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Full Length Research Paper

Growth, ectomycorrhization and biochemical parameters of *Quercus suber* L. seedlings under drought conditions

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Drought conditions are the major constraint to the early establishment of *Quercus suber* species. However, drought responses of this species depend on provenances. The objective of this study was to obtain more comprehensive knowledge on the influence of drought conditions on the response of *Q. suber* L. seedlings originating from Algeria. Soil water status in soil (SWC) and relative water content (RWC) in leaves, morphological parameters for growth evaluation and physiological parameters, and ectomycorrhization were evaluated in two plots of seedlings: watered (W) and none watered (NW). Ten weeks of water starvation induced a reduction in RWC, ectomycorrhization rate and height and diameter growth of none watered seedlings. Under drought conditions, both soluble sugars and proteins were enhanced while polyphenols and %N decreased. The reduction of soil water content was negatively correlated with carbon isotope discrimination. The survival of the seedlings under these drought conditions was due to two strategies: osmotic adjustment through soluble sugars and proteins accumulations in leaves and an increase in carbon discrimination which enhances the water use efficiency (WUE).

Key words: *Quercus suber*, growth, drought, ectomycorrhization, pigments, sugars, starch, plyphenols, δ13C.

INTRODUCTION

Summer drought in the Mediterranean region is characterized by 4 months dry period with little or no precipitations, high temperatures and high irradiance, considering the major constraints for vegetation (Aussenac, 2000; Faria et al., 1999). Mediterranean species are thus, often exposed to water stress during summer (Pardos et al., 2005). Mediterranean oak species have developed mechanisms to avoid excessive loss of

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cell water and to maintain growth (Caritat et al., 2006). Increased drought severity is expected in the Mediterranean basin over the twenty-first century (Aussenac, 2002; Ramirez-Valentine, 2011) and the increase in the length of the dry season may lead to severe water deficit and tree mortality (Kurze-Besson et al., 2006). Priority should be given to ecophysiological research for the study of the adaptative plants Mediterranean behavior under these predicted summer conditions.

The cork oak (*Quercus suber*) is a western Mediterranean species, widely distributed in Mediterranean forest, thus presenting significant ecological and economic interests. It is an evergreen and sclerophyllous species growing from the sea level up to 700 m in altitude (Quezel and Médail, 2003). This plant is well adapted to summer conditions because it maintains a favorable ratio between water loss and uptake during the dry period. Also, *Q. suber* is considered as a drought tolerant species (Nardini et al., 1999; Nardini and Tyree, 1999). Maintaining a favorable water status in tissues, with a high relative water content (RWC) during summer drought, is ensured by deep roots and/or osmotic adjustment through accumulation of molecules such as proteins, sugars and proline (Kwak et al., 2011; Otieno et al., 2006; Pardos et al., 2005). A decrease in shoots and leaves biomass by reducing growth and root drop is also noted (Kurze-Besson et al., 2006; Ksontini et al., 1998).

Cork oak is an ectomycorrhizal species. Molecular and morphological approaches analysis led to the identification of fifty-five taxa of fungi forming wide diversity of ectmycorrhizas (Azul et al., 2010). Under drought conditions, controlled ectomycorrhization increases the performance of *Q. suber* L. (cork oak) nursery and field seedlings (Sebastiana et al., 2013) but little is known about the effect of drought on ectomycorrhization (Richard, 2011; Shahin, 2012).

The carbon isotope discrimination (δ13C) of foliage integrates signals resulting from environmental constraints like water availability (Warren and Adams, 2000). Therefore, δ13C may be a useful indicator of drought stress in seasonally dry climates (Warren and McGrath, 2001) because water supply affects the stomatal conductance and photosynthesis of plants, which changes 13/12C ratios in the synthesized carbohydrates (Du et al., 2015).

Although, *Q. suber* is well adapted to dry conditions, it remains that its natural regeneration is low. This seedlings recruitment limitation probably will be amplified with the predicted global changes in Mediterranean region. Muhamed et al. (2013) showed that spatial patterns of association between understory shrubs and oak seedlings are very sensitive to increasing drought under climate change, while it is known that plants neighbors can promote oak regeneration.

Many studies on *Q. suber* trees and seedlings behavior to water stress on the northern Mediterranean shore exist (Nardini and Tyree, 1999; Nardini et al., 1999; Otieno et al., 2006); however, few studies on the responses of this oak species to water stress were performed in Algeria (Acherar et al., 1992).

Understanding the responses of cork oak to actual and predicted summer conditions is essential to determine the future sustainability of cork oak woodlands. In this context, the effects of water scarcity on *Q. suber* were studied through growth, water relations, ectomycorrhization, photosynthetic pigments, sugars, starch, protein, polyphenols, δ13C and % N contents.

MATERIALS AND METHODS

The experimental setting

Acorns of cork oak (*Quercus suber*) were collected, in November 2010, from Beni Ghobri forest located in Azazga (Tizi-Ouzou, Algeria) (36°42' to 36°47' N; 4°22' to 4°27' W longitude, 620 m altitude). After one month stratification at 4°C, seeds germinated at 20°C. To limit the effect of acorn variation in the seedlings, the seedlings with similar shoot height were transplanted to plastic bags (30 x15 cm) filled with 1.5 kg of soil substrate. Soil substrate consisted of a mixture of natural soil from Beni Ghobri forest (2/3) as source of ectomycorrhizal inoculums and washed sand (1/3). The plants were grown in a greenhouse, localized at Tizi-Ouzou (140 m altitude, 36° 42' 12886" N and 4° 2' 53 3339" E) characterized by a Mediterranean climate, and were watered regularly (three fold a week) until mid-July. Then, two plots (30 seedlings and 3 replicates/ plot) of seedlings were constituted: watered (W) and none watered (NW). Ten weeks after end of September, seedlings of the two treatments (W, NW) were harvested for determination of morphological and physiological parameters.

Measurement of substrate moisture contents

Measurements of substrate moisture volumetric content were made for the plots. Soil samples of 1 g were taken from the plastic bags at a depth of 10 cm and dried at 105°C for 72 h and then water content was calculated using the formula: (FW- DW)/FW (Mathieu and Pieltain, 2003) where FW and DW are the fresh and dry weight, respectively.

Measurement of relative water content (RWC)

The RWC was measured on fully expanded leaves as described by Nardini et al. (1999). Five plants and three leaves per plants were examined. Fresh weight (FW) of the leaves was determined immediately after harvesting and then allowed to float on distilled water during 24 h at 4°C, then turgid weight (TW) of the leaves was determined. The dry weight (DW) of the leaves was determined after drying at 75°C during 72 h. The RWC were calculated as:

$$RWC = (FW - DW) / (TW - DW) \times 100$$

Morphological parameters

Morphological parameters were determined on ten seedlings per treatment. Stem length and collar diameter were measured. Shoots, roots and leaves biomass were determined after drying at 75°C for 72 h. Then, the root to shoot ratio was estimated.

Physiological parameters

Chlorophylls, sugar, starch, proteins and total polyphenols contents were determined on five seedlings per treatment and 2 to 3 leaves/seedling.

Chlorophylls contents of fresh leaf discs (0.1 g) were determined spectrophotometrically after extraction in acetone 80% in the dark and the chlorophyll contents were calculated as proposed by Lichtenthaler and Buschmann (2001):

$$Cha+b= 7.15 \times A663 - 18.71 \times A647$$

Soluble sugars were extracted from fresh leaves in ethanol (70%), the residues was incubated in HCl (1. 1% v/v) for 30 min at 95°C for starch extraction. Then, soluble sugars and starch were quantified colorimetrically at 625 nm with anthrone reagent following Cerning-Berorard (1975) method.

Soluble proteins contents were determined following Bradford (1976) method. They were extracted from fresh leaves in distilled water and then quantified spectrophotmetrically at 595 nm after colorimetric reaction with Bioard reagent.

Total polyphenols were determined spectrophotometrically following the method described by Peñuelas et al. (1996). 2.5 g powder per sample of dry leaves were extracted with 20 ml of 70% aqueous methanol (v/v) acidified with some concentrated HCl drops. The samples were left at ambient temperature in the dark for an hour and a half. The extracts were filtered and the total polyphenols were quantified after colorimetric reaction using Folin-Ciocalteu reagent during one hour in dark at 765 nm. The total polyphenols content was calculated as gallic acid equivalent from the calibration curve of gallic standard solutions and expressed as mg gallic acid equivalent/g of dry weight.

Foliage samples for analysis of carbon isotopic discrimination δ13C, C% and N% were dried at 70°C for 72 h and ground to a fine powder. The abundance in combusted samples was performed using a mass spectrometer (Finnigan, Delta-S, Bremen, Germany) in CNRS UMR 7266 LIENSS with a precision of 0.1‰. δ13C (‰) was calculated with respect to the PDB Pee Dee Belemnite standard:

$$\delta^{13}C = (Rsample/Rstandard - 1)1000$$

where *Rsample* and *Rstandard* are the 13C/12C ratios in a sample and the standard (Pee Dee Belemnite), respectively (Warren and Adams, 2000).

Ectomycorrhizal colonization assessment

Percentage of ectomycorrhization was determined with a binocular and calculated as mycorrhizal root apex/total root apex (mycorrhizal and non mycorrhizal) of all the root systems (Parke et al., 1983).

Statistics analysis

Statistical analysis was performed using STATISTICA software (Version 7.1; StatSoft Inc.). The differences between the two lots (watered and none watered) for all recorded data were compared by the student test where the conditions of normality and equality of variances are checked. Otherwise, a Mann Whitney none parametric test was achieved. The significance level for all the tests was $P < 0.05$.

RESULTS

The seedlings of *Q. suber* were grown in irrigated and water starvation conditions for 10 weeks in greenhouse prior to morphological and physiological data measurements. At the end of the culture, significant changes were shown from the two lots in terms of substrate water contents (Figure 1a). The reduction of water availability was accompanied by a significant decrease in leaf relative water status (RWC), the RWC values were 85.43 and 70.78% in watered and none watered seedlings, respectively (Figure 1b).

The soil of Beni Ghobri forest was rich in ectomycorrhizal inoculum. Approximately eight month's cork oak seedlings were colonized by ectomycorrhizal fungi. The difference in ECM rate between the two treatments was significant. ECM decreased in drought conditions (<9%) while the well watered seedlings showed higher ECM (>39%) (Figure 1c). So, the ectomycorrhization was approximately 5-fold higher in well watered seedlings.

Length of the main stem was reduced in none irrigated lot (Figure 2a). The leaf biomass and root/shoot ratios did not differ significantly between the two batches studied (W and NW) (Figures 2b and c). The statistic analysis showed that the diameter of the stem at the base was significantly higher in the control watered plants as compared to the none watered ones (Figure 2d).

Majority of the physiological variables investigated were statistically different (Figures 3 and 4) between the two lots (W and NW). Total chlorophyll contents showed no significant difference between watered and non-watered seedlings (Figure 3a). Leaves protein content recorded in none watered samples were higher than in the well watered seedlings (Figure 3b). The levels of soluble sugars tend to increase with drought conditions. Especially, this drop was approximately 3-fold in none watered conditions as compared to the watered conditions (Figure 3c). The starch content showed a little increase in the none watered seedlings (1.026 fold higher) (Figure 3d).

On the contrary, total polyphenols synthesis and/or accumulation generally decrease. A significant decrease was observed (1.5-fold) in none watered seedlings as compared to the control individuals (Figure 3e). Non watering induced a significant increase of δ13 C contents in leaves; the δ13C were higher in none watered (-32.04) than in watered (-32.79) *Q. suber* seedlings (Figure 4a). The total nitrogen concentrations (N%) in leaves were different between the two treatments; N content was reduced in drought conditions (Figure 4b).

The C% in leaves were similar in watered conditions,

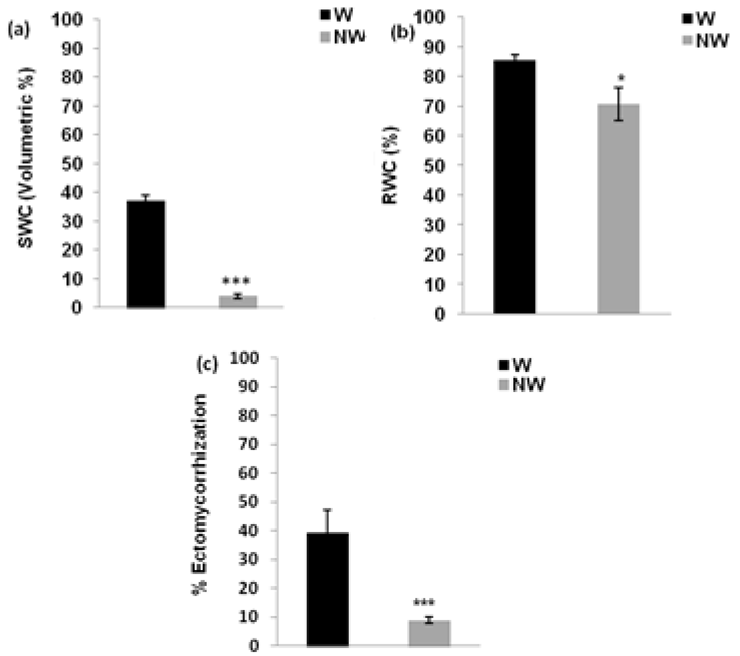

Figure 1. Effects of drought conditions on water status and ectomycorrhization of *Quercus suber* seedlings: (a) soil water content (SWC) and (b) leaf relative water content (RWC) (c) ectomycorrhization rate (ER). Means±SE. for RWC n=5, SWC n=10, and ER n=10. (*: p<0.05, ***: p<0.001).

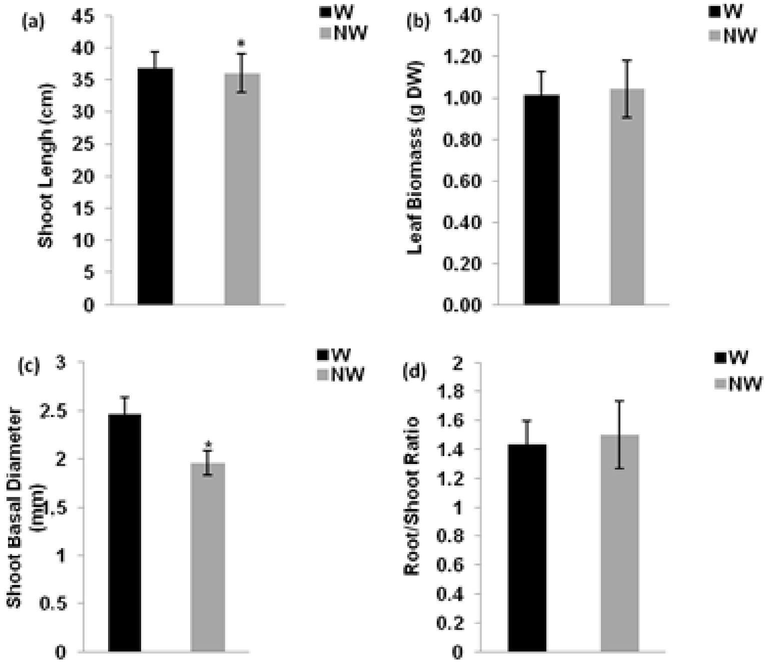

Figure 2. The effects of drought conditions on morphological traits of *Quercus suber* seedlings. (a) Shoot height, (b) Leaf biomass, (c) shoot basal diameter and (d) root/shoot ratio. Means±SE. for shoot height n=20; leaf biomass n=12; shoot basal diameter n=13 and shoot/root ratio n=10. (*: p<0.05).

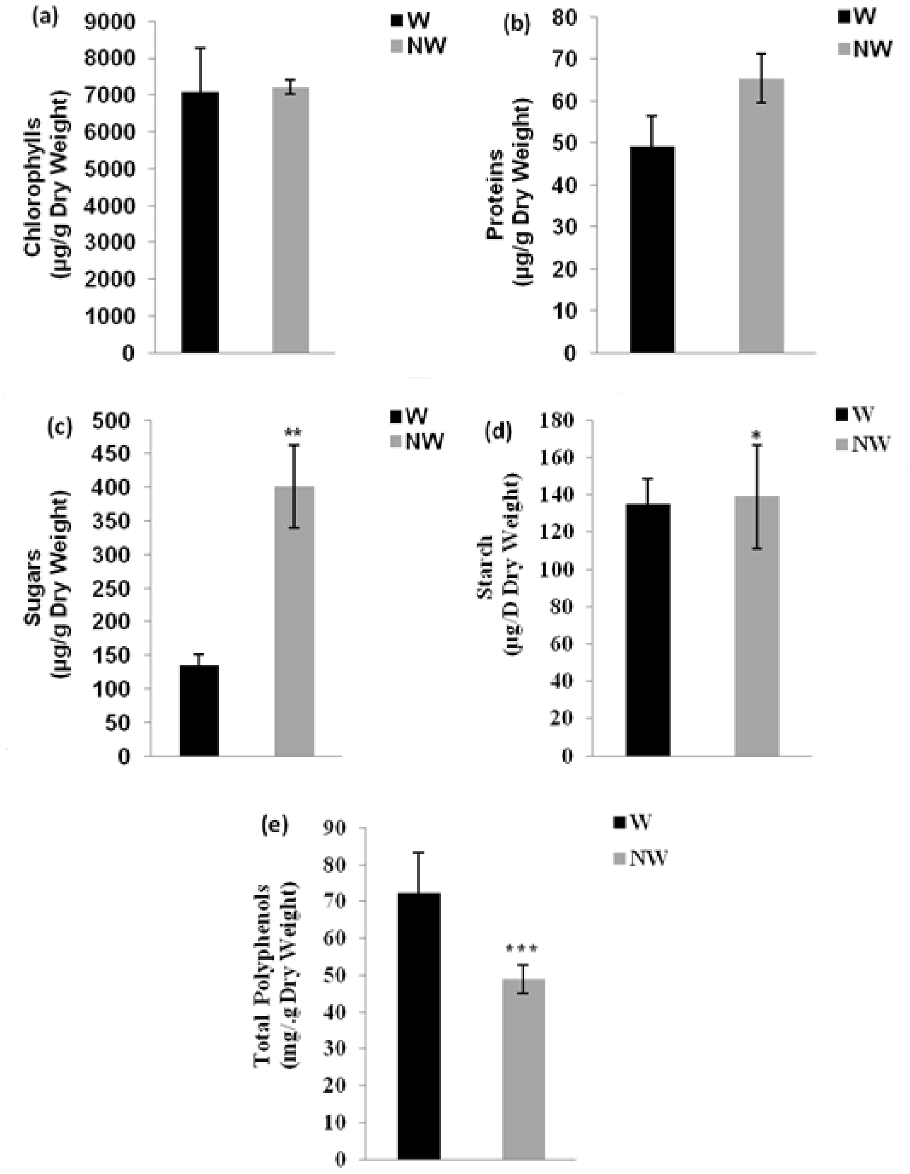

Figure 3. The effects of drought conditions on biochemical traits of *Quercus suber* seedlings. (a) Chlorophylls, (b) proteins, (c) sugars, (d) starch and (d) total polyphenols leaves contents. Means±SE. for all variables n=5. (*: $p<0.05$, ***: $p<0.001$).

thus, drought had no negative effect on this parameter (Figure 4c). The correlations between SWC and ectomycorhization, δ13C, N% and C% parameters were evaluated (Figure 5). The δ13C leaves contents showed a significant negative correlation with SWC; the reduction of SWC enhanced δ13C leaves contents ($r^2 = 0.5829$; $r = -0.7635$; $p = 0.0006$; $y = -31.918 - 0.0293*x$) (Figure 5a), while the total nitrogen (N%) and C% leaves concentration did not show correlation with the SWC ($p> 0.05$) (Figures 5b and c). A positive correlation exists between SWC and ECM rate ($r^2 = 0.3109$; $r = 0.5576$; $p = 0.0162$; $y = 8.245 + 0.7775*x$) and ECM decreases with SWC (Figure 5d).

DISCUSSION

When comparing the two treatments, soil water status in none watered pots was about 9.5-fold lower as compared to the watered substrates (Figure 1). This decrease was

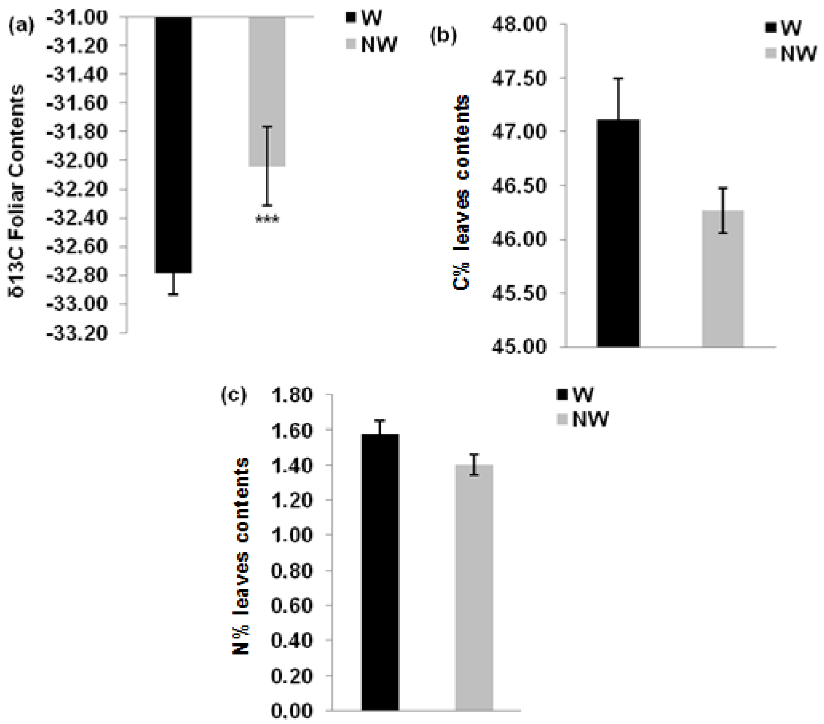

Figure 4. The effects of drought conditions on δ13C, C% and N% leaves contents of *Quercus suber* seedlings. (a) δ13C, (b) C% and N%. Means±SE. for all variables n=5 (***: $p<0.001$).

accompanied by a significant reduction in leaf relative water content (RWC) of the eight-month old seedlings (from 85.43 to 70.78%). In summer, the soil moisture volumetric content dropped to 5-7 % along the upper 0.6 m explored and leaf RWC decreased to nearly a constant level of about 82% that were only 8% less than those recorded in the spring (Nardini et al., 1999). The low values of RWC recorded in the current study may be due to more arid climatic conditions. Favorable tissue water status in *Q. suber* during summer drought was achieved through deep rooting which facilitate soil water uptake (Otieno et al., 2006).

The lower ECM colonization in *Q. suber* seedlings in drought conditions was previously obtained by Shahin (2012) in *Q. ilex* and *Q. pubescens* seedlings. The lower ECM colonization in dry conditions can be explained by the fact that photosynthates quantities are insufficient to both plant and fungal, thus they are preferentially allocated to plant growth or ectomycorrhizal inoculums was less abundant in none watered lot (Shahin et al., 2012). Some studies reported that seedlings inoculated with ectomycorrhizal fungi enhanced plant tolerance to water stress due to their less resistance to water flow from soil to roots, by increasing the absorbing surface and the ability of the fungus to penetrate finer pores as those operated by hairy roots (Parke et al., 1983).

Lengh and Diameter growth were reduced by drought conditions (Figure 2). While primary growth is sensitive to winter-spring warming, secondary growth is sensitive to summer-autumn warming (Camarero et al., 2015). Cambium activity is known to be negatively influenced by drought (Caritat et al., 2000). Interruption of shoot growth in the dry summer is a significant adaptation trait (Kurze-Besson et al., 2006) and thus, water stress tends to decrease the ratio of shoot biomass/root biomass (Ksontini et al., 1998).

Regarding the physiological parameters investigated, differences were shown (Figures 3 and 4). Drought generally causes decrease in photosynthetic pigments (Rajasekar and Manivannan, 2015). Decrease in chlorophyll contents was due to decrease in chlorophyll biosynthesis rather than its degradation (Jain et al., 2013). Vaz et al. (2010) showed no difference in *Q. suber* tree leaves from summer to autumn.

Osmotic adjustment is found to help cork oak seedlings to maintain turgor during moderate stress. To maintain water uptake, plants increased the water potential gradient between the plant cells and soil by increasing solute concentrations in the root cells. Like the present study results, in *Quercus* seedlings species, soluble sugars increased in drought-treated plants relative to control well watered plants and the opposite pattern was

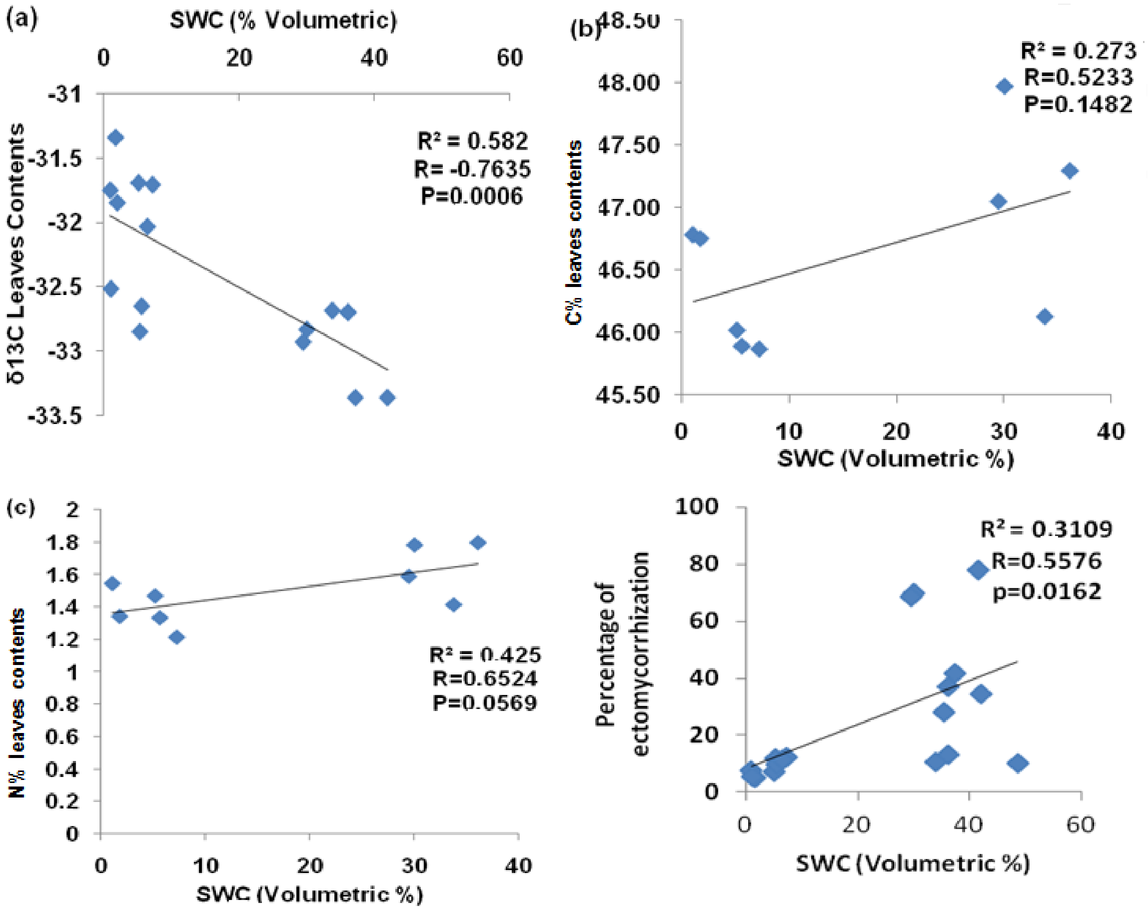

Figure 5. Correlations between SWC and δ13C, C%, N% and ectomycorrhization parameters of *Quercus suber* seedlings. (a) δ13C, (b) C%, (c) N% leaves contents and (d) percentage ectomycorrhization.

found in starch (Gonzalez-Rodríguez et al., 2011). Higher soluble sugars concentrations in cork oak seedlings grown under moderate water stress provoked a decrease of the osmotic potential and are at least partly responsible for osmotic adjustment which maintain RWC >80% (Pardos et al., 2005). Soluble proteins also contribute to osmotic adjustment (Kwak et al., 2011).

Polyphenols contents decreased in non-watered seedlings of *Q. suber*. Moderate experimental drought increased the concentrations of polyphenolic compounds with antioxidant function in the leaves of *Q. ilex* trees (Rivas-Ubach et al., 2014). Polyphenols are synthesized in large quantities during water stress, high temperatures or exposure to solar radiation, which are conditions that characterize the Mediterranean summer (Hernandez et al., 2009). Sometimes, the quantities were higher in watered plants like in loblolly pine needles (Booker and Maier, 2001).

Secondary metabolism in plants remains unclear; different regulation responses in the polyphenols pathway probably exist varying with species, development stage of the plant, intensity, duration and rate of progression of the stress (Liu et al., 2011). The increased carbon discrimination recorded in drought conditions was previously reported in *Q. suber* trees by Gouveia and Fortas (2009) which shows that trees subjected to greater water stress had δ13C enriched leaves, reflecting the trade-off between assimilation rate and water loss. The increase of carbon isotope discrimination with drought induces an increase in water-use efficiency (WUE) (Shahin et al., 2011).

These results showed that drought conditions reduced N% leaves contents while previous results have reported that leaf total nitrogen did not show a significant change in water stressed treatments (Kwak et al., 2011). In *Q. suber* trees, significant correlations were not found between total N and rainfall (Gouveia and Fortas, 2009).

Conclusion

This study carried out in semi-controlled conditions, shows that *Q. suber* seedlings are affected by 10 weeks of summer drought. The survival of the seedlings under these conditions is due to two strategies: osmotic adjustment through soluble sugars and proteins accumulations in leaves and an increase in carbon discrimination which enhances the water use efficiency (WUE). Some responses (stability of chlorophylls contents and R/S ratios and decrease in polyphenols contents) obtained in this study with Algerian *Q. suber*, are contrary to other *Q. suber* provenances responses (Europe and Tunisia).

Further studies could involve the responses of different provenances to actual and future water stress; this will permit understanding the mechanisms of *Q. suber* tolerance to drought conditions. The success of reforestation in the perspective of Mediterranean climate aridity which is scheduled for the next decades needs provenances well adapted to these future conditions.

Conflict of interests

The authors have not declared any conflict of interests.

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