Title

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Rapid recovery of photosynthesis and water relations following soil drying and re-watering is related to the adaptation of desert shrub *Ephedra alata* subsp. *alenda* (Ephedraceae) to arid environments

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ABSTRACT

Ephedra alata subsp. *alenda* is the most important pioneer plant of the moving and semi-stable sand dunes in the deserts and steppes of south Tunisia and occurs naturally in the Grand Erg Oriental, one of the most extreme habitats for plant growth on the planet. A new analysis of physiological performance of this medically important and internationally threatened xerophytic shrub was conducted to assess possible mechanisms of drought tolerance and how these relate to its ecological success. Five-month old plants, grown under controlled climatic conditions, were subjected to a well-watered control treatment or progressive drought by withholding water for 14 d with subsequent recovery for 7 d. Soil water depletion significantly reduced stem relative water content (RWC) water potential (Ψ_w) and osmotic potential (Ψ_π). *Ephedra* displayed more negative Ψ_w and Ψ_π values of ca. −3.5 and −4.1 MPa, respectively, at the end of the drought treatment, and were associated with turgor loss. Low stem Ψ_w reduced stomatal conductance (g_s), photosynthetic CO_2 assimilation rates (A_{CO_2}), transpiration (E) and internal CO_2 concentration (C_i). However, instantaneous (WUE; $A_{CO_2}\,E^{-1}$) and intrinsic (WUE$_i$; $A_{CO_2}\,g_s^{-1}$) water use efficiency (WUE) increased gradually as water deficit was intensified. Stomatal closure therefore only exerted limited control against dehydration and could not compensate for decreases in soil water status, typical of anisohydric behavior. Drought-stressed stems accumulated high levels of proline up to 480% of control values, highlighting a pivotal role in osmotic adjustment during intense water deficit. In contrast, the osmotic adaptation to soluble sugars was limited. Drought-stressed plants increased A_{CO_2}, E, g_s and C_i and decreased WUE and WUE$_i$ during the first 48 h after re-watering, such that they reached similar values to those of control plants by the end of the experiment. Stem proline levels of drought-stressed plants returned to near control values with re-watering. Overall, rapid recovery of photosynthesis following drought-breaking moisture appears to be a critical mechanism allowing *E. alata* to withstand and survive dry environments.

1. Introduction

Plants growing in Mediterranean-type ecosystems are subjected to prolonged periods of high temperatures and water deficit during annual summer drought, interspersed by short cool winters with variable precipitation (Cowling et al., 1996). Currently, the faster-than-predicted change in global climate (IPCC, 2007) and a variety of climate change scenarios suggest an increase in aridity for the semi-arid regions of the globe and the Mediterranean region in the near future. Under these conditions, the mechanisms by which different species respond to the summer drought largely determine their success and regeneration (Hernàndez et al., 2010).

Drought stress is known to shape many physiological and biochemical attributes in plants, especially in desert habitats. The most dominant growth forms in the Mediterranean ecosystem are evergreen and semi-deciduous plants. They adapt to their environment through physiological responses, in addition to ecological strategies, coping with water shortages by either (i) stress avoidance (so-called "drought avoiding species") or (ii) stress tolerance ("drought tolerant species") (Levitt, 1980). This classification roughly corresponds to the actually widespread categorization of plants across the continuum of stomatal regulation of water

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status in isohydric vs anisohydric species (Tardieu and Simonneau, 1998). Isohydric species maintain plant midday water potential at relatively constant values regardless of drought conditions via stomatal closure potentially resulting in mortality by carbon starvation during extreme drought, whereas anisohydric species display large midday water potential fluctuations by maintaining relatively high gas exchange rates even during arid season potentially leading to hydraulic failure during extreme drought (McDowell et al., 2008; Nardini et al., 2014). Recent progress in plant hydraulics has generated a coupled hypothesis of carbon starvation and hydraulic failure as mechanisms that explain how plants succumb to drought-induced mortality (McDowell, 2011). However, the outcome of such responses depends on several interacting variables, including the frequency and severity of the stress, plant genotype and plant developmental stage (Alpert, 2000; Otte, 2001; Chaves et al., 2003). Desert plants regulate their water status and adapt to their heterogeneously stressful environment through a multiplicity of ecophysiological features by following two major strategies in relation to drought, based upon variation in phenology (deciduousness) and ability to tolerate water deficit (persistence) (Ackerly, 2004). Soil depth and texture are considered to be the most important edaphic properties that influence plant moisture regimes in arid environments with episodic rainfall (Grigg et al., 2008; Dhief et al., 2009).

Ephedra alata Decne. subsp. *alenda* (Stapf) Trab., a dioecious species, is the most important pioneer plant of the moving and semi-stable sand dunes in the deserts and steppes of south Tunisia and occurs naturally in the Grand Erg Oriental (Nabli, 1989) and grows up to 5 m tall near Rjim Mâatoug, Tunisa (Chaieb and Boukhris, 1998). Other than its sand stabilization value, *E. alata* has gained popularity because of its secondary product chemistry—it is an important source of pharmaceutical compounds (Smith, 1977; Nawwar et al., 1984, 1985; Abourashed et al., 2003; Hayashi et al., 2010). Local nomadic populations also use it for pasture and for fuel, but overuse in this respect has contributed to its decrease (Nabli, 1989; Chaieb and Boukhris, 1998). Recently, Al-Qarawi et al. (2011) reported that seed borne fungi play an important role in deterioration of seed quality, and therefore declines in *E. alata* populations. In natural habitats, *E. alata* flowers during late winter and spring, and seeds start to mature from mid-May to early August (Derbel and Chaieb, 2013), after which they are dispersed by wind. In arid areas, the ability to overcome multiple and simultaneous stresses is of great importance for successful establishment of plants. Seeds of *E. alata* germinate best in spring season at a fluctuating regime of 28 °C day and 13 °C night temperatures, whereas seed germination decreases with increasing osmolality of growth media (Al-Taisan et al., 2010). Detailed knowledge of how *E. alata* responds to drought is vital for protection of its natural populations, and equally important for conserving its ecological restoration value as a stabilizer of shifting sand substrates and its cultural value as a medicinal remedy. In particular, we address the ecophysiological traits that determine the ability of a species to cope with drought and attempt to explain its distribution and ecological fitness in the Mediterranean region (Lambers et al., 1998).

Mediterranean shrubs represent excellent models for studying plant responses to drought because they are generally tolerant and well adapted to decreased soil water availability during the seasonal drought. Several native species are potentially interesting under aspects of dune stabilization and extension of plant cover, including *E. alata*. The coordinated changes in water (osmotic) relations and net CO_2 assimilation rate are key issues for understanding the tolerance of *E. alata* to drought stress. We present detailed physiological mechanisms for coping with extreme seasonal water deficit on this evergreen Mediterranean shrub species for the first time. In this context, we evaluated stem gas exchange properties, stem water relations and the role of organic solute accumulation in osmotic adjustment of *E. alata* seedlings exposed to low soil water availability, and during a period of subsequent recovery from drought stress. The relationship between stem water relations and stomatal behavior was also explored, to test the hypothesis that maintaining turgor potential may provide an indication of the capacity of a species to access groundwater during the summer drought, which characterizes the Mediterranean-type climate of southern Tunisia. Basic physiological responses of this important regional species are not well understood, and such information is indispensable to improve the basis for management of this plant species in the delicate shifting sands ecosystems of Northern Africa.

2. Materials and methods

2.1. Study species

Ephedra is the only genus in the family Ephedraceae within the Gnetales order, the closest living relatives of the Angiosperms (Friedman, 1996, 1998). It comprises ca. 50 species native to arid and semi-arid regions of North Africa, Asia, Europe and North- and Central America (Price, 1996; Caveney et al., 2001). Most of these species are perennial evergreen shrubs that can exceed 1 m in height, with slender and joined photosynthetic stems. Of these, only *E. alata* subsp. *alenda* (Stapf) Trab., *Ephedra altissima* Desf., *Ephedra fragilis* Desf. and *Ephedra nebrodensis* Guss. are found in Tunisia (Cuénod et al., 1954; Le Floc'h et al., 2010). The genus is characterized by scale-like or needle-like leaves, which are either decussate or in whorls of three and are fused at the base to form a nodal sheath (Price, 1996).

2.2. Seed collection and site description

Seeds of *E. alata* were collected from natural populations at Tiert, southern Tunisia in July 2008 (30°52′N, 10°09′E). They were dried in open air, cleaned and then stored in the seed bank at the Laboratoire d'Ecologie Pastorale (IRA-Médenine, Tunisia) until their use. The site is characterized by an arid-type climate with dry and hot summers and cold winters. The annual rainfall is low, rarely exceeding 100 mm. The rains are infrequent and irregular, sometimes with no rain during long periods of several years. Mean annual temperature is 20 °C. The soil texture is sandy with increasing particle diameters with depth. The dunes reach a height of approx. 8–12 m.

The site of Tiert is dominated by *E. alata*, *Calligonum azel*, *Calligonum comosum*, *Cornulaca monacantha*, *Genista saharae*, *Helianthemum confertum*, *Limoniastrum monopetalum*, *Retama raetam*, *Stipagrostis pungens*, *Traganum nidatum* and *Zygophyllum album*. These species play a significant role in the vegetation physiognomy in these sites where the vegetation cover is approximately 4–11%. The vegetation is composed of three strata: a phanerophyte stratum with a height exceeding two meters, a nanophanerophyte stratum of less than two meters to which *E. alata* belongs; and a chamaephyte stratum of less than 0.5 m height.

2.3. Treatments and experimental set-up

Seeds were surface sterilized in 5% sodium hypochlorite for 5 min, subsequently washed with deionized water and air-dried before being used in experiments to avoid fungal infection. Germination experiments were conducted in an incubator set at 15 °C. Three germinated seeds were transplanted into each pot (18 × 20.6 cm height), which contained a 1:2 mixture of peat and sand with a drainage layer at the bottom of the pot. Each pot was irrigated every two days with rain water to field capacity (FC). Thirty days after emergence, seedlings were thinned to one per pot and irrigated with a half-strength Hewitt's solution (Hewitt, 1966).

Plants were grown in a growth chamber with a constant 25 ± 1 °C temperature, a relative humidity of 50% during the day and 75% at night and a 16 h light/8 h dark regime with 250 μmol m^{-2} s^{-1} photosynthetic photon flux density (PFD) during the day period.

Five-month old plants (36.1 ± 1.3 cm) were divided into two groups. The first group was kept under well-watered conditions (as explained above, 100% FC) throughout the entire experimental period (control), while the second was subjected to water deficit stress by withholding water during 14-d, and after full recovery during the following 7-d (drought-recovery). Four harvests were made at the beginning of treatment, after 7 d, after 14 d, and after a 7-d recovery period (day 21). Overall, the experimental design included two levels of irrigation treatments (well irrigated or drought stressed) × two durations of treatment (7 and 14 days), and a recovery for 14 days of drought stress with 6 replicate plants in each treatment.

2.4. CO_2 and H_2O gas-exchange

Net assimilation of CO_2 (A_{CO_2}), stomatal conductance (g_s), transpiration rate (E), internal CO_2 concentration (C_i), water use efficiency ($WUE = A_{CO_2} E^{-1}$) and intrinsic WUE ($WUE_i = A_{CO_2} g_s^{-1}$) were measured periodically during the experiment using the longest stem in an LCpro+ portable photosynthesis system equipped with a conifer leaf chamber (ADC BioScientific Ltd, UK). All measurements were made between 10:00 and 12:00 h. Ten sampling dates were chosen during the period of drought stress (days 0, 2, 4, 6, 9, 11 and 14) and the recovery period (days 16, 18 and 21) and five plants per treatment were selected randomly on each date. We measured the width and length of stems using vernier calipers, and then calculated the net photosynthetic rate per unit area. The stems were irradiated with a PFD of 1500 μmol m^{-2} s^{-1} of internal light source and the CO_2 concentration in the chamber was set at 360 μmol mol^{-1}. Readings were logged every 30 s until we obtained stable values.

2.5. Plant water relations

After 7 and 14 days of drought stress and a recovery of previously stressed plants, water potential (Ψ_w) was determined using a Scholander pressure chamber (PMS Instruments, Corvallis, Oregon, USA) (Scholander et al., 1965) on the same stems after measurements of CO_2 and H_2O exchange. After Ψ_w measurement, stems were tightly enclosed in aluminium foil, frozen by immersing in liquid nitrogen and stored in a freezer at −20 °C. After thawing, a vapor pressure osmometer (Wescor 5520, Logan, UT, USA) was used to determine osmolality of the sap expressed from stems, which was converted to Ψ_π, by the van't Hoff equation: $\Psi_\pi = -nRT$, where n is the value reading from the instrument, R is the ideal gas constant and T is the absolute temperature (Nobel, 1991). Turgor potential (Ψ_p) was calculated as the difference between Ψ_w and Ψ_π values.

At the end of the water deficit period (days 7 and 14) and again after the recovery period (day 21), the relative water content (RWC) was calculated as RWC (%) = (FM − DM)/(TM − DM) × 100 according to Morgan (1984), where FM, DM, and TM are the fresh, dry and turgid masses, respectively. FM was determined immediately after sampling, while TM was determined after incubating the stem discs in distilled water overnight. DM was determined from oven-dried material after 48 h at 70 °C.

2.6. Biochemical analysis

Stems lyophilized with a LCD-1 lyophilizer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) were used to determine biochemical analysis.

Proline concentration was quantified spectrophotometrically by the ninhydrin method according to Bates et al. (1973). The plant material was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 24,000 × g. The supernatant was used for the estimation of the proline concentration. The reaction mixture consisted of 1 ml of acid ninhydrin and 1 ml of glacial acetic acid, which was boiled at 100 °C for 1 h. After termination of reaction in ice bath, the reaction mixture was extracted with 2 ml of toluene, and absorbance was read at 520 nm. Proline concentration was expressed as μmol proline g^{-1} DM.

Soluble sugars were quantified following the phenol–sulfuric acid method as described by Dubois et al. (1956). 100 mg of dry stem was extracted in 80% (v/v) methanol. The extract was then centrifuged at 5000 × g for 10 min. The supernatant was used for the estimation of soluble sugars concentrations. The reaction mixture consisted of 0.5 ml 5% phenol and 2.5 ml 98% sulphuric acid. Once the extract had cooled, its absorbance was determined at 590 nm. Glucose was used as reference standard. Soluble sugars concentration was expressed as mmol glucose g^{-1} DM.

2.7. Statistical analysis

Data were analyzed by repeated-measures ANOVA or *t*-Student tests and differences were considered as significant when $P < 0.05$. We used repeated-measures ANOVA analysis over time to evaluate physiological responses of *E. alata* seedlings to decreased water availability, where water treatment was the between subject effect and time was the within subject effect. Days since the onset of drought (0, 7, or 14 for water relations, proline, and soluble sugars, and 0, 2, 4, 7, 9, 11, or 14 for photosynthesis) were specified as the within-subject factor. Post hoc pairwise comparisons (LSD test) were performed to identify specific differences between treatment pairs and day of drought. A Student's *t*-test analysis on independent samples was performed for all physiological traits, testing for significant differences between recovered and control seedlings on each day measurements were taken. All statistical analyses were performed with SPSS v.20.0 statistical package (SPSS Inc., Chicago, USA).

3. Results

3.1. Plant water relations

During the drought-stress period (days 7–14), stem RWC was significantly lower in stressed plants compared to control plants as water deficit intensified (repeated-measures ANOVA, time × drought interaction, $F_{2,16} = 8.23$, $P < 0.01$; Fig. 1a). RWC decreased by 17% and 30% compared with control plants after 7-d and 14-d of the drought stress treatment, respectively. By the end of the drought-recovery period, RWC in previously stressed plants reached similar values to those of control plants.

Stem Ψ_w and Ψ_π decreased gradually with on-going drought stress (Fig. 1b and c), and repeated-measures ANOVA revealed a significant time × drought interaction on both variables ($F_{2,16} = 7.39$ and 9.89, respectively; $P < 0.01$). The largest reduction in Ψ_w occurred in drought-stressed stems on day 14, when it dropped to −3.5 MPa. A similar pattern was observed in Ψ_π but there was an additional decline to −4.1 MPa. After the 7-d drought-recovery period, Ψ_w and Ψ_π increased slightly compared with stressed plants at 14 d of withholding water, but they remained significantly lower than control plants (t test, both $P < 0.05$).

As shown in Fig. 2a, Ψ_w was linearly related to RWC, indicating that a decrease in Ψ_w during the drought stress period was associated with a decrease in RWC. Both parameters reached similar values to those of control plants at the end of the drought-recovery

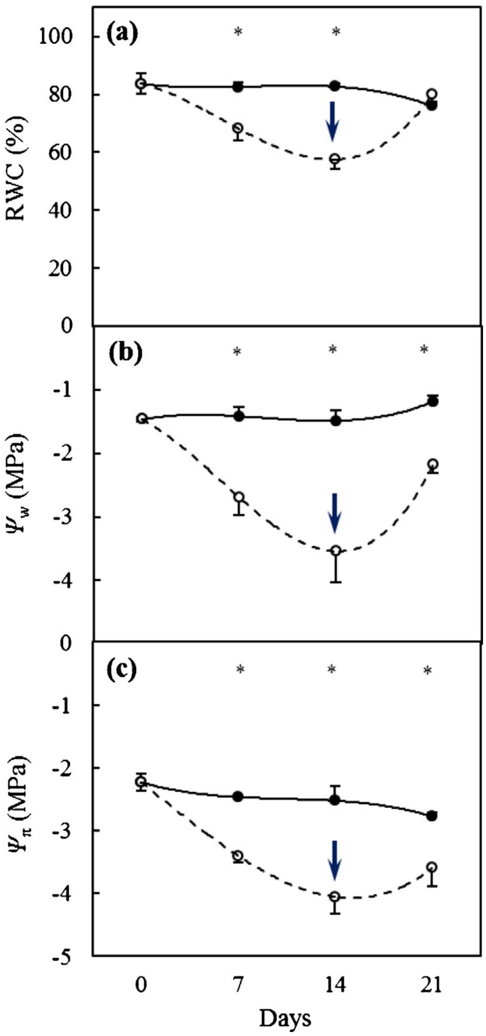

Fig. 1. Changes in (a) relative water content (RWC, %), (b) water potential (Ψ_w, MPa) and (c) osmotic potential (Ψ_π, MPa) of *Ephedra alata* stems in control (●) and drought (○) treatments throughout the experimental period. Values represent means ± SE, $n = 5$. Arrows indicate the beginning of the drought-recovery period. Lines describing the dependencies were obtained using a polynomial regression. Asterisks denote significant treatment differences from pairwise comparisons ($P < 0.05$).

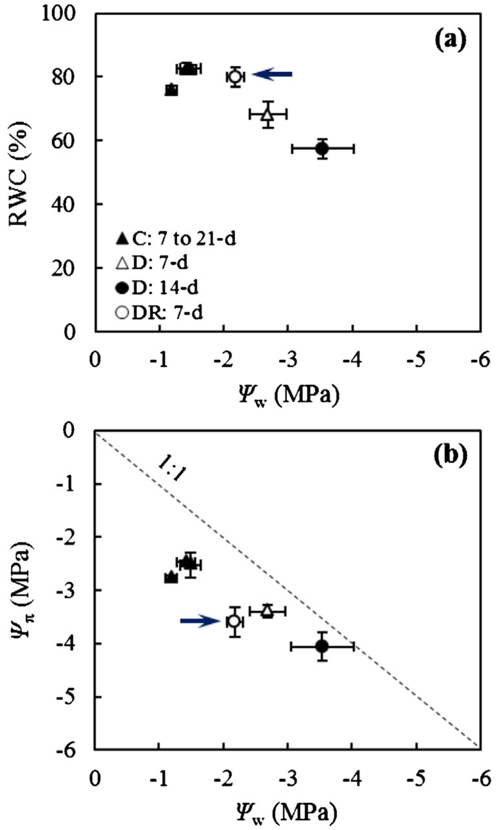

Fig. 2. Relationship between RWC and Ψ_w (a), and between Ψ_π and Ψ_w (b) for *Ephedra alata* plants in control (C) and drought (S) treatments throughout the experimental period. Values represent means ± SE, $n = 5$. The 1:1 line indicates $\Psi_\pi = \Psi_w$ and hence turgor potential = 0. Arrows indicate the beginning of the drought-recovery period. C: 7, 14 and 21-d control plants (▲); D: 7- and 14-d drought period (△ and ●, respectively); DR: 7-d drought-recovery period after 14-d water deficit period (○).

period. Stem turgor values for *E. alata* were derived from plots of Ψ_w and Ψ_π recorded during the experiment (Fig. 2b). According to the relationship $\Psi_w = \Psi_\pi + \Psi_p$, Ψ_p is taken to be the difference between each point response and the line of equality at any value of Ψ_w. Stem Ψ_π decreased with decreases in Ψ_w and the proximity of points to the zero line showed that variations in Ψ_p were largest for well-irrigated plants and smallest for drought stressed plants, but the difference among treatments was not statistically significant (repeated-measures ANOVA, time × drought interaction, $F_{2,16} = 0.81$, $P > 0.05$). Ψ_p of previously stressed plants recovered to values near control plants after their re-watering (Fig. 2b).

3.2. CO_2 and H_2O gas-exchange

Withholding water was followed by a gradual decline in net assimilation of CO_2 (A_{CO_2}), stomatal conductance (g_s) and transpiration rate (E), as shown in Fig. 3a–c (repeated-measures ANOVA, time × drought interaction, $F_{6,48} = 6.78$, 2.94 and 2.57, respectively; $P < 0.05$). The values of A_{CO_2} of well-watered plants ranged predominantly between 13.5 and 16.5 μmol CO_2 m^{-2} s^{-1}; however, there is a decrease in the A_{CO_2} of stressed plants which becomes significant after 2 d reaching a value of around 11 μmol CO_2 m^{-2} s^{-1}. Differences in A_{CO_2} increase as water deficit was intensified and the lowest value (6.2 μmol CO_2 m^{-2} s^{-1}) was reached after 2 weeks (Fig. 3a). Similarly, E reached the highest values in control plants (3.6 to 6.1 mmol H_2O m^{-2}), and was almost completely inhibited by 43% after withholding water for 7 d, and to 72% after 14 d (Fig. 3b). Stressed plants closed their stomata slightly after withholding water for 2 d compared to control plants. Stomatal closure increased by 50% of the control values after 7 d of drought, and by 77% after 14 d of drought (Fig. 3c). The values of internal CO_2 concentration (C_i) of stressed and control plants did not differ significantly after 9 d treatment period. However, after 9 d, C_i in drought-stressed plants became significantly lower than well-watered plants (Fig. 3d).

Water use efficiency (WUE = $A_{CO_2}\,E^{-1}$) and intrinsic WUE (WUE$_i$ = $A_{CO_2}\,g_s{}^{-1}$) showed similar patterns of increasing divergence between drought-stressed and well-watered plants as the experiment progressed, especially after 7 d. By prolonging water deficit, stressed plants displayed higher WUE and WUE$_i$ values than in control plants, where the differences become significantly only after 11 d (repeated-measures ANOVA, time × drought interaction, $F_{6,48} = 3.01$ and 4.67, respectively; $P < 0.05$; Fig. 3e–f).

During the drought-recovery period (days 14–21), previously stressed plants began to increase A_{CO_2}, E, g_s and C_i along with decreases in WUE and WUE$_i$ during the first 48 h after re-watering such that they reached similar values to those of control plants by the end of the recovery period (Fig. 3a–f).

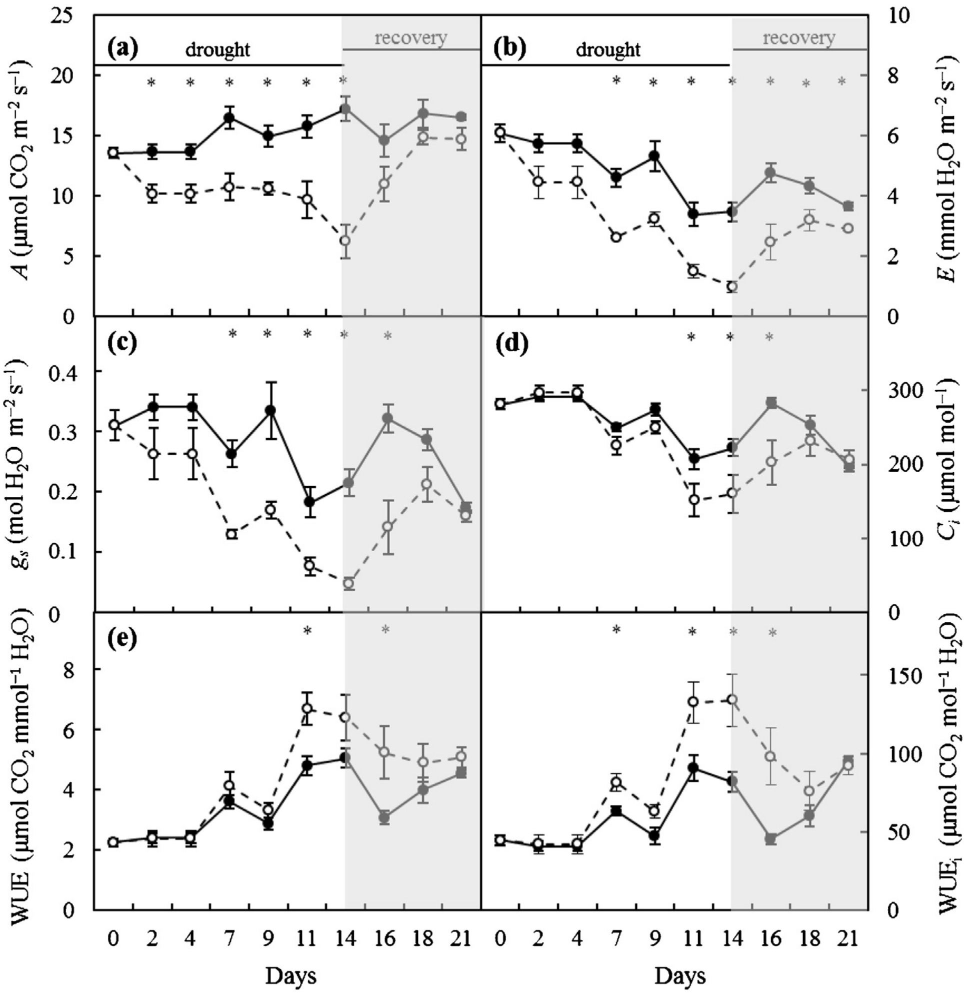

Fig. 3. Changes in (a) net CO_2 assimilation rate (A, µmol m^{-2} s^{-1}), (b) transpiration rate (E, mmol m^{-2} s^{-1}), (c) internal CO_2 concentration (C_i, µmol mol^{-1}), (d) stomatal conductance (g_s, mol m^{-2} s^{-1}), (e) water use efficiency (WUE, µmol CO_2 mmol^{-1} H_2O), and (f) intrinsic WUE (WUE$_i$, µmol CO_2 mmol^{-1} H_2O), for *Ephedra alata* plants in control (●) and drought (○) treatments throughout the experimental period. Values represent means ± SE, $n = 5$. The gray area indicates the 7-d drought-recovery period. Asterisks denote significant treatment differences from pairwise comparisons ($P < 0.05$).

3.3. Accumulation of organic solutes

The repeated measures ANOVA used to examine the effect of time on accumulation of proline indicated highly significant time and time × drought effects ($F_{2,12} = 87.68$ and 55.55, respectively; $P < 0.001$; Fig. 4a). Proline concentration in stems of control plants remained almost stable throughout the experiment reaching an average value of 7.5 µmol g^{-1} DM. Differences in proline accumulation increased with ongoing drought stress. Proline concentration after 7 and 14 days increased significantly by 267% and 480% of the control value, respectively. Stem proline accumulation of previously stressed plants recovered gradually after re-watering toward control values.

The accumulation of soluble sugars in drought-stressed stems increased by 26% and 15% compared to control values after 7 and 14 d of withholding water, respectively. However, repeated-measures ANOVA revealed that differences in soluble sugar concentration did not differ significantly between and within subject effects. The values of soluble sugars were restored to control values after 7 d of drought-recovery (Fig. 4b).

4. Discussion

Results from this study indicate that increasing water use efficiency allowed photosynthesis to continue in drought-stressed plants, albeit at lower rates than well-watered plants, while water potential slowly declined. The data presented demonstrate that coordination of water loss and carbon gain, combined with proline accumulation function as mechanisms behind its successful distribution across arid regions. Most previous studies on the genus *Ephedra* have focused on the pharmacological and medical aspects of the species (Smith, 1977; Nawwar et al., 1984, 1985; Abourashed et al., 2003; Hayashi et al., 2010), and studies on the ecophysiology of *Ephedra* have long been needed to improve the basis for management of this important regional plant species. Analysis of the relationship between water relations and gas exchange characteristics of this xerophytic species in this study provide a tool for understanding how plants cope with extreme drought, and how the mechanisms of adaptation to dry conditions may affect its prospects for cultivation, restoration, and persistence in natural ecosystems in the face of climate change.

Water deficit markedly reduced A_{CO_2} and E in *E. alata* plants. This pattern in gas exchange of stems is due to the cessation of plant growth associated with reduced availability of photoassimilates to apical sink region Gorai (unpublished data). The relationship between A_{CO_2} and g_s have received increasing recent attention due to the trade-off between water loss and carbon gain experienced by plants during extreme, climate change-type drought (McDowell et al., 2008). Our data show that both parameters are involved in decreasing gradually with on-going drought stress in a manner that

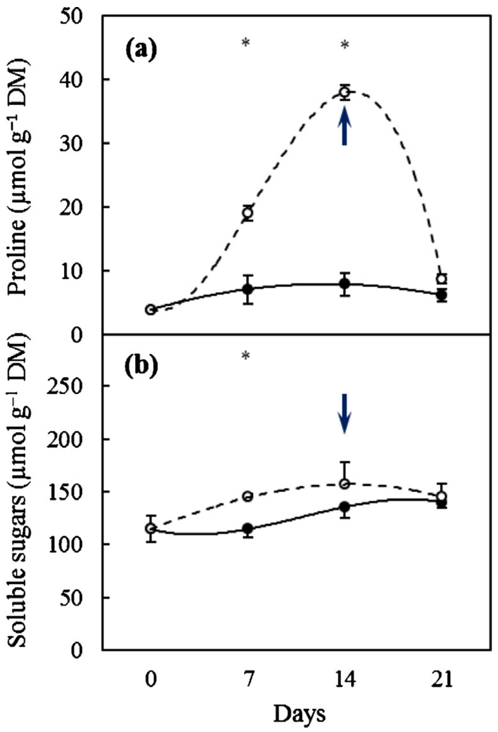

Fig. 4. Changes in (a) proline content (µmol g^{-1} DM) and (b) soluble sugar contents (µmol g^{-1} DM) in stems of *Ephedra alata* in control (●) and drought (○) treatments throughout the experimental period. Values represent means ± SE, $n = 4$. Arrows indicate the beginning of the drought-recovery period. Lines describing the dependencies were obtained using a polynomial regression. Asterisks denote significant treatment differences from pairwise comparisons ($P < 0.05$).

improves water use efficiency (Fig. 5a). The values of g_s and A_{CO_2} in control plants reached the highest values and varied slightly over time (7 to 21 d). As A_{CO_2} decreased in parallel with g_s, stomatal limitations seemed to account mainly for this reduction of photosynthesis. However, it is important to note that our results are probably most indicative of how seedlings behave during drought because of the nature of pot studies and the small size of our plants. The behavior of mature plants is likely mediated by their size and the extension of their roots.

Seedling establishment is a dynamic and critical stage in a plant's life history, being decisive for its performance and success in stressful environments. Drought is one of the major causes of seedling mortality (Moles and Westoby, 2004) and in arid environments soil moisture is likely to be one of the more important factors upon which predictive germination is based (Gutterman, 1993). Based on a 3-year field experiment in a hyper-arid region of Southern Tunisia, Derbel and Chaieb (2013) characterized the seedling emergence and survival of four Saharan shrubby species including *E. alata*. These authors found that seedling survival rates at the end of the experiment ranged between 11% and 27%. In the case of *E. alata*, seedling emergence rate was only 50% which was significantly higher in spring and by the end of the experiment seedling mortality was 80% with the highest value reached during the summer of the first sowing year (47%). It has been reported that the first summer rainless period after seedling establishment is a major limiting factor when seedling roots are not fully developed (Pratt et al., 2007). According to McDowell et al. (2008), the limited rooting volume explored by seedlings exposes them to more negative soil water potentials than plants with larger root systems, decreasing leaf-specific hydraulic conductance of the soil-plant continuum and hence the safety margin between transpiration (E) and critical rates (E_{crit}).

The relationship between g_s and Ψ_w appears to be community-specific and may reflect differential water use by shrubs in arid environments (Jacobsen et al., 2008). As shown in Fig. 5b, *E. alata* displayed sensitive stomatal responses which result from lowering stem Ψ_w. This is consistent with previously reported correlations on Mojave Desert species, like *Coleogyne ramosissima* and *Larrea tridentate*, that displayed relatively steep declines in stomatal conductance with declining water potential (Jacobsen et al., 2008). At the end of the recovery period, previously stressed plants quickly restored their water status by increasing Ψ_w, coupled with increasing g_s. This type of stomatal control has been extensively studied and is mostly involved in the regulation of plant water status (Li, 2002; Schroeder et al., 2001; Laporte et al., 2002; Luan, 2002).

Many authors use the internal CO_2 concentration (C_i) to describe the limitations applied to the photosynthesis (Lawlor and Cornic, 2002; Medrano et al., 2002). Gas exchange in *E. alata* shows a reduction in CO_2 assimilation associated with an increase in stomatal resistance, which decreases the availability of internal CO_2 and reduces water loss through transpiration. Similar results have been obtained on olive plants (Bacelar et al., 2007; Giorio et al., 1999). It is predominately diffusive resistance that limits photosynthesis under mild to moderate drought conditions, whereas under severe drought, when stomatal conductance (g_s) drops below 0.1–0.05 mol H_2O m^{-2} s^{-1}, metabolic limitations become dominant (Flexas et al., 2004, 2006). By referring to this widely accepted theory, the inhibition of carbon assimilation in *E. alata* is associated with limitations in diffusive intercellular CO_2 which is

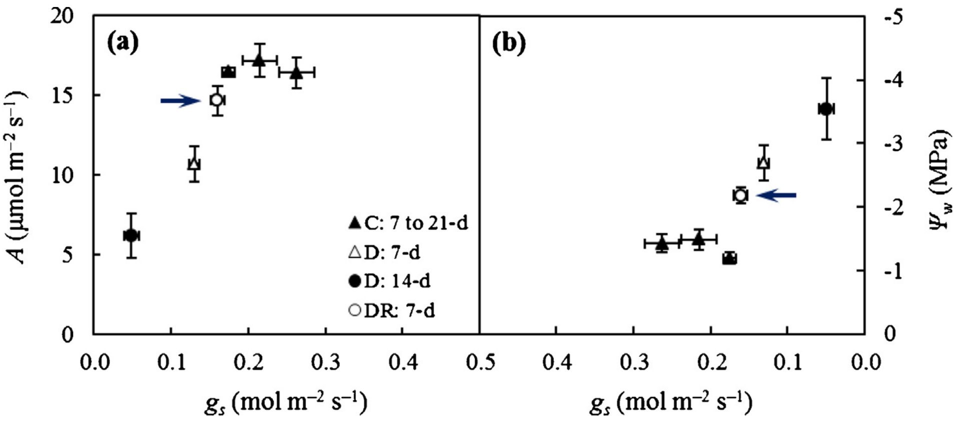

Fig. 5. Relationship between A and g_s (a), and between Ψ_w and g_s (b) for *Ephedra alata* plants in control and drought treatments throughout the experimental period. Values represent means ± SE. Arrows indicate the beginning of the drought-recovery period. C: 7, 14 and 21-d control plants (▲); D: 7- and 14-d drought period (△ and ●, respectively); DR: 7-d drought-recovery period after 14-d water deficit period (○).

controlled by stomata (stomatal effect). Furthermore, soluble sugar concentration in drought-stressed plants increased during drought treatments, suggesting that the limitation of carbon gain by partial stomatal closure was minimal. Stomatal closure therefore only exerted limited control against dehydration and could not compensate for decreases in soil water status, typical of anisohydric behavior.

Drought adaptation of Mediterranean plants relies on a variety of mechanisms including deep rooting patterns, avoidance or resistance of cavitation-induced embolism, and compensation or repair of embolism-induced hydraulic damage (Nardini et al., 2014). It was shown (Derbel and Chaieb, 2013) that *E. alata* stood out by a dual strategy with the development of a deep taproot reaching a depth more than 1.5 m, and superficial lateral roots running horizontally 10 m from the tap root. However, mature *Ephedra* plants avoid rooting in the top 30 cm of the soil (Derbel and Chaieb, 2013), except close to the stem where they may receive increased water infiltration from stem flow, and where shading from the main body of the plant offers protection to the roots (Schwinning and Hooten, 2009). This rooting pattern affirms the association of deep roots with the evergreen life history and Evenari et al. (1982) categorize it as aridoactive plant. With respect to comparative hydraulic strategies among species and their relationship with mortality mechanisms (McDowell et al., 2008), intense drought is predicted to lead, in anisohydric species, to hydraulic and symplastic failure whereas long-lasting moderate drought would cause carbon starvation in isohydric species. *E. alata* could be characterized as an anisohydric species. Our data show that drought-stressed seedlings of *E. alata* reduced their g_s associated with reduced water availability, indicating that even typically anisohydric plants must constrain g_s at some point to avoid hydraulic failure (McDowell et al., 2008).

The finding in the present study that drought-stressed seedlings reached relatively less negative stem Ψ_{w} of −3.5 MPa, at least compared with species in other drylands, can be attributed to their occurrence on sandy soils. This value is lower than the across-species mean midday Ψ_{w} (−4.6 MPa) from three semi-arid shrub communities (29 species) in southern California including chaparral, coastal sage scrub, and Mojave Desert scrub (Jacobsen et al., 2008). In soils with such a high sand content (88–98% sand; Dhief et al., 2009), decreased midday Ψ_{w} below −3 MPa would confer little advantage steepening the soil-root water potential gradient (Lambers et al., 1998). Compared with other leafless perennial species of the shrubby genus *Calligonum* found in the same environment (Grand Erg Oriental), Dhief et al. (2009) reported that all species reached less negative Ψ_{w} values (≈−2.5 MPa) during the hot and dry summer, with extremes in July. The findings are comparable with those of Grigg et al. (2008) on desert plants inhabiting sandy soils where *Acacia* species typically displayed more negative leaf Ψ_{w} values, approaching −3.0 MPa for *Acacia jensenii* in the dry season. Plants on the dunes would display more favourable water relations (higher g_s and less negative leaf Ψ_{w}) than closely related plant species in the interdunes because of the greater soil depth and associated water storage capacity within the sandy dune ridges (Grigg et al., 2008; Dhief et al., 2009). In the case of *Ephedra*, the 10–12 m high dunes provide a large volume with a reasonable matric potential to store rainfall. Yair et al. (1997) showed that in the sand dunes of Nizzana water can infiltrate in sand layers deeper than 3 m.

Due to differences in the hydraulic architecture of gymnosperm wood containing only short tracheids, like *E. alata*, and angiosperm wood containing long vessels, gymnosperms attain relatively lower levels of hydraulic conductivity for a given level of cavitation resistance compared with angiosperms, or else have to invest much more carbon to achieve the same level of hydraulic conductivity (Hacke et al., 2004). The relatively drought-tolerant anisohydric species are more likely to die from hydraulic failure than carbon starvation because they maintain a thin margin of hydraulic safety (McDowell et al., 2008). *Ephedra* reached Ψ_{w} of ca. −3.5 MPa, which is closely related to the mean cavitation resistance (Ψ_{50}) of approx. −4 to −4.8 MPa which has been found across Mediterranean-type and Desert ecosystems (Choat et al., 2012). This demonstrates that *Ephedra* appears to operate with narrow safety margins in terms of the difference between Ψ_{50} and the stem Ψ_{w}.

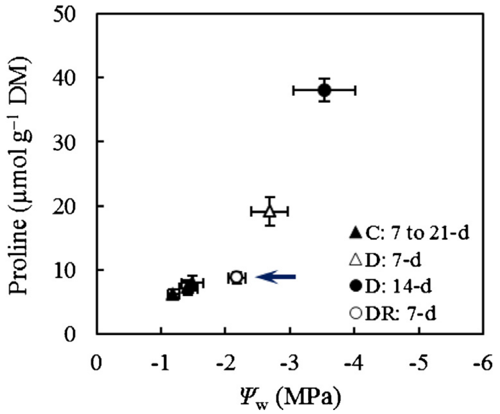

Fig. 6. Relationship between stem proline (μmol g^{-1} DM) and stem Ψ_{w} for *Ephedra alata* plants in control and drought treatments by withholding watering throughout the experimental period. Values represent means ± SE. Arrows indicate the beginning of the drought-recovery period. C: 7, 14 and 21-d control plants (▲); D: 7- and 14-d drought period (△ and ●, respectively); DR: 7-d drought-recovery period after 14-d water deficit period (○).

E. alata displayed more negative values of Ψ_{w} as soil water status declines. Bittman and Simpson (1989) reported that low Ψ_{w} may be the consequence of low water uptake and hydraulic flow rates within the plants, or high water loss rates. The relationship between RWC and Ψ_{w} has often been used to quantify the dehydration tolerance: tissues which maintain a high RWC as Ψ_{w} decreases are more tolerant to dehydration. In our experiment, stems of *E. alata* decrease their RWC values from 84% to 58%, which is a typical characteristic of drought-tolerant species as RWC dropped below 60% (Kawaguchi et al., 2004; Gorantla et al., 2007; Perera et al., 2008). This is consistent with the findings of Sade et al. (2012), who reported that isohydric vs. anisohydric behavior should ultimately be reflected in RWC (Sade et al., 2009). Furthermore, the existence of a significant relationship between Ψ_{w} and RWC values suggests the importance of solute accumulation process in maintaining low Ψ_{w}, which could involve selective increase of particular solutes. As withholding water was prolonged, *E. alata* displayed more negative Ψ_{π} values, and consequently different Ψ_{p} values for a wide range of Ψ_{w}, demonstrating its capacity to osmoregulate.

A very common response to drought is an increased production of compatible organic solutes such as soluble sugars, sugar alcohols, proline and betaines (Serraj and Sinclair, 2002). These compatible solutes contribute to cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins (Bohnert and Jenson, 1996; Attipali et al., 2004). In our experiments, accumulation of proline in drought-stressed stems was significantly increased by withholding water to 480% of control value at the end of drought period. However, proline content restored at the end of recovery period reaching similar control values. Synthesis of this amino acid under drought conditions was inversely proportional to the water status of plants (Fig. 6). It was found that proline accumulation was significantly correlated to low water potentials suggesting the involvement of this solute in osmotic adjustment.

In conclusion, the results obtained suggest that *E. alata* was able to overcome drought stress by reducing its photosynthetic performance and water relations. Consequently, an osmotic adjustment was developed by this desert shrub as a tolerance mechanism to confront drought stress. Thus, a rapid recovery of photosynthetic apparatus was achieved characterizing the capacity of *E. alata* to withstand and survive in dry environments. Further investigations are necessary to understand the ecophysiological strategies of *Ephedra* plants for survival under natural environmental conditions.

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