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## Research paper

# Impact of root growth and hydraulic conductance on canopy carbon-water relations of young walnut trees (*Juglans regia* L.) under drought



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## ABSTRACT

In addition to soil characteristics, two plant traits control the supply of water from the soil to the canopy: root growth and plant hydraulic conductance. Here we examine the impact of root growth and hydraulic conductance on water uptake and transpiration of walnut under deficit irrigation. A greenhouse experiment was conducted using nine young walnut trees (*Juglans regia* L.) grown for three months in transparent pots, equipped with: (i) rhizotron tubes, which allowed for non-invasive monitoring of root growth; (ii) pressure transducer tensiometers, recording soil water potential at soil-root interfaces; (iii) psychrometers attached to mature leaves, measuring stem water potential; and (iv) weighing scales used to determine total plant transpiration. Irrigation treatments consisted of different replenishment levels (100%, 75%, and 50%) of potential transpiration replicated over time. Walnut trees showed rapid physiological acclimation characterized by a fast decline and subsequent stabilization of transpiration rates soon after the beginning of drought stress treatments. We also observed a significant decrease in plant hydraulic conductance with decreasing soil and stem water potential under drought stress. At the end of the experiment, isotopic measurements revealed the integrated effect of physiological acclimation on canopy carbon-water relations. Leaf carbon isotope ratios showed significant increases in water-use efficiency with deficit irrigation levels. Leaf water hydrogen and oxygen isotope ratios confirmed that changes in water use-efficiency were caused by decreases in transpiration. Conversely, root growth was highest under low stress (T100) and lowest under high stress (T50). These results indicate the existence of a fundamental tradeoff between water-use efficiency and root growth, which will be useful to optimize the application of water and improve the design of irrigation systems in walnut orchards.

## 1. Introduction

Walnut (*Juglans regia* L.) is a tree species of great economic importance, particularly in the Central Valley of California (DANR/UC, 2014), which provides 99% of the US commercial supply and 66% of the worldwide production of walnut kernels (California Walnut Board, 2015). In California, the majority of walnut orchards are located in areas that are periodically affected by drought. In recent years, drought stress has led to increased tree mortality and a decline in walnut productivity across the state (USDA, 2014). Identifying how plant traits control the supply of water from the soil to the canopy is of high

relevance in order to optimize water application while maintaining orchard productivity under increasing climatic variability.

Walnut trees have high water requirements. Their growth is strongly affected by water deficit, which results in decreased yield, deep bark canker, and low kernel size and quality, among other issues (Lampinen et al., 2003; Buchner et al., 2008). In contrast, early seasonal over-irrigation can cause *Phytophthora* root rot and dieback (Lampinen et al., 2003). In addition, both nitrate deficit (e.g. after its leaching due to excessive irrigation) and climate seasonality can alter root-to-shoot growth allocation (Silva et al., 2015a; Sperling et al., 2017) jeopardizing the sustainability of tree growing operations (Prichard, 1998;

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Di and Cameron, 2002). As in other parts of the world currently experiencing changes in climate, the increasingly frequent drought events in California call for adjusted water management, which requires understanding of the relationship between water application and tree transpiration to avoid the undesirable effects of limited and excessive irrigation.

The soil water that is available for plants is held by soil matric forces between field capacity (i.e.  $-0.01$  MPa for coarse textured soils and  $-0.03$  MPa for fine textured soils) and the permanent plant wilting point (i.e. from  $-1.5$  MPa up to  $-6$  MPa, depending on plant species and its capacity to take up water from soil) (Salisbury and Ross, 1978; Veihmeyer and Hendrickson, 1927). This notion has been revised due to the fact that only a fraction of the total available water in the root zone is “readily” available (Allen et al., 1998), while another fraction of soil water is available at longer-term. In other words, from a hydrological perspective, plant water availability is “rate limited” by hydraulic impedances on the pathways of water (Couvreur et al., 2014b). Three main properties are thought to control the flow rate-limitation. The first one is the soil hydraulic conductivity, which strongly depends on soil water content, texture and structure (Vereecken et al., 2015). The hydraulic conductivity of a drying soil decreases by orders of magnitude, relative to a saturated soil, limiting the water movement from the bulk soil to the soil-root interface (Gardner, 1960). The second property that affects the flow rate limitation is the distribution of roots. The number of roots in each soil layer defines the length of the pathway (Gardner, 1964), with shorter pathways resulting in higher plant water availability. The third property defining the readily available water is the plant hydraulic conductance (Couvreur et al., 2014a). The maximal water flow rate that can be sent to the shoot to supply transpiration is limited by plant hydraulic conductance, which is mainly controlled by root radial conductivity (Couvreur et al., 2012; Hachez et al., 2012) and total root length (Alsina et al., 2011), though cavitation may limit the axial transfer of water under drought (Sperry et al., 2008).

While root growth affects plant water availability as mentioned above, soil water content can, in turn, affect root growth in many ways. A first feedback is the closure of stomata in conditions of low soil water availability, which limits photosynthesis and thus decreases the amount of carbon available to be invested in root biomass (Jackson et al., 2000). In tress, the higher root-to-shoot ratios and rooting depth, and the decrease of the biomass of fine roots and root length under water deficit it's well documented in field and laboratory experiments (Hartmann, 2011; Kozłowski and Pallardy, 2002; Mainiero and Kazda, 2006; Mokany et al., 2006; Poorter et al., 2012). Accordingly, the growth response is strongly influenced by the severity of the stress (Zang et al., 2014). Even a considerable amount of the available energy is invested to the growth of new roots, these young roots take up water more efficiently representing a suitable plant strategy under water deficit (Arend et al., 2011). However, other root traits, such as root density, specific root length and root area are only slightly affected (Eissenstat et al., 2000). Also, both high and low soil water contents limit root growth; the former through hypoxia and the latter through soil mechanical impedance (Bengough et al., 2006). Finally, soil water potential and soil temperature appear to be major factors influencing root growth (Teskey and Hinckley, 2006). Otherwise, at canopy level, many plant physiological processes may be related to the control of water status, and the shifting in isotope composition of plant compounds have been related as an interesting plant signaling of water stress, and described as a different approach for measurement of drought impact on the terrestrial ecosystems (i.e., oxygen, hydrogen and carbon isotope composition of plant tissues) (Burgess and Huang, 2014; De Jong Van Lier et al., 2006; Maxwell et al., 2014; Sun et al., 2011).

The understanding of processes affecting plant water availability has fundamental and applied implications. Recent studies have recognized the key role of roots in promoting acclimation to different types of stress; mainly through preferential growth and control of

hydraulic properties that regulate transpiration (Alsina et al., 2011; Schoppach et al., 2014; Silva et al., 2015b). A better understanding of root response is, therefore, key for understanding water fluxes through the soil-plant-atmosphere continuum. Accordingly, here we examine the effect of root growth and plant hydraulic conductance on water availability for canopy transpiration of young walnut trees (*J. regia*) under different levels of water stress.

## 2. Materials and methods

### 2.1. Growth conditions and treatments in the greenhouse

The study was conducted from April 2015 to July 2015, using nine 8-month-old potted walnut trees (*J. regia*) cv. Chandler, grafted onto Paradox rootstock (*J. hindsii* x *J. regia*) in an experimental greenhouse at the University of California, Davis. Plants were grown in  $0.02$  m<sup>3</sup> pots filled with a 1:3 (v/v) mixture of a fine sand and organic compost. As the experiment was conducted over a short period and the plants were young, the size of the pots was considered suitable. Pots were kept covered with aluminum foil to avoid soil evaporation and their transparent walls were covered with plastic sheets that were black inside and white outside, to protect roots from light exposure. All plants received equal daily fertilizer application in water (6 ppm of  $\text{NH}_4^+$ , 96 ppm of  $\text{NO}_3^-$ , 26 ppm of P, 124 ppm of K, 90 ppm of Ca, 24 ppm of Mg, 16 ppm of S, 1.6 ppm of Fe; and < 1 ppm of Mn, B, Cu, Zn and Mo) (DANR/UC, 2014). Irrigation treatments were defined relative to each plant's “standard daily transpiration” observed under field capacity conditions one day before the experiment began (TD\*) normalized to the control for that date and on subsequent dates (see below): (i) 100% of TD\* (no water limitation) (T100); (ii) 75% of TD\* (moderate water limitation) (T75) and (iii) 50% of TD\* (strong water limitation) (T50) for a period of 10-days over three different time periods (April, April/May, and July). All pots were maintained at field capacity for at least a week (any water excess was drained from the bottom of the pot) before the beginning of each 10-days period experiment. Replicates were monitored over time due to the careful tracking of soil-plant properties and limited availability of leaf psychrometers and high precision weighing scales for all individuals. Hence, the experiment was replicated using three different plants per treatment monitored over 10-days in three different time periods (April, April/May, and July), for a total of nine receiving one of the irrigation treatments and three control plants. While temporal replications integrate the effect of different insolation and temperature conditions in the greenhouse at each 10-day sampling event, we expect to observe consistent shifts between T100, T75, T50 throughout the experiment.

### 2.2. Soil-plant water status monitoring

Stem water potential ( $\psi_{stem}$ ) was measured on expanded terminal leaflets located close to the trunk, every 15 min and averaged to hourly values, with a psychrometer/hygrometer (one per plant), model PSY-1 (ICT International Pty, Australia). The leaflet equipped with the psychrometer was fully covered with an insulation capsule limiting temperature fluctuations (see Fig. 1). As the monitored leaf did not transpire, the measurement was representative of stem rather than leaf water potential. An independent measurement of stem water potential was carried out weekly on fully expanded leaflets with a pressure chamber (PMS Instrument Company, Albany, OR). Prior to this destructive measurement, leaflets were enclosed in foil-laminate bags for at least 10 min (Fulton et al., 2001).

Plant transpiration rate (TR) was quantified by automatic weighing of pots on a high precision weighing scale (0.001 kg, Mettler Toledo PBA430) every ten minutes, averaged to hourly values. Draining water was collected daily in plastic reservoirs attached laterally to the bottom of the pots by flexible rubber tubing. Hence, the weight of leaching water did not affect the weighing scale reading until its collection. Both



Fig. 1. Pots equipped with minirhizotrons tubes, on the weighing scales with the tensiometer and plastic reservoirs to collect the leached water (A); and a leaflet equipped with the psychrometer and the insulation capsule (B) Photo: Daniela Jerszurki.

the added irrigation water and collected leachate were weighed and removed from the water balance in order to evaluate the weight loss due to  $TR$  (note that plant weight fluctuation was considered to be insignificant for the duration of each 10-day repetition).

Bulk soil water potential at soil-root interface ( $\psi_{soil}$ ) was monitored by one tensiometer per pot, placed at approximately the midpoint of the root system at 0.2 m depth, and recording data every ten minutes to generate average hourly values. Its porous ceramic cup was connected through a water-filled PVC (polyvinyl chloride) tube and a smaller acrylic glass tube equipped with a pressure transducer. A rubber cap on top of the tensiometer ensured its airtightness. All plant and soil measurements were continuously recorded with a data logger (Campbell Scientific, Logan, USA; Campbell CR1000) located inside the greenhouse. Hourly average air temperature ( $^{\circ}\text{C}$ ) and relative humidity (%) were obtained in an automatic micrometeorological station placed inside the greenhouse. The reference evapotranspiration was obtained by use of an atmometer Model E (ETgage Company, Loveland, USA), that gives one pulse at each 0.254 mm of evaporated water (ET<sub>0</sub>). Hourly vapor pressure deficit (MPa) was estimated by the difference between saturated and actual vapor pressure. Saturated vapor pressure was calculated using air temperature based on the Tetens formula (Murray, 1967). Actual vapor pressure was obtained by saturated vapor pressure multiplied by fractional humidity.

We used an empirical water stress indicator ( $\alpha$ , ranging from 1 to 0) based on plant relative transpiration (Gardner and Ehlig, 1963; Schoppach and Sadok, 2012; Tanner, 1967; Wesseling, 1991). For each plant, the potential daily transpiration was estimated as a product of the plant standard daily transpiration ( $TD^*$ ) by the ratio of the actual daily transpiration ( $TD$ ) to  $TD^*$  of the unstressed plant (T100). The water stress indicator was simply calculated as the ratio of  $TD$  to plant potential daily transpiration. For instance, the following equation applies to each  $i$ -treatment over time ( $t$ ) in days:

$$\alpha_{i,t} = \frac{TD_{i,t}}{TD_i^*} \times \frac{TD_{T100}^*}{TD_{T100,t}} \quad (1)$$

Note that by definition, trees in the T100 treatment have  $\alpha$  values equal

to 1 (no stress).

From the same principles, plant potential transpiration rate ( $TR_{pot}$ ) can be calculated for each plant and time, by assuming that the T100 treatment meets its  $TR_{pot}$ , and that  $TR_{pot}$  scales proportionally for the different plants:

$$TR_{pot\ i,t} = TR_{T100\ t} \times \frac{TD_i^*}{TD_{T100}^*} \quad (2)$$

### 2.3. Leaf isotopic signals

An undisturbed (not covered) leaf was harvested and water extracted using a custom-made cryogenic distillation system suitable for isotopic analysis, adapted from previous studies of this kind (e.g., Vendramini and Sternberg, 2007). Briefly, the leaves were transferred to individually cut 1.27 cm diameter pyrex tubes (Wale Apparatus) where the leaf material was held in place by stainless steel wool. After attachment to a vacuum manifold, leaves were frozen in liquid nitrogen and air evacuated to 100 mTorr. The tube was then flame sealed to preserve the vacuum, and subjected to gravity assisted cryogenic distillation, the top of the tube at  $110^{\circ}\text{C}$ , bottom at  $-20^{\circ}\text{C}$ . After distillation, the tube was removed and ice water isolated by flame sealing the tube again to separate water and leaf material. Leaf material was separated and ground to a powder using liquid nitrogen in a mortar and pestle. 3 mg samples were submitted for  $\delta^{13}\text{C}$  determination at the UC Davis Stable Isotope Facility by continuous flow GC-IRMS on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The water samples were transferred to 2 mL vials and was analyzed for  $\delta\text{D}$  by equilibration with water vapor and added hydrogen gas, assisted by a platinum black powder catalyst. Next,  $\text{CO}_2$  was added to the system and equilibrated with water vapor for  $\delta^{18}\text{O}$  analysis. Water analysis was performed at the University of Miami by using multiflow system connected to an Isoprime mass spectrometer (GV, Manchester, UK).

To standardize isotopic data, values are reported in del notation with reference standards as in the equation below. Our values are

calibrated to Vienna Belemnite of the Pee Dee (VPDB) formation for  $\delta^{13}\text{C}$ , and the Vienna Standard Mean Ocean Water (VSMOW) for  $\delta^{18}\text{O}$  and  $\delta\text{D}$  of water.

$$\delta^yX = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \quad (3)$$

Intrinsic water-use efficiency (iWUE) is an isotopically derived measure of physiologically mediated gas exchange of water and  $\text{CO}_2$  through stomata between leaf and air. This measure has been used to assess water stress, and productivity-efficiency tradeoffs in a variety of agronomic settings (Chaves et al., 2003; Farquhar, 1989; Farquhar et al., 1992). First isotopic discrimination ( $\Delta^{13}\text{C}$ ) between the source (atmosphere) and plant tissue must be derived as below.

$$\Delta^{13}\text{C} = (\delta^{13}\text{C}_a - \delta^{13}\text{C}_p) / \left( 1 + \frac{\delta^{13}\text{C}_p}{1000} \right) \quad (4)$$

Then given previously determined (Farquhar, 1989) diffusive and bio-synthetic fractionation factors,  $a$ , and  $b$  (4.4‰, and 27‰, respectively) are used with  $\Delta^{13}\text{C}$  to calculate  $c_i/c_a$ , or the ratio of internal ( $c_i$ ), to atmospheric ( $c_a$ ) partial pressure of  $\text{CO}_2$ .

$$\Delta^{13}\text{C} = a + (b - a) \frac{c_i}{c_a} \quad (5)$$

Finally, according to Fick's law and a scaling factor of 1.6 for relative diffusivity rates of  $\text{CO}_2$  and water through air, iWUE ( $\frac{\text{mmolC}}{\text{molH}_2\text{O}}$ ) can be calculated yielding a value for the relative amount of carbon assimilated by photosynthesis per water lost by transpiration.

$$iWUE = \frac{c_a \left( 1 - \frac{c_i}{c_a} \right)}{1.6} \quad (6)$$

#### 2.4. Plant hydraulic conductance ( $K_h$ )

Using the physical principles of water flow in plant hydraulic architectures (Couvreur et al., 2012), the stem xylem water potential ( $\psi_{\text{stem}}$ ) can be related to plant hydraulic conductance ( $K_h$ ) and  $TR$  as follows:

$$\psi_{\text{stem}} = \bar{\psi}_{\text{sri}} - \frac{TR}{K_h} \quad (7)$$

Where  $\bar{\psi}_{\text{sri}}$  is the average soil water potential at soil-root interfaces, which is a good approximation of the bulk soil water potential and relatively stable in time when  $TR$  is low, rooting density is high, or when the soil is wet (Silva et al., 2015a; Javaux et al., 2013).

As in this experiment VPD is lower than 1.5 kPa,  $K_h$  does not fluctuate significantly between morning ( $t_1$ ) and noon ( $t_2$ ) (Caldeira et al., 2014). We thus obtain the following expression of the plant hydraulic conductance from Eq. (7):

$$K_h = \frac{TR_{t_2} - TR_{t_1}}{\psi_{\text{stem } t_1} - \psi_{\text{stem } t_2}} \quad (8)$$

Note that this relation does not mean that  $K_h$  controls  $TR$  or  $\psi_{\text{stem}}$  but only that the latter two variables scale linearly with each other ( $K_h$  being the slope of the linear relation), for a given soil water potential.

Such a property can be used to approximate the maximal supply rate of water to the leaves ( $Q_{\text{avail}}$ ). When  $Q_{\text{avail}}$  is higher than the plant potential transpiration ( $TR_{\text{pot}}$ ), other factors such as the light environment limit transpiration. But when  $TR_{\text{pot}}$  surpasses  $Q_{\text{avail}}$ , transpiration is supply limited (or co-limited).  $Q_{\text{avail}}$  was calculated for each plant and time as follows:

$$Q_{\text{avail } i,t} = K_{h \text{ } i,t} (\bar{\psi}_{\text{sri } i,t} - \psi_{\text{stem } \text{min}}) \quad (9)$$

Where  $\psi_{\text{stem } \text{min}}$  is the minimum stem water potential (set at  $-1.8$  MPa) used to determine the maximum water supply (McDowell et al., 2008; McElrone et al., 2010). Accordingly, beyond  $\psi_{\text{stem } \text{min}}$ , a dramatic

lowering of  $K_h$  due to embolism is expected to provoke hydraulic failure.

#### 2.5. Visible root length ( $R_l$ )

The visible root length ( $R_l$ ) was monitored weekly over five weeks from the beginning of each 10-days period experiment by combining root mapping on the transparent walls of the pots (external  $R_l$ ) and observation of inner root length with minirhizotrons (internal  $R_l$ ), which provide a nondestructive method for repeated root observations (Rewald and Ephrath, 2013). In addition, weekly root length observations started five weeks before each 10-days period experiment in order to follow the  $R_l$  pattern through time. Minirhizotrons consisted of transparent acrylic tubes with an inner diameter of 50 mm, and wall thickness of 3 mm. We used one tube per pot, installed at an angle of  $45^\circ$ , and sealed with silicon. Analyses of  $R_l$  were performed weekly with a BTC minirhizotron digital image capture system (Bartz Technology Co., Carpinteria, CA, USA), located inside the minirhizotron tube. Each observation consisted of systematically taking pictures at one-centimeter intervals from the top to the bottom of the pot in three dimensions, totaling approximately 90 pictures per tube. The Rootfly software (ROOTFLY, 2014) was used to analyze root length semi-automatically.

#### 2.6. Statistical analysis

Statistical analysis of  $\psi_{\text{stem}}$ ,  $\psi_{\text{soil}}$ ,  $TR$ ,  $R_l$  and  $K_h$  obtained at each sampling event were performed using a restricted maximum likelihood (REML) estimator of the mixed linear model procedure using SAS 9.1 (SAS Institute Inc., Cary, NC) considering the interaction effect between treatments and sampling events. Least square (LS) means were used to distinguish between individual treatments. When appropriate, analysis of covariance (ANCOVA) was used to compare stable isotope regression lines with respect to their slope and intercept (Ellsworth and Sternberg, 2014). Significance levels were set at  $\alpha = 0.05$ . To investigate the timing of physiological acclimation, segmented regression analysis (Motulsky, 1999) was carried out using Prism 6.07 (GraphPad, 2007) in order to explain daily water stress indicator as a non-linear function of  $\psi_{\text{soil}}$  and  $\psi_{\text{stem}}$ .

### 3. Results

#### 3.1. Stem water potential ( $\psi_{\text{stem}}$ ), soil water potential ( $\psi_{\text{soil}}$ ) and transpiration rate ( $TR$ )

Stem water potential and transpiration rate differed significantly between treatments ( $P < 0.05$ ). The averaged midday transpiration rate for well-watered plants was 62% higher than in T50 and 33% higher than in T75 (Fig. 2c). Under strong water limitation, the average midday  $\psi_{\text{stem}}$  decreased by 55% relative to that observed under well-watered conditions (Fig. 2b). As expected, average  $\psi_{\text{soil}}$  differed significantly over treatments (Fig. 2a) ( $P < 0.05$ ), ranging between  $-0.025$  MPa and  $-0.07$  MPa under strong water limitation.

The ratio between actual daily transpiration (TD) and plant standard daily transpiration (TD\*) for T100 was higher than T75 and T50, which both decreased mainly in the first days of the experiment (Fig. 3e). In T100 the ratio between TD and TD\* was mostly stable over time with small fluctuations attributed to changes in the atmospheric demand as the pot was kept at pot capacity. The resulting  $TR$  pattern was dependent on daily variations of vapor pressure deficit (VPD) and air temperature ( $T_{\text{air}}$ ) for the well-watered plants (Fig. 3c and 3d).

Our empirical water stress index ( $\alpha$ ) decreased as water became limiting in the soil (Fig. 3a). Rapid decreases in  $TR$  were observed following a  $\psi_{\text{soil}}$  threshold that delineates a non-linear relationship between these state variables, indicating a transition from a large to a small decrease of  $TR$ . Accordingly, the segmented regression analysis

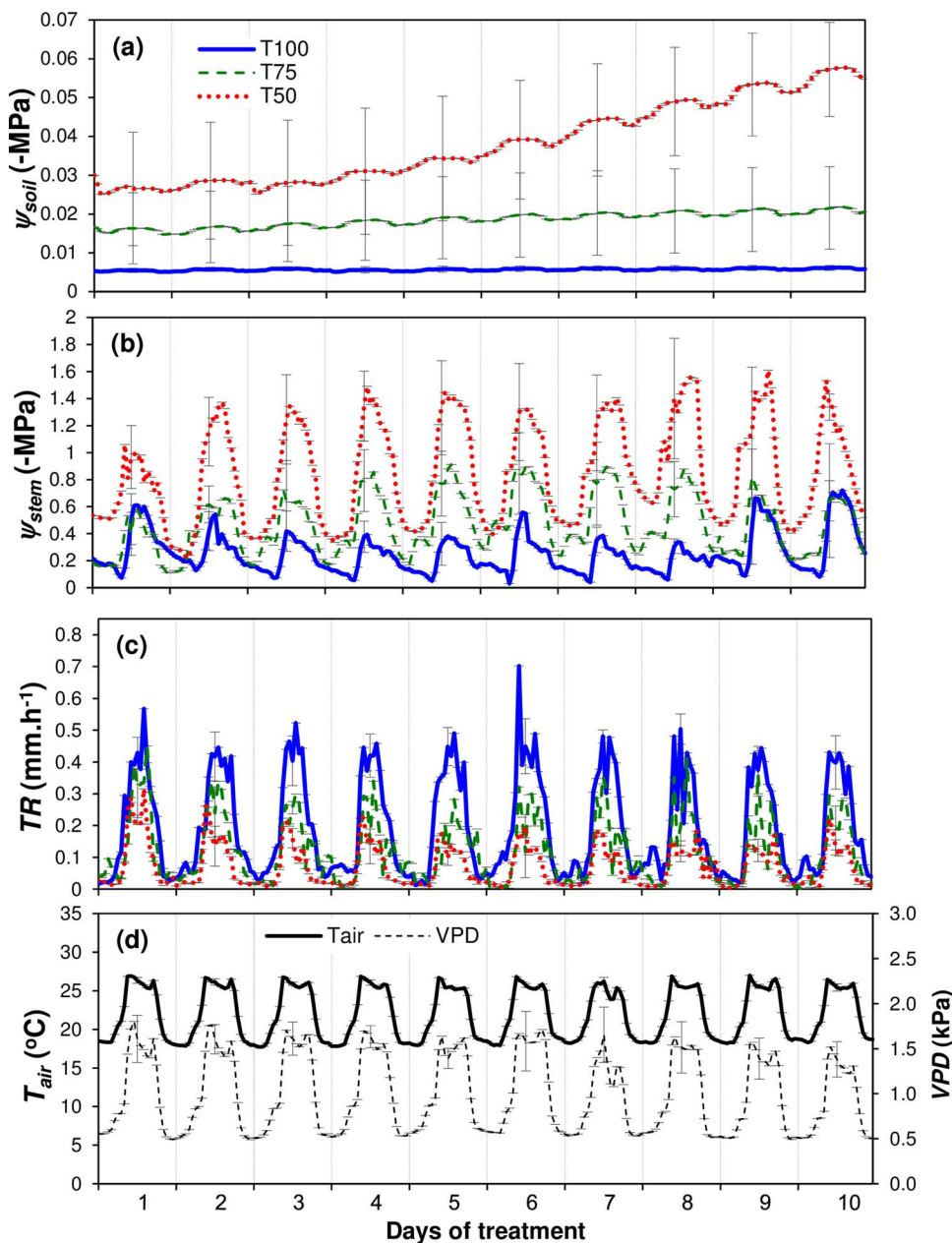


Fig. 2. Daily evolution of soil water potential (a), stem water potential (b), actual transpiration rate (c), vapor pressure deficit and air temperature (d) for T100, T75 and T50. Each point represents average values of the repetitions. Standard error bars are shown ( $n = 3$ ). For  $\psi_{soil}$ ,  $\psi_{stem}$  and  $TR$ , the differences between treatments are significant at the  $P = 0.05$  level.

shows a clear breakpoint ( $\psi_{soil\ BP}$ ) at around  $-0.02$  MPa ( $R^2 = 0.93$ ) (Fig. 3a) found in the  $-0.02 \pm 0.002$  MPa  $\psi_{soil}$  confidence interval ( $P < 0.05$ ). After this  $\psi_{soil\ BP}$  threshold,  $TR$  continued decreasing but at lower rates ( $0.4 < \alpha < 0.6$ ) relative to the initial stress response ( $0.6 < \alpha < 1$ ).

As for  $\psi_{soil}$ , we observed a nonlinear relationship between  $\alpha$  and  $\psi_{stem}$ , with a significant breakpoint ( $\psi_{stem\ BP}$ ) at around  $-0.46$  MPa ( $R^2 = 0.85$ ) (Fig. 3a) found in the  $-0.46 \pm 0.03$  MPa  $\psi_{stem}$  confidence interval ( $P < 0.05$ ). After the  $\psi_{stem\ BP}$  threshold, the  $TR$  continued to decrease but at lower rates than those observed initially.

### 3.2. Isotopic measurements

Analysis of covariance of linear regressions between hydrogen and oxygen isotope ratios of leaf water showed significant differences in intercept between treatments (i.e., d-excess), but no differences in slope (Fig. 4a and b). All experimental pots were covered to suppress soil evaporation, therefore, differences between treatment regression lines relative to the source water line are attributed to changes in leaf

transpiration. Differences in intercept tracked expected declines in transpiration rates under drought stress and are consistent with changes in  $iWUE$  inferred from carbon isotope ratios (Fig. 4c). There was no difference between T100 and T75 with respect to  $iWUE$  or d-excess, indicating physiological acclimation and maintenance of a steady balance between photosynthesis and transpiration. However,  $iWUE$  and d-excess of T50 trees was significantly different from the others, indicating low stomatal conductance ( $P < 0.05$ ).

### 3.3. Plant hydraulic conductance ( $K_h$ ) and maximum water supply ( $Q_{avail}$ )

In T75 and T50 treatments, plant hydraulic conductances ( $K_h$ ) significantly decreased ( $P < 0.05$ ) between the first and last halves of the 10-day observation period (Fig. 5a). Maximal supply rates of water to the leaves  $Q_{avail}$  were largely higher than plant water needs in the T100 and T75 treatments, while it decreased critically close to  $TR$  in the T50 treatment. In T75 and T50,  $Q_{avail}$  was respectively 45% and 60% lower than in T100, due to the combined effects of more negative soil water potential and lower  $K_h$  (Fig. 5b–d).

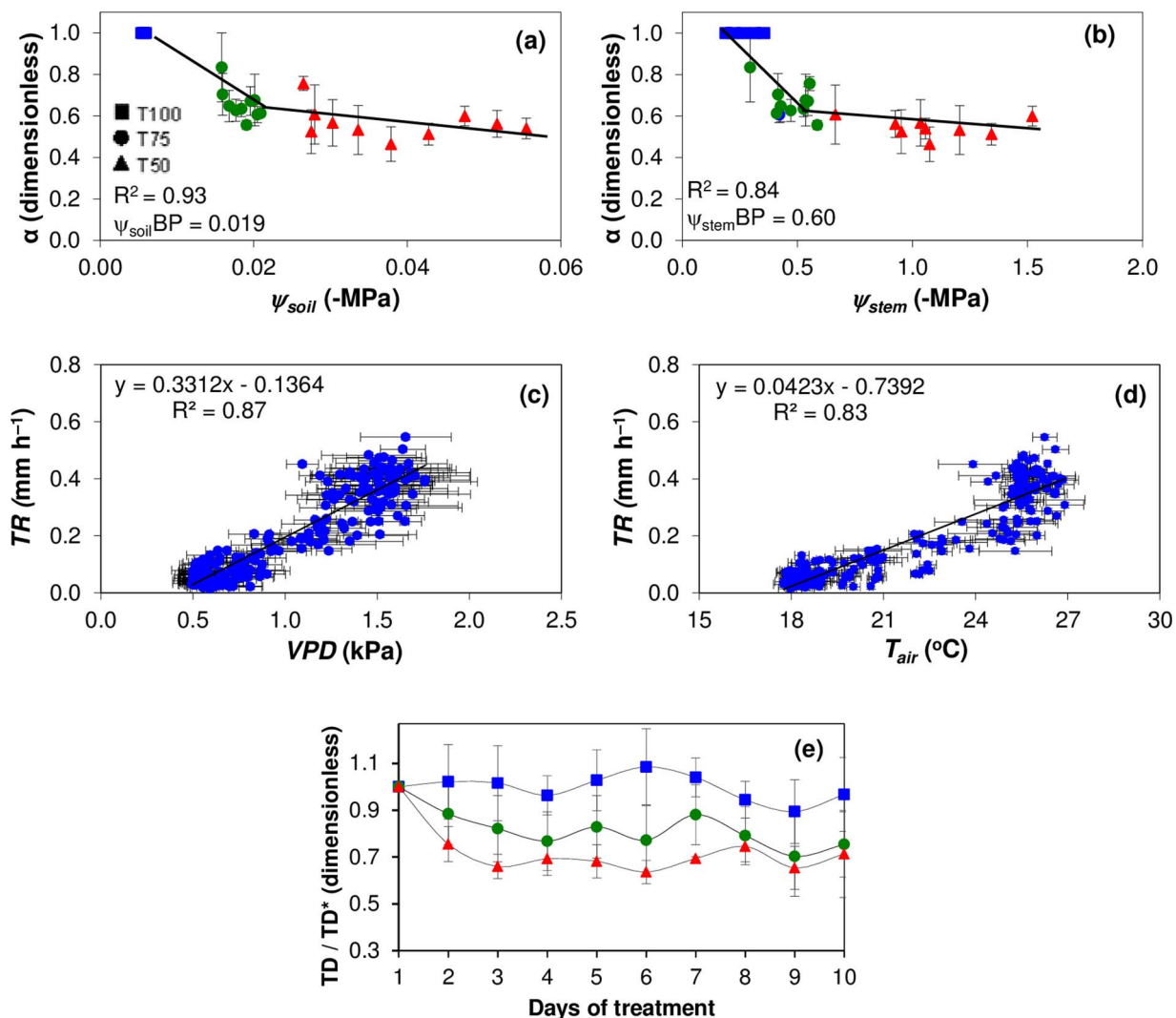


Fig. 3. Average daily water stress indicator represented as a function of average daily  $\psi_{soil}$  (a) and  $\psi_{stem}$  (b);  $TR$  under no water limitation (T100) represented as a function of  $VPD$  (c) and  $T_{air}$  (d); ratio between actual daily transpiration and plant standard daily transpiration (e). Each point represents the average values of the repetitions. Standard error bars are shown ( $n = 3$ ). The values for the  $\psi_{soil}$  and  $\psi_{stem}$  breakpoints ( $\psi_{soil}^{BP}$  and  $\psi_{stem}^{BP}$ ) and  $R^2$  are indicated.

### 3.4. Visible root length ( $R_v$ )

Snapshots of root growth over time are shown in Fig. 6. In general, under well-watered conditions, new roots (blue colors) started to grow before the old roots died (green, yellow and brown colors) and were more frequently observed (Fig. 6).

Large variability was recorded for relative external (Fig. 7a) and internal (Fig. 7b) patterns of root growth at each sampling event. However, the cumulative total and living root growth detected by the minirhizotron showed significant changes with greater growth observed in the well-watered treatment (Fig. 7c). Crucially, root growth patterns were proportionally and positively related with d-excess (Fig. 4a). This indicates the existence of a fundamental tradeoff between root growth and iWUE (Fig. 4c), by which canopy transpiration and root development can be estimated based on changes in leaf stable isotope ratios. It is important to note, however, that differences between T100 and T75 with respect to either root growth or iWUE were not statistically significant. Therefore, acclimation is possible at that level and high physiological stress seems to be required to study costs and benefits of such a tradeoff with respect to changes in water supply.

## 4. Discussion

Our observations (Fig. 2b) confirmed the decreasing  $TR$  as a response of midday depressions of leaf water potential (Simonin et al., 2015), showing the minimum  $\psi_{stem}$  in T50 (around midday) between  $-1.0$  MPa and  $-2.0$  MPa, which was strongly and positively correlated with  $\psi_{soil}$ , explaining low  $TR$  under deficit irrigation ( $P < 0.05$ ) (Fig. 2c). Indeed, stomata are expected to be completely closed in walnut trees when leaf water potential reaches  $-1.6$  MPa (Cochard et al., 2002) and similar  $\psi_{stem}$  values and associated stomatal closure have been previously reported in stressed walnut trees (McElrone et al., 2010), as transpiration rates decrease to prevent leaf dehydration under moderate to high  $T_{air}$  and  $VPD$  ( $25$ – $28$   $^{\circ}\text{C}$  and  $0.95$ – $2.4$  kPa, respectively, Fig. 2d). Otherwise, the strong and positive correlation between  $TR$  and evaporative demand was noticed for well-watered plants ( $R = 0.93$ ), as observed in previous studies (Duursma et al., 2014; Franks et al., 1997; Gholipour et al., 2010; Kholová et al., 2010; Monteith, 1995), followed by strong and moderate water limitation ( $R = 0.85$  and  $R = 0.70$ , respectively; data not shown).

Multiple lines of isotopic evidence integrate the effect of physiological responses to treatments during the entire experiment and corroborate a significant decline in  $TR$  under deficit irrigation. Leaf water regressions show significant deviation from source water with

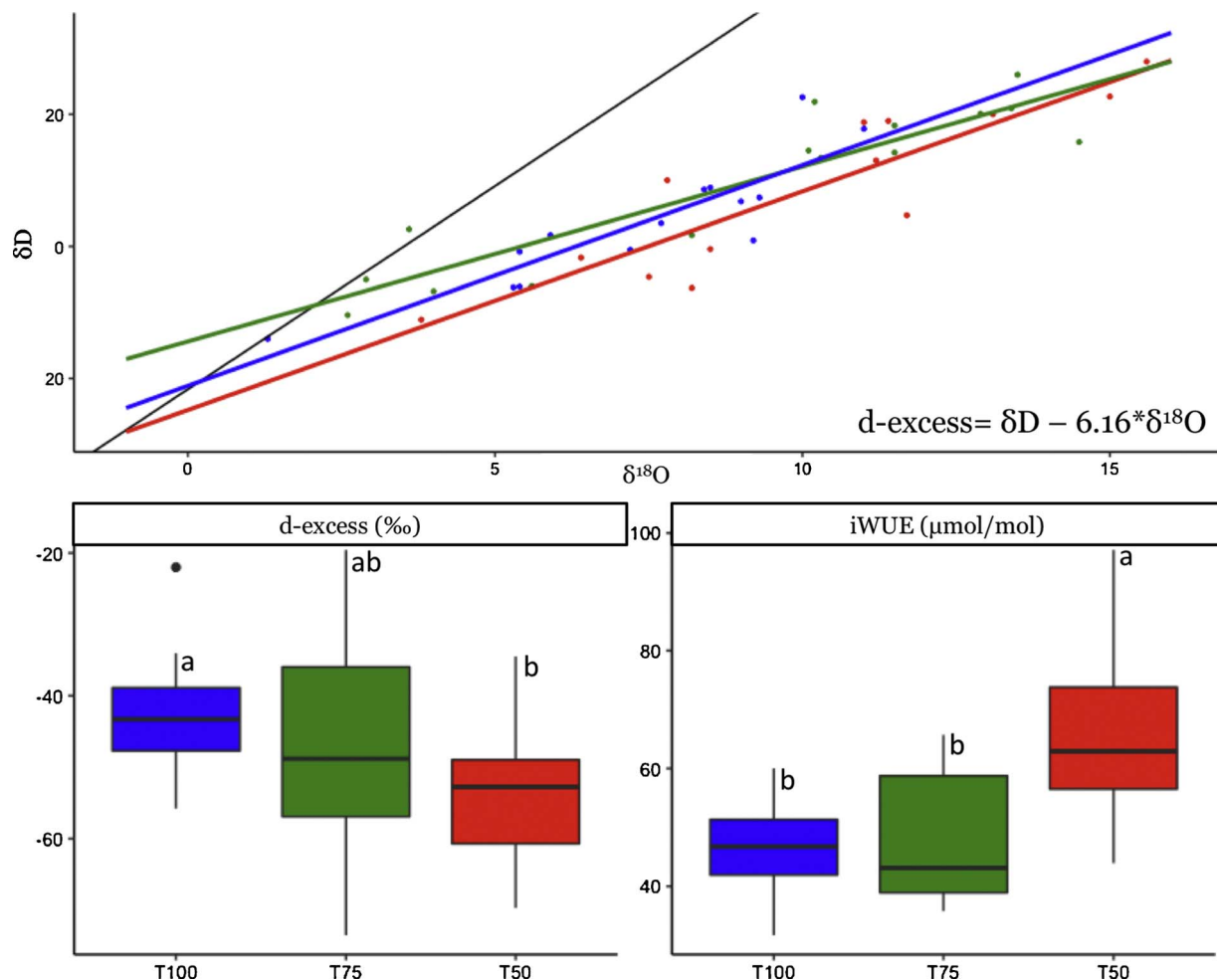


Fig. 4. Linear regressions of leaf water hydrogen and oxygen stable isotope ratios compared to source water (black line; slope 6.16) (a); d-excess, calculated as a function of the source-water line and slope:  $\delta D - 6.16 * \delta^{18}O$  (b); and, whole season iWUE calculated from leaf carbon isotope ratios (c). Different letters show significant difference ( $P < 0.05$ ).

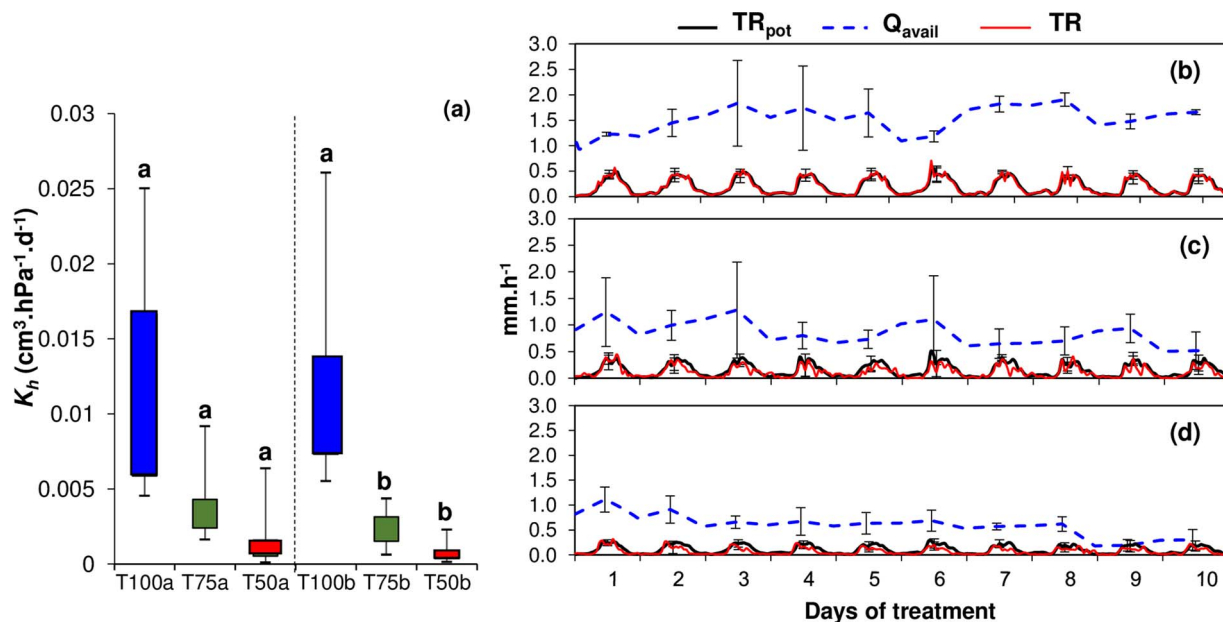


Fig. 5.  $K_h$  boxplot of early (first five days) (T100a, T75a and T50a) and late phases (last five days) of the experiment (T100b, T75b and T50b) (a) and hourly  $TR_{pot}$ ,  $TR$  and  $Q_{avail}$  for T100 (b), T75 (c) and T50 (d). Different letters above the bars refer to significant difference ( $P < 0.05$ ) between treatments (T100, T75 and T50) within each period of five days (early and late phases). For  $TR_{pot}$ ,  $TR$  and  $Q_{avail}$ , the differences between treatments are significant at the  $P = 0.05$  level. Each point represents average values of the repetitions. Standard error bars are shown ( $n = 3$ ).



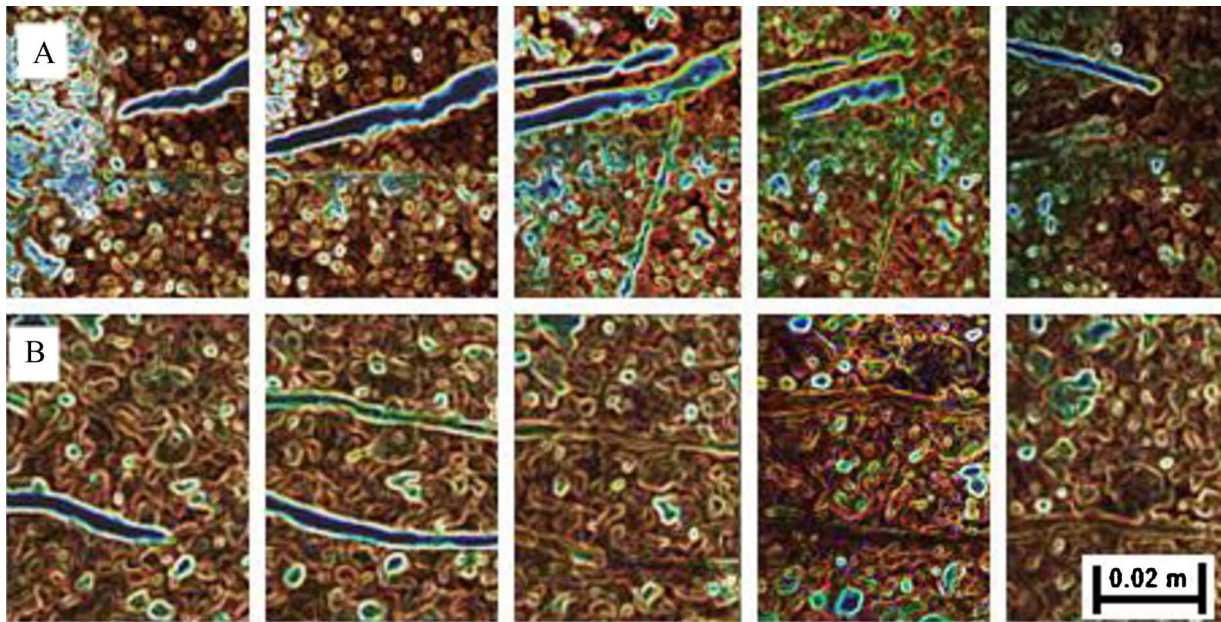


Fig. 6. Visible root length of walnut trees over time for T100 (a) and T50 (b), from minirhizotron technique. Each section represents a weekly measurement from the beginning of the first sampling period (March). Similar sectional images were used to quantify the root length of each plant at each treatment and sampling period.

regression intercepts varying between T100 and T50, but with undistinguished differences between T100 and T75. Deviations in slope relative to source water are governed by vapor pressure deficit and related to equilibrium fractionation both at the soil surface and leaf surface (Larcher et al., 2015). As the experimental pots were covered, all leaf water fractionations from source water are attributed solely to leaf transpiration. Additionally, there are significant changes in the hydrogen to oxygen isotope regression intercepts, which correspond to changes in deuterium excess (d-excess). Notably, decreasing d-excess pairs with increasing iWUE in response to deficit irrigation. This result demonstrates that a proportional increase in  $\delta D$  relative to  $\delta^{18}O$  ratios occurs in association with a drought-induced decline in stomatal conductance (Voelker et al., 2014). Although instantaneous measurements

of stem water potential often overlap across treatments, the isotopic data verify that integrated differences in water potential during the whole the experiment were significant between T100 and T50 treatments. Independently determined changes in iWUE and d-excess are consistent with previous observations of stomatal regulation of carbon-water relations (Werner et al., 2012), which in our experiment were also associated with changes in biomass allocation. Most notably, T50 plants operated at a significantly higher stress level than T75 and T100, showing the highest levels of iWUE as well as the most significant decline in root growth.

In a recent study, the declining TR for wheat under water limitation closely matched the fraction of “transpirable” (as opposed to total) soil water (Schoppach and Sadok, 2012). This general mechanism of

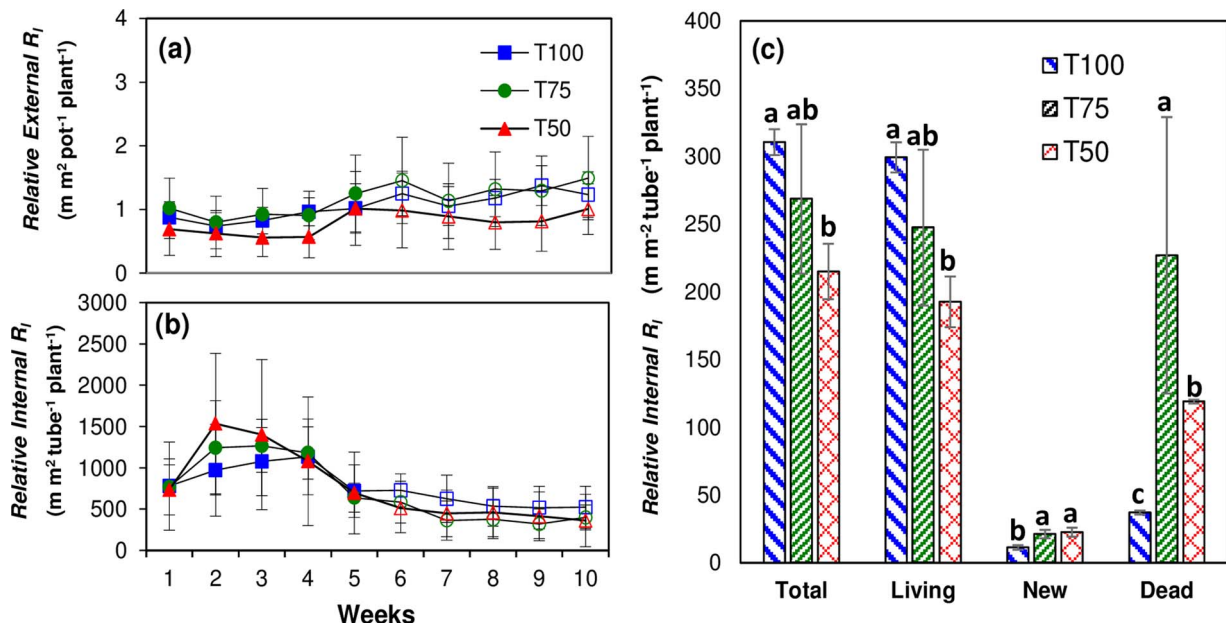


Fig. 7. Relative external (a) and internal (b) visible root length, and dynamics of internal visible root length during treatments (c) detected by the minirhizotron technique. Each point represents average values of the repetitions. Standard error bars are shown ( $n = 3$ ). Different letters above the bars refer to significant difference ( $P < 0.05$ ) between treatments (T100, T75 and T50). Closed and opened markers represent the five weeks before and five weeks after the beginning of treatments, respectively.

reducing water loss by transpiration earlier under water stress has also been recognized in peanut (Devi et al., 2009) and pearl millet (Kholová et al., 2010). Here, our results showed an early and rapid decline in transpiration followed by stabilization of water loss in stressed trees, which is consistent with the fraction of “transpirable” soil water general mechanism of declining  $TR$  (Schoppach and Sadok, 2012) and with the classic descriptions of the plant water stress function (Gardner and Ehlig, 1963; Tanner, 1967; Wesseling, 1991). The nonlinear decrease of  $TR$  as a function of  $\psi_{soil}$  and  $\psi_{stem}$  can be seen as a water conservative strategy to prevent water loss and leaf dehydration (McElrone et al., 2010) long before being limited by water supply from the soil-root system (see for instance  $Q_{avail}$  in Fig. 5c). Such a strategy lowers the risk of hydraulic failure and increases the  $iWUE$ . Considering that the major part of the walnut orchards are located in areas periodically affected by drought and due to its high water requirement over seasons, this observed trend and its further understanding has a key role in the identification and use of relevant physiological traits in plant breeding programs, allowing greater water-use efficiency under deficit conditions.

The observed values of  $K_h$  (Fig. 5a) fall in the typical range ( $10^{-4}$ – $10^{-2}$   $\text{cm}^3 \text{hPa}^{-1} \text{d}^{-1}$ ) reported for young tree species (Lo Gullo et al., 1998; Tyree et al., 1998) and annual crops (Jackson et al., 1996). Our results highlight the decrease of  $K_h$  under moderate and strong water limitation (Fig. 5b). Water deficit is one of the most important factors affecting  $K_h$  (Hernandez et al., 2009), and its decline in response to decreasing stem water potential under water deficit has been reported in walnut at  $\psi_{stem}$  approaching  $-1.8$  MPa due to cavitation (Burgess and Huang, 2014; McElrone et al., 2010). However, we observed reduced  $K_h$  long before reaching such negative stem water potentials (Fig. 3b). As our  $K_h$  only includes hydraulic resistances between the stem and the soil-root interface, its reduction might have been fostered by a combination of poor soil-root contact under lower soil water content (Carminati et al., 2013) and altered root permeability (Hachez et al., 2012) that were described in other species.

The  $Q_{avail}$  conceptualizes the maximum limit to water supply from the soil-root-stem system (flow rate threshold beyond which substantial cavitation would occur). It turns out that in the T75 treatment, a reduction of stomatal opening due to water limitation occurred long before transpiration was limited by  $Q_{avail}$ . Functionally, such stomatal regulation might play the role of extra security margin against hydraulic failure (Sperry et al., 2008) and translate into a so-called water saving behavior at longer term (Schoppach et al., 2014). The results also suggest that the supply-demand view in plant transpiration modeling is inappropriate for walnut, so that more complex models are needed (Tardieu et al., 2015; Huber et al., 2015).

Despite the significant effect of water deficit on various plant properties, root growth responses over time did not correlate with any other recorded variable, and could thus not explain changes of  $K_h$ . However, our observations suggest that healthy roots (blue color, Fig. 6b) rapidly shifted to decaying roots (green, yellow and brown colors, Fig. 6b) with the continuity of water stress, which means a reduction of root activity and less capacity to take up water (Hendrick and Pregitzer, 1992). Root growth is extremely variable, presenting great sensitivity to the growth environment (Qiao et al., 2010) which could explain the non-significant differences in root growth over treatments and the differences between field (Burgess and Huang, 2014) and pot observations. Several studies described the impact of water deficit on root growth (Klepper, 1987; Weir and Barraclough, 1986) and the variability of root length for annual crops, such as wheat (Asseng et al., 1998), cotton (Taylor and Klepper, 1975), sorghum (Robertson et al., 1993) and tree species (Burgess and Huang, 2014; Kuhns et al., 1985; Larson, 1974). These results encourage the application of long-term experiments in order to clarify the link between root growth and canopy transpiration under water stress.

## 5. Conclusions

The objective of this work was to clarify the effect of root traits on canopy transpiration of young walnut trees under water deficit. We demonstrate how plant hydraulic conductance can be determined by both soil and plant water potential under well-watered and water deficit conditions, and their relation with canopy transpiration. Our observations confirmed the significant influence of soil and stem water potential on water readily available for canopy transpiration. A nonlinear relationship between water stress indicators and stem and soil water potential showed a rapid acclimation characterized by a significant decline in both transpiration and root growth, followed by stabilization of water loss by stressed plants. Leaf carbon, hydrogen, and oxygen stable isotope ratios integrated the effect of increased  $iWUE$  due to declines in transpiration under imposed drought stress. A tradeoff was identified between root growth and  $iWUE$ , as both these variables changed proportionally and in opposite directions in response to deficit irrigation. This response was connected with soil and stem water potential and isotopic signatures of changes in carbon-water relations. These results can be leveraged to improve models that couple water availability and root growth, needed to understand soil-plant water balance under drought stress. Furthermore, these results will be useful to optimize the application of water and improve the design of irrigation systems in young walnut orchards, by achieving a balance between irrigation and plant water use that ensures the sustainable use of water resources.

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