with approximately 33% reduction of initial basal area, and a final tree basal area of 6.6 m² ha⁻¹ left standing. Thinning treatments were applied between August and November 2004. After thinning, some of the slash was left on site. Final tree density was approximately 770 trees ha⁻¹ in the heavy thinning, versus approximately 550 trees ha⁻¹ in the moderate thinning --1 in the moderate thinning treatment.

Experimental and sampling design

The forest thinning experiment is a randomized block design with one factor (thinning intensity) and three replicate blocks. All replicate blocks (60 × 120 m each) are located on moderately steep (less than 20%) slopes. One replicate block is located on a site with north-west (NW) aspect and the other two on sites with north-east (NE) aspect. Each replicate block was divided into two adjacent plots of 60 × 60 m each, and each plot was assigned one of two different thinning treatments: heavy thinning, with approximately 50% reduction of initial basal area, and a final tree basal area of 4.5 m² ha⁻¹ left standing; or moderate thinning, with approximately 33% reduction of initial basal area, and a final tree basal area of 6.6 m² ha⁻¹ left standing. Thinning treatments were applied between August and November 2004. After thinning, some of the slash was left on site. Final tree density was approximately 770 trees ha⁻¹ in the moderate thinning treatment, versus approximately 550 trees ha⁻¹ in the heavy thinning treatment. The effects of thinning treatments on the carbon and water relations of the remaining trees were measured using a telescopic pruner. In November 2007, current-year needles from the 2005 leaf cohort and lignified stem sections were collected from 10 trees in each plot (10 trees × two treatments × three replicate blocks = 60 trees, including the same 36 trees sampled in 2005). New leaves of *P. halepensis* flush in spring and grow throughout the summer and into early fall (Weinstein 1989; Dougherty, Whitehead & Vose 1994). Foliar samples were taken in November, when all the pine needles were fully expanded. In May 2009, leaf gas exchange measurements (*A*, *g*, *WUE*) were conducted in order to validate the interpretation of isotopic data. Leaf gas exchange measurements were made on 9–12 remaining trees per treatment in each replicate block (~30 trees per thinning treatment overall). In October 2010, two increment cores were taken at ~1.3 m height from each of the same 36 pines sampled in 2005, for tree ring growth analysis.

Leaf elongation, nutrient concentrations and isotopic composition

The length of 10 randomly selected pine needles per sampled tree was measured with a precision of 1 mm for each leaf cohort (2004, 2005 and 2007). Bulk leaf samples were oven-dried at 60 °C and ground to a fine powder using a ball mill. Finely ground bulk leaf samples were weighed using a precision balance, and encapsulated in tin capsules for stable isotope analyses. Bulk leaf material contains varying proportions of organic compounds with different isotopic signatures; however, several studies measuring δ¹⁸O and δ¹³C in both bulk leaf material and leaf cellulose have found that the stable isotope compositions of these materials are strongly positively correlated (e.g. Barbour et al. 2000; Sullivan & Welker 2007; Powers et al. 2008; although cellulose extraction may be necessary when comparing plants grown under different environmental conditions, as in Grams et al. 2007).

The carbon isotope ratio of bulk leaf material (δ¹³C) was analysed using elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS, Bremen, Germany). The oxygen isotope ratio of the bulk leaf material (δ¹⁸O) was determined with a Finnigan MAT Delta Plus XL following the method described in Farquhar, Henry & Styles (1997). This method uses a high-purity alumina pyrolysis tube, a pyrolysis temperature of 1130 °C, chloropentane doping of the carrier gas and a Porapak Q gas chromatography (GC) column before the molecular sieve column. The Porapak Q column separates N₂/CO₂ from any organic compounds, the N₂ and CO₂ is allowed to proceed to and be separated on the molecular sieve column, then both columns are back flushed to remove any contaminants before the next sample is analysed. Isotope analyses were conducted at the Center for Stable Isotope Biogeochemistry, University of California, Berkeley (USA). Isotope ratios are expressed in delta notation (‰), where the isotopic composition of a material relative to that of an internationally accepted reference standard is given by

\[
\delta^X = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000
\]

where \( R \) is the molecular ratio of heavy to light isotope forms (Dawson et al. 2002). The standard used for δ¹³C is PeeDee Belemnite (V-PDB) and the standard for δ¹⁸O is Vienna-standard mean ocean water (V-SMOW). Long-term (3+ years) external precisions for carbon and oxygen isotope analyses are 0.14 and 0.23‰, respectively.

Figure 1. Monthly rainfall and mean monthly temperatures between January 2004 and December 2009 (data from Murcia weather station provided by the Spanish Agencia Estatal de Meteorología).
Foliar nitrogen and carbon concentrations were determined with a Thermo Finnigan Flash 1112 elemental analyzer (Franklin, MA, USA). Foliar phosphorus and potassium concentrations were measured at Centro de Edafología y Biología Aplicada del Segura-Consell Superior de Investigaciones Científicas (CEBAS-CSIC) by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Elemental Iris Intrepid II XDL, Franklin, MA, USA) after a microwave-assisted digestion with HNO₃:H₂O₂ (4:1, v:v).

**Stem water content and isotopic composition**

In November 2007, lignified stem sections were collected from the same trees sampled for bulk leaf material. Lignified stem sections from remaining trees in both treatments were also collected in May 2008 (n = 24) and September 2008 (n = 14) in order to obtain stem water content and isotopic composition values representative of the growing season. Two stem sections per tree, approximately 10 mm in diameter and 20 mm long, were collected for stem water extractions, and all leaf and green stem tissue was removed from them to avoid contamination of xylem water with isotopically enriched water (Ehleringer & Dawson 1992). Upon collection, stem samples were immediately placed in capped vials, wrapped with parafilm, and stored in the freezer until water extraction. Stem water was extracted using a cryogenic vacuum distillation line (Ehleringer, Roden & Dawson 2000). Stem water content was calculated from the weight difference before and after oven-drying stem samples at 100 °C. In semiarid ecosystems, interplant differences in stem water content often reflect differences in plant water status (e.g. plant water potential; Querejeta, Egerton-Warburton & Allen 2009).

Analysis of stem water δ¹⁸O (δ¹⁸Ostem water) was conducted at the Stable Isotope Laboratory of the Department of Earth and Planetary Sciences, University of New Mexico (USA), using the CO₂ equilibrium technique. The water samples (1 mL each) were injected into borosilicate vials equipped with rubber septa, which were previously purged with He–CO₂ gas mixture (0.5% CO₂). After 24 h equilibration at 25 °C, the CO₂ was measured by continuous flow isotope ratio mass spectrometry using an automated CombiPal–Gas Bench system coupled to a Finnigan Mat Delta Plus mass spectrometer. The results were corrected using three laboratory standards (calibrated against international water standards). Reproducibility was better than 0.1% based on repeats of laboratory standards. δ¹⁸O values are expressed in delta notation (‰) relative to the international standard V-SMOW.

Since leaf δ¹⁸O is strongly influenced by the isotopic signature of the source water used by the plant (Dawson et al. 2002; Barbour 2007), it is standard practice to report the oxygen isotopic composition of leaf tissue as oxygen isotope enrichment above that of source water, in order to account for this effect. Leaf oxygen isotope enrichment above source water (Δ¹⁸O; Barbour et al. 2000) is calculated as:

\[ \Delta^{18}O = \frac{\delta^{18}O_{bulk~leaf} - \delta^{18}O_{stem~water}}{\delta^{18}O_{stem~water}} \times 1000 \]

We used the δ¹⁸Ostem water values measured in November 2007 to calculate leaf Δ¹⁸O, although it should be noted that these δ¹⁸Ostem water values may not be exactly representative of mean source water δ¹⁸O integrated over the entire leaf growing season (spring to fall).

**Leaf gas exchange measurements**

A and gₑ were measured in May 2009 with a portable photosynthesis system (LI-6400, Li-Cor, Inc., Lincoln, NE, USA) equipped with a LI-6400–40 Leaf Chamber Fluorometer (LI-6400, Li-Cor) and a Li-Cor 6400–01 CO₂ injector. Gas exchange was measured on 1-year-old sunlit needles from apical shoots in southerly oriented branches in the middle part of the crown. Approximately 20 attached needles were placed in a 2 cm² leaf cuvette for gas exchange measurements. The CO₂ concentration in the cuvette was maintained at 380 μmol mol⁻¹ CO₂. Measurements were done at saturating light of 1.500 μmol m⁻² s⁻¹, and at ambient air temperature and relative humidity. The leaf-to-air water vapour pressure difference was between 0.75 and 1.2 mmol mol⁻¹ for all measurements and the air flow was set to 350 μmol s⁻¹. All leaf gas exchange measurements were conducted at mid-morning between 9:00 and 11:00 h (local standard time; 7:00–9:00 GMT) on sunny days. Pine needles were collected after leaf gas exchange measurements, and the leaf sections enclosed in the leaf cuvette of the Li-Cor 6400 were digitized by scanning on A3 flatbed scanner (HP Deskscan) fitted with a transparency adaptor at 300 dpi, using an 8-bit greyscale. We analyzed the images with specific software (WinRhizo, Regent Instruments Inc., Québec, Canada) to obtain needle surface area (and needle average diameter; Li, Kräuchi & Dobbertin 2006; Fuentes et al. 2007). Total needle surface area values measured by this method were on average 7.5% higher (2.15 cm²) than the area of the leaf cuvette (2.00 cm²). All gas exchange parameters were expressed on a total needle surface area basis. WUE, was calculated as A/gₑ.

**Tree ring growth analysis**

Increment cores taken in October 2010 were mounted, air dried and fine-sanded until tree rings were clearly visible under a binocular microscope. Tree rings were visually cross-dated and measured to the nearest 0.01 mm with a measuring table (LINTAB, Frank Rinn, Heidelberg, Germany) coupled with the TSAP software package (Frank Rinn, Heidelberg, Germany) (Rinn 1996). Obtained tree ring series were compared with each other and with a previously constructed chronology for the same species in a neighbouring site (de Luis et al. 2009) to ensure accuracy of cross-dating. Finally, cross-dating was statistically verified using the program COFECHA (Laboratory of Tree-Ring Research, University of Arizona, Tucson, AZ, USA) (Holmes 1983). For each tree, ring widths of years 2004 to 2009 were converted to standardized index values [tree ring width (TRW index)] by dividing them by the average
annual radial growth increment of that tree during the period 1983–2003 (pre-thinning reference period).

Statistical analyses

All statistical analyses were performed with SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). Repeated measures analysis of variance (ANOVA) was used to evaluate the effects of thinning intensity, replicate block (between-subject factors), annual leaf cohort/year (within-subject factor) and their interactions on the dependent variables. Individual trees were considered subjects. The dependent variables were pine needle length, foliar nutrient concentrations and foliar stable isotopes ratios in the two annual leaf cohorts (2005, 2007) produced after application of the thinning treatments (2004) and the natural logarithm of the TRW index of years 2005 to 2009. Treatment effects on leaf gas exchange parameters, stem water content and stem water δ18O (measured in a single year) were tested using two-way ANOVAs (considering thinning intensity as fixed factor and replicate block as random factor). Mean values ± standard error of measured variables in each treatment are presented. For each leaf cohort/year, linear regression analyses were used to examine the relationships between measured variables across all sampled individuals from both thinning treatments.

RESULTS

Foliar δ18O and δ13C

Repeated measures ANOVA indicates that thinning intensity significantly affected the foliar δ18O of remaining trees (Table 1; Fig. 2a). Mean bulk leaf δ18O and δ13C in the 2004 leaf cohort (fully formed before thinning) were indistinguishable between trees that were to be assigned to different thinning treatments. However, a striking difference between treatments became apparent shortly (12 months) after thinning: foliar δ18O in the 2005 leaf cohort (newly formed after thinning) was significantly less enriched in the heavily thinned than in the moderately thinned stands (Fig. 2a, \( P = 0.010 \)). This difference in foliar δ18O between thinning treatments was still apparent in the 2007 leaf cohort (Fig. 2a, \( P = 0.035 \)). Interestingly, the difference in bulk leaf δ18O between heavily and moderately thinned stands was largest for the 2005 leaf cohort, which was produced during the driest year of the study period (Fig. 2a).

In contrast to δ18O, bulk leaf δ13C was unaffected by thinning intensity (Table 1; Fig. 2b). Foliar δ13C in the 2007 leaf cohort (but not in the 2005 cohort) was positively correlated with foliar δ18O across individuals from both thinning treatments (Fig. 3a).

Leaf growth and elemental concentrations

Repeated measures ANOVA showed no significant difference in pine needle length between thinning treatments (Table 1). However, remaining pines in the heavily thinned stands tended to have somewhat longer needles than those in the moderately thinned stands, and this difference became marginally significant for the 2007 leaf cohort (\( P = 0.080 \)), when average needle length was 7.1 ± 0.1 cm in heavily thinned stands versus 6.8 ± 0.1 cm in moderately thinned stands (Fig. 2c). In both thinning treatments, pine needle length was much shorter in the 2005 cohort than in the other cohorts due to severe drought. In the 2005 leaf cohort, pine needle length was weakly negatively correlated with foliar δ18O across individuals from both thinning treatments (\( r^2 = 0.127; P = 0.038 \)).

Foliar N concentration was highest in 2005 for both thinning treatments, possibly due to a pulse of nitrogen availability derived from decomposition of dead fine roots following tree thinning. However, poor leaf growth due to severe drought may have also contributed to altered leaf stoichiometry and increased foliar N concentration in the 2005 leaf cohort. Foliar elemental concentrations in remaining trees were not significantly different between thinning treatments according to repeated measures ANOVA (Table 1; Fig. 2d–h). However, foliar N concentration became transiently higher in the heavily thinned than in the moderately thinned stands for the 2005 leaf cohort (produced shortly after thinning), whereas the reverse pattern was found in the 2004 (pre-thinning) and 2007 leaf cohorts.

Stem water content and isotopic composition

The δ18O of stem water in the remaining trees did not differ between moderately and heavily thinned stands in

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**Table 1.** Levels of significance of main effects (thinning intensity, replicate block, leaf cohort and the interaction between thinning intensity × leaf cohort) on the leaf stable isotope composition, needle length and leaf nutrient concentrations of two leaf cohorts produced after thinning (2005 and 2007) in *Pinus halepensis*, as determined by repeated measures analysis of variance

<table>
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<th></th>
<th>df</th>
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<th>δ18O</th>
<th>Needle length</th>
<th>C</th>
<th>N</th>
<th>C/N</th>
<th>P</th>
<th>K</th>
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<tr>
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<td>&lt;0.001</td>
<td>&lt;0.005</td>
<td>0.269</td>
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<tr>
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<td>0.034</td>
<td>0.086</td>
<td>0.427</td>
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</tbody>
</table>

Values of \( P < 0.05 \) appear in bold.
Figure 2. Mean values of (a) bulk leaf $\delta^{18}O$, (b) bulk leaf $\delta^{13}C$, (c) needle length, (d) leaf carbon concentration (C), (e) leaf nitrogen concentration (N), (f) leaf carbon to nitrogen ratio (C/N), (g) leaf phosphorus concentration (P) and (h) leaf potassium concentration (K) in remaining Pinus halepensis trees of the heavy and moderate thinning treatments for the 2004, 2005 and 2007 leaf cohorts (error bars indicate ± one SE). Thinning treatments were applied in the fall of 2004.

Figure 3. Relationships between (a) bulk leaf $\delta^{13}C$ and $\delta^{18}O$ ($\delta^{13}C = -29.70 + 0.17\delta^{18}O; r = 0.46, P < 0.01, r^2 = 0.21, n = 56$), and (b) bulk leaf $\delta^{13}C$ and foliar oxygen isotope enrichment above source water ($\Delta^{18}O$) ($\delta^{13}C = -31.12 + 0.19\Delta^{18}O; r = 0.46, P < 0.01, r^2 = 0.21, n = 55$) in the 2007 leaf cohort of remaining Pinus halepensis trees, across individuals from both thinning treatments.
November 2007 (−5.38 ± 0.11‰ and −5.28 ± 0.10‰, respectively; \( P = 0.459 \)), May 2008 (−3.57 ± 0.08‰ and −3.39 ± 0.15‰, respectively; \( P = 0.366 \)) or September 2008 (−2.61 ± 0.32‰ and −2.20 ± 0.36‰, respectively; \( P = 0.503 \)). This indicates that the sources of water used by the remaining pines were indistinguishable between the two thinning treatments. There were no significant differences in stem water content between moderately and heavily thinned stands in November 2007 (51.36 ± 0.49% and 52.00 ± 0.39%, respectively; \( P = 0.289 \)) or May 2008 (49.30 ± 0.43% and 49.97 ± 0.58, respectively; \( P = 0.354 \)). However, mean stem water content was significantly higher in heavily than in moderately thinned stands (49.68 ± 0.68% versus 46.45 ± 1.03%, respectively; \( P < 0.01 \)) in late summer (September 2008).

Oxygen isotope enrichment of bulk leaf material above source water (\( \Delta^{18}O \)) was not significantly different between thinning treatments in November 2007 (35.32 ± 0.24‰ in heavily thinned stands versus 35.69 ± 0.17‰ in moderately thinned stands; \( P = 0.168 \)). Across individuals from both thinning treatments, foliar \( \Delta^{18}O \) was positively correlated with foliar \( \delta^{13}C \) (Fig. 3b), whereas stem water content was weakly negatively correlated with bulk leaf \( \delta^{18}O \) (\( r^2 = 0.204; P < 0.001 \)) and \( \Delta^{18}O \) (\( r^2 = 0.142; P = 0.004 \)). Stem water content was not correlated with stem water \( \delta^{18}O \).

**Leaf gas exchange measurements**

\( A \) and \( g_s \) in the remaining trees were both significantly higher in heavily thinned stands than in moderately thinned stands (\( P = 0.008 \) and \( P = 0.001 \), respectively; Fig. 4a,b). However, \( WUE_i \) was not significantly different between thinning treatments (Fig. 4c), thus suggesting roughly parallel increments of similar magnitude in both \( A \) and \( g_s \) after heavy thinning. There was a strong positive correlation between \( A \) and \( g_s \) across individuals from the two thinning treatments (Fig. 5), thus indicating tight stomatal control of both transpiration and carbon assimilation rate in *P. halepensis*.

**Tree ring growth**

Repeated measures ANOVA indicates that the remaining trees in heavily thinned stands had significantly greater radial growth than those in moderately thinned stands during the period 2005 to 2009 (Fig. 6, \( P < 0.001 \)). Mean annual TRW from 2005 to 2009 were 1.64 ± 0.12 mm in heavily thinned stands versus 1.14 ± 0.11 mm in moderately thinned stands. For the 1983–2003 period (before thinning), there were no significant differences in TRW between stands assigned to different thinning intensities (mean annual TRW for this period is 0.66 ± 0.04 mm in plots assigned to heavy thinning versus 0.71 ± 0.06 mm in plots assigned to moderate thinning; repeated measures ANOVA, \( P = 0.520 \)). Although the thinning treatments were applied in the fall of 2004, significant differences in TRW index between thinning intensities did not appear until 2006 (Fig. 6).

Figure 4. Effects of thinning intensity on the net photosynthetic rate (\( A \)), stomatal conductance (\( g_s \)) and intrinsic water-use efficiency (\( WUE_i \)) of remaining *Pinus halepensis* trees. Different letters above columns indicate significant differences between thinning treatments (\( P < 0.05 \)); error bars represent one SE.

In 2007, TRW index was negatively correlated with foliar \( \Delta^{18}O \) (Fig. 7a) and foliar \( \delta^{18}O \) (Fig. 7b) across individuals from both thinning treatments. TRW index was also significantly correlated with foliar \( \delta^{13}C \) (\( r = -0.44, P = 0.01, r^2 = 0.20 \)) and stem water content (\( r = 0.45, P < 0.01, r^2 = 0.20 \)), but not with stem water \( \delta^{18}O \).
Differences in the foliar $\delta^{18}O$ of the remaining trees between heavily and moderately thinned stands cannot be plausibly explained in terms of differences in microclimatic conditions. Reducing tree density opens the canopy and changes microclimatic conditions by allowing greater penetration of solar radiation and wind into forest stands (Brèda et al. 1995; Aussenac 2000). Thinning leads to significant changes in light availability, temperature and humidity in the forest environment, and these changes in microclimatic conditions could affect the foliar $\delta^{18}O$ of remaining trees (Yakir 1992; Barbour 2007). Forest sites with more open canopies generally show greater light intensity, wider temperature fluctuations and lower air relative humidity than neighbouring sites with more closed canopies (Aussenac 2000). In water-limited conifer forests, low-density stands with more open canopies are usually sunnier, hotter and drier than neighbouring stands with denser canopies (Meyer, Sisk & Covington 2001; Rambo & North 2009; Ma et al. 2010; Martín-Benito et al. 2010). Higher temperature and lower air humidity [higher vapour pressure deficit (VPD)] would be expected to lead to more enriched foliar $\delta^{18}O$ in semiarid forest with more open canopies (Burk & Stuiver 1981; Yakir 1992; Roden & Ehleringer 1999; Barbour 2007). However, we found that the remaining trees in low-density stands (heavily thinned) with more open canopies showed consistently lower foliar $\delta^{18}O$ values than those in moderate-density stands (moderately thinned) with denser canopies.

Lower foliar $\delta^{18}O$ in heavily thinned stands supports our prediction that a greater reduction in inter-tree competition

**Figure 5.** Relationship between net photosynthetic rate ($A$) and stomatal conductance ($g_s$) in remaining *Pinus halepensis* trees across individuals from both thinning treatments ($A = 2.56 + 60.18 g_s; r = 0.78, P < 0.01, r^2 = 0.61, n = 68$). The $A/g_s$ regression equation for the heavy thinning treatment was $A = 2.97 + 55.07 g_s (r = 0.57; P < 0.01, n = 35)$ versus $A = 1.77 + 72.10 g_s (r = 0.59; P < 0.01, n = 33)$ for the moderate thinning treatment.

**DISCUSSION**

Figure 6. Mean tree ring width index (TRW index) of *Pinus halepensis* trees in the heavily and moderately thinned stands from 2004 to 2009 ($n = 18$; error bars indicate ± one SE). Thinning treatments were applied in the fall of 2004. TRW index is calculated by dividing each annual tree ring width by the average annual radial growth increment of that tree during the period 1983–2003 (pre-thinning reference period).

Figure 7. Relationship of tree ring width index (TRW index) in 2007 with (a) foliar oxygen isotope enrichment above source water of the 2007 leaf cohort ($\Delta^{18}O; r = -0.45, P < 0.01, r^2 = 0.20, n = 34; \Delta^{18}O = 36.85 – 0.29 \text{TRW index}$) and (b) bulk leaf $\delta^{18}O$ in the 2007 leaf cohort ($r = -0.43, P = 0.01, r^2 = 0.18, n = 35, \delta^{18}O = 31.54 – 0.29 \text{TRW index}$) across individuals from both thinning treatments.
for soil resources would be more effective at enhancing the water status of remaining trees (Fig. 2a). *P. halepensis* is a drought-avoiding species with tight stomatal control of transpiration and photosynthesis under water-limiting conditions (Ferrio et al. 2003; Klein et al. 2005; Maseyk et al. 2008; Voltas et al. 2008). Lower foliar δ18O suggests higher leaf-level g, in stands subjected to heavy thinning than in stands subjected to moderate thinning (Barbour et al. 2000; Barbour 2007; Farquhar et al. 2007), and this interpretation of isotopic data is well supported by leaf gas exchange data (Fig. 4b). As expected, a greater decrease in canopy interception of rainfall and inter-tree competition for soil water led to higher g, in the remaining trees of heavily thinned stands (Brèda et al. 1995). Whereas foliar δ18O in the 2004 leaf cohort (produced before thinning) was indistinguishable between plots assigned to different thinning treatments, significant differences were apparent in the 2005 leaf cohort (produced after thinning, Fig. 2a), thus indicating a short response time (~12 months) of bulk leaf δ18O to differences in competition intensity in *P. halepensis*. Higher g, in the remaining trees of heavily thinned stands apparently overwhelmed any potential effects of drier and warmer microclimatic conditions on foliar δ18O.

Differences in bulk leaf δ18O between treatments were largest in a severe drought year (2005, with annual rainfall of only 159 mm; Fig. 2a). Intense drought likely aggravates the negative effects of inter-tree competition on tree water status in semiarid forests, so heavy thinning may have been particularly beneficial to the water relations of remaining trees during a very dry year. The difference in foliar δ18O between heavily and moderately thinned stands was less pronounced (but still significant) in a relatively wet year (2007, 364 mm annual rainfall), when inter-tree competition for soil water may have been less intense.

Stem water δ18O was unaffected by forest stand density, thus indicating that the source water used by the remaining *P. halepensis* trees was indistinguishable between thinning treatments. Stem water δ18O reflects the isotopic signature of the soil water sources used by the plant, as no fractionation occurs during water uptake by plant roots (Dawson & Ehleringer 1991; Barbour 2007; Ellsworth & Williams 2007). Since pines used soil water of nearly identical δ18O in heavily and moderately thinned stands, the possibility that differences in the isotopic signature of source water might have been responsible for differences in bulk leaf δ18O between thinning treatments (Barbour 2007) can probably be ruled out.

Foliar δ13C did not differ between thinning treatments, thus suggesting that time-integrated WUE, in the remaining trees was unaffected by forest stand density. This interpretation is well supported by leaf gas exchange data showing no difference in WUE (WUE = A/gs) between heavily and moderately thinned stands despite significantly higher A and gs in the former (Fig. 4). Lack of response of foliar δ13C to forest thinning in spite of enhanced A and gs in remaining trees was reported by Sala et al. (2005) and McDowell et al. (2006), who attributed this result to parallel increases of similar magnitude in both parameters. According to the semi-quantitative dual isotope models developed by Scheidegger et al. (2000) and Grams et al. (2007), similar foliar δ13C combined with lower foliar δ18O in heavily than in moderately thinned stands must be interpreted as indicating higher g, and A in the former. Once again, this interpretation of isotopic data is in good agreement with leaf gas exchange data (Fig. 4). Furthermore, higher carbon assimilation rate translated into greater radial growth of the remaining trees in heavily thinned stands than in moderately thinned stands (Fig. 6). Interestingly, bulk leaf δ18O responded to heavy thinning much earlier (Fig. 2a) than tree radial growth.

Foliar δ13C was positively correlated with both leaf δ18O and Δ18O in the 2007 leaf cohort, thus suggesting that δ13C is (at least partly) under stomatal control in *P. halepensis* (Scheidegger et al. 2000; Barbour, Walcroft & Farquhar 2002; Keitel et al. 2003; Voltas et al. 2008). The strong positive correlation between photosynthetic rate and g, (Fig. 5) and the negative relationships of TRW index with Δ18O and δ18O in 2007 (Fig. 7) across individuals from both thinning treatments further indicate that carbon assimilation rate in *P. halepensis* is largely regulated by changes in stomatal aperture in response to soil water availability. Thus, our data suggest that higher photosynthetic rates in the remaining pines of heavily thinned stands resulted primarily from higher g, (and therefore lower stomatal limitation to carbon fixation), as compared to moderately thinned stands. However, possible differences in light availability and/or in the biochemical photosynthetic capacity of leaves between thinning treatments may have also played a role (Martín-Benito et al. 2010). The photosynthetic capacity of leaves is positively related to plant nutrient status and foliar N concentration because it depends on the concentration of N-containing enzymes, pigments and electron transporters in leaf tissue (Reich et al. 1995; Dawson et al. 2002). In 2005, the foliar N concentration reached the highest values in both treatments (Fig. 2c), probably as a result of impaired needle growth during this drought year. However, foliar N was transiently higher in heavily than in moderately thinned stands in 2005 (Fig. 2c); since there was no significant difference in mean needle length between treatments, this may have been the result of a larger ‘pulse’ of nitrogen availability in soil shortly after heavy thinning. Higher carbon assimilation rate in heavily thinned stands may thus have been partly attributable to enhanced biochemical photosynthetic capacity of pine needles due to improved plant N status.

In conclusion, heavy thinning reduced inter-tree competition for water more effectively than moderate thinning, thus resulting in higher gs, photosynthetic activity and radial growth in the remaining trees. Simultaneous measurement of δ18O and δ13C in bulk leaf material helped characterize the leaf-level physiological response of the remaining trees to different thinning intensities at an early stage (~12 months after stand density manipulation). To the best of our knowledge, this is the first study reporting a significant response of foliar δ18O to silvicultural thinning intensity. Our results show that the dual isotope approach is
particularly useful in situations where foliar $\delta^{13}C$ is unaffected by thinning treatments due to parallel increases of similar magnitude in both photosynthetic activity and $g_s$. Bulk leaf $\delta^{18}O$ has a short response time to thinning, which may be an important consideration for managers interested in assessing the effectiveness of silvicultural practices in low productivity forests where tree radial growth responses may take longer to become detectable.

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