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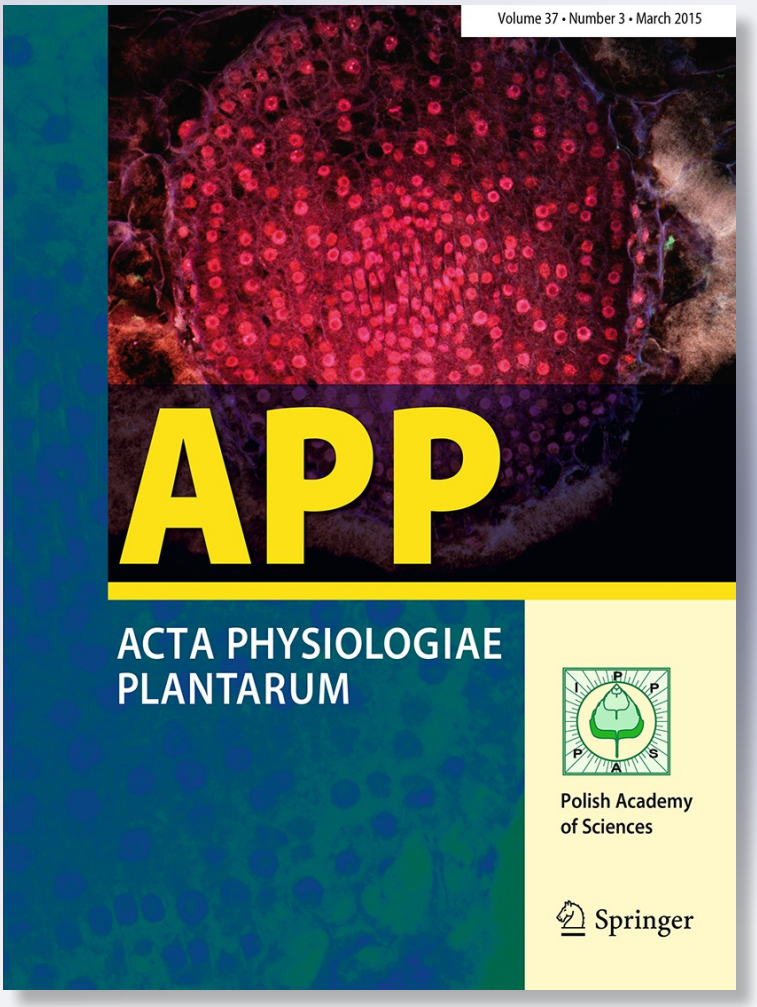

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ORIGINAL PAPER

Leaf water status, osmoregulation and secondary metabolism as a model for depicting drought tolerance in *Argania spinosa*

Abdelghani Chakhchar[1] · Mouna Lamaoui[1] · Said Wahbi[2] · Abderrahim Ferradous[3] · Abdelhamid El Mousadik[4] · Saad Ibnsouda-Koraichi[5] · Abdelkarim Filali-Maltouf[6] · Cherkaoui El Modafar[1]

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Abstract The present investigation was undertaken to characterise and to distinguish four contrasting *Argania spinosa* ecotypes in terms of drought tolerance by exploring the changes of leaf water status, osmoregulation and secondary metabolism. *A. spinosa* plants corresponding to four contrasting ecotypes (Lks, Alz, Rab and Adm) were subjected to drought stress. The results exhibited that there was a significant decrease in predawn leaf water potential (Ψ_{pd}), stomatal conductance (g_s) and leaf relative water content under the influence of the intensity and duration of drought stress. Negative and significant correlations were recorded between epicuticular wax load (EWL) and residual transpiration rate. Electrolyte leakage (EL) increased significantly in leaves of plants under drought stress treatment compared to control plants. Furthermore, our data revealed that drought stress can induce shikimate and phenylpropanoid pathways in *A. spinosa*. A significant induction of phenylalanine ammonium lyase (PAL), shikimate dehydrogenase (SKDH) and cinnamate 4-hydroxylase (C4H) enzymes and an increase in polyphenol content were recorded, of which Lks showed the highest induction and accumulation among ecotypes. Accumulation of polyphenols was positively correlated with the SKDH, PAL and C4H activities. The strong induction of secondary metabolism in Lks might be linked to its better ability of drought tolerance. The proline and soluble sugar content in leaves of all ecotypes increased substantially in parallel with the severity of stress-induced. According to canonical discriminant analysis of our data, the four ecotypes were separated by the following physiological and biochemical parameters: EL, g_s, EWL, soluble sugars and polyphenols.

Keywords *Argania spinosa* · Drought stress · Shikimate and phenylpropanoid pathways · Polyphenols

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✉ Abdelghani Chakhchar
chakhchar.ckr@gmail.com

[1] Laboratoire de Biotechnologie Valorisation et Protection des Agroressources, Faculté des Sciences et Techniques Guéliz, Université Cadi Ayyad, 40 000 Marrakech, Morocco

[2] Laboratoire de Biotechnologie et Physiologie Végétale, Faculté des Sciences Semlalia, Université Cadi Ayyad, 40 000 Marrakech, Morocco

[3] Centre Régional de la Recherche Forestière Marrakech, BP 12360 Ain Itti Ennakhil, Marrakech, Morocco

[4] Laboratoire de Biotechnologie et Valorisation des Ressources Naturelles, Faculté des Sciences, Université Ibn Zohr, Agadir, Morocco

[5] Laboratoire de Biotechnologie Microbienne, Faculté des Sciences et Techniques, Université Sidi Mohamed Ben Abdellah, Fès, Morocco

[6] Laboratoire de Microbiologie et Biologie Moléculaire, Faculté des Sciences, Université Mohammed V Agdal, Rabat, Morocco

Introduction

The ways in which individual plants respond to abiotic stress are complex and varied (Rodziewicz et al. 2014). In Mediterranean ecosystem, the plants are subjected to a continuous and severe drought stress (Nogués and Baker 2000). Some endemic species are well adapted to these conditions like argan tree in Morocco (Msanda et al. 2005; Díaz-Barradas et al. 2010). Responses to drought are species- and genotype-dependent characteristics. Unlike annual plants, trees inevitably suffer drought periods; thus,

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they have evolved to incorporate various mechanisms to resist drought effects (Pita et al. 2005). In addition, the reactions of the plants to drought stress differ significantly in function of drought density and duration. Water-stress tolerance involves subtle changes in the physiological and biochemical processes of plants. Leaf water potential and stomatal conductance are considered important components for improving drought tolerance in plants (Pita et al. 2005; Chaves et al. 2003). Drought stress causes water loss within the plant and therefore a reduction in its relative water content. The membranes of plants cells are sensitive to environmental stresses such as drought stress. As the cells are subjected to drought stress, electrolytes leak into the surrounding tissues. This is due to increases in permeability and loss of integrity in plant membranes (Quan et al. 2004). During drought conditions, epicuticular wax load (EWL) increases, thereby minimising cuticular transpiration and maximising leaf water retention (Zhang et al. 2005; Goodwin and Jenks 2005; Seo and Park 2011). A positive correlation between EWL and residual transpiration rate (RTR) has previously been demonstrated (Premachandra et al. 1992). The reduction of RTR, based on genetic selection, is now a challenge to improve cereal crops' tolerance in improvement of cereals for cultivation in environments where water is a growth-limiting factor (González and Ayerbe 2010). Furthermore, the polyphenols comprise the major category of secondary metabolites in plants, with the shikimate pathway commonly used for biosynthesis. Biosynthesis of polyphenol compounds is stimulated by biotic and abiotic stresses. Large class of phenolic compounds is derived from phenylpropanoid pathway starting from phenylalanine via the actions of some intermediates, such as cinnamate 4-hydroxylase (C4H) and phenylalanine ammonia lyase (PAL). Thus, one mechanism utilised by the plants to overcome the drought stress effects might be via accumulation of compatible solutes, such as proline (Kavi-Kishor et al. 2005) and soluble sugars (Ashraf and Harris 2003).

Nevertheless, there is still insufficient knowledge on the physiological and biochemical mechanisms describing the responses of *Argania spinosa* to drought stress. The argan tree (*A. spinosa* (L.) Skeels; synonyms *Argania sideroxylon* Roem. & Schult.) is endemic to Southwestern part of Morocco, where it grows over about 800,000 hectares (Msanda et al. 2005). The understanding of the physiological and the biochemical adaptive mechanisms controlling drought tolerance of *A. spinosa*, in a pot-study, is our major aim to suggest criteria for the early selection of the most tolerant ecotypes, after validation by field study, to be planted in areas exposed to drought risk. The objectives of this work were (1) to test some physiological traits related the tolerance of *A. spinosa* and the damages caused by exposure to controlled drought stress, (2) to determine drought stress effects on polyphenols as well as the enzymes related to its biosynthesis and (3) to assess differences in physiological and biochemical parameters studied between four contrasting *A. spinosa* ecotypes. To our knowledge, this is the first time that the secondary metabolism of *A. spinosa* has been assessed under drought stress.

Materials and methods

Study site and sampling procedures

Sampling of *A. spinosa* seeds was conducted in four regions of the argan tree forest in the southwest of Morocco. Seed collection for this study was conducted in the southwest region of Morocco. Climatic, geographical and hydrological conditions of these four regions are markedly different (Table 1). From these contrasting regions, we chose two contrasting coastal ecotypes [site 1 and 2: Rabia (Rab) and Admine (Adm), respectively] and two inland ecotypes [site 3 and 4: Aoulouz (Alz) and Lakhssas (Lks), respectively] for a better interpretation of the mechanisms regulating the biochemical and physiological processes. After stratification in moist compost (3 weeks), pre-germinated seeds were transferred into alveolate containers with rigid walls characterised and specified in the special conditions relating to both production works of nursery plants as those of regeneration of the argan tree planting in a greenhouse under semi-controlled conditions. Uniform seedlings with a single stem and similar height were selected, transplanted into 15-cm diameter plastic pots containing a mixture of soil and peat 1/1 (v/v) to which 10 %

Table 1 The geographical and climatic characteristics of the sites of *A. spinosa* ecotypes studied

Origin site	Province	Territory	Temperature (°C)	Rainfall (mm)	Altitude (m)	Humidity (%)	*m* (°C)	*M* (°C)
Rabia	Essaouira	Coastal	17–18	295	181–226	80–90	9.6	22.2
Admin	Agadir	Coastal	18–19	225	275–430	75–85	7.2	27.1
Aoulouz	Taroudante	Inland	19–20	232	700–850	60–70	5.6	35.7
Lakhssas	Tiznit	Inland	21–22	189	916–988	50–60	7.3	31.2

m the mean minimum temperature of the coldest month and *M* the mean maximum temperature of the coldest month of the year

of perlite was added to improve field capacity. Each pot contained one seedling. Seedlings of four contrasting ecotypes of *A. spinosa* were grown in growth chamber. The environmental conditions in the chamber during the experiment were maintained at 28 ± 1 °C temperatures during day and 25 ± 1 °C during night in a 16:8 photoperiod and the relative humidity ranged between 65 and 70 %. The average maximum photosynthetically active radiation (PAR) was 400 μmol m^{-2} s^{-1} provided by a combination of fluorescent and incandescent lamps. The environmental conditions of the growth chamber are those described and applied in the forestry centres in Morocco for multiplication and regeneration of the argan tree. Uniform young *A. spinosa* plants of similar height, aged 18 months, were selected for the experiment for each ecotype. The experimental layout was completely randomized with three factors (ecotype, watering regime and time). We adopted three distinct watering regimes as follows: two drought-stressed treatments (50 and 25 % of field capacity (FC)) which correspond to medium and severe stress, respectively, and one control treatment (100 % of FC).The treatments were applied for 2 months and each treatment included fifteen plants for each ecotype. In each replication, we harvested the fully expanded young leaves from plants every fortnight during the experimental period to determine physiological and biochemical parameters.

Physiological parameters measurement

Leaf water potential

The predawn leaf water potential (Ψ_{pd}) was determined with the pressure chamber technique (SKPD 1400, Skye Instruments, Powys, UK) at predawn (05:00–06:00 h). Measurements were taken in the upper part of stem (3 cm) with five newly expanded leaves per plant (five plants/treatment).

Stomatal conductance

Using a portable steady-state diffusion porometer (Model Sc-1, Decagon Devices, Pullman, USA), the stomatal conductance (g_s) was determined. Measurements were made on fully exposed leaves between 10:00 and 12:00 h. The data were collected from two leaf samples per plant (five plants/treatment).

Relative water content

Leaf relative water content (RWC)calculations were made according to the formula: FWC (%) = (FW − DW)/(SW − DW) × 100, where FW, SW and DW refer to fresh weight, saturated weight (after 24 h rehydration on distilled water) and dry weight (after oven drying for 48 h at 70 °C), respectively. The data were collected from five leaves per plant (five plants/treatment).

Residual transpiration rate

The Residual transpiration calculations were made following the method of McCaig and Romagosa (1989). The collected leaves from *A. spinosa* plants were weighed immediately after excision as fresh weight. These leaves were then kept in a controlled environment (relative humidity of 50 % and 22 °C in the dark). Two hours later, the loss of water in the leaves was evaluated by sequential measurement of weight, approximately every half hour for 6 h. The dry weight of leaves was then measured (drying overnight at 60 °C). RTR calculations were made considering the linear phase of water loss through transpiration between 120 and 360 min. RTR results were expressed in g H_2O kg^{-1} dry weight min^{-1}. There were 5 replicates per treatment (one plant per replicate).

Electrolyte leakage

Leaf membrane injury was determined by recording of electrolyte leakage (EL) as described by Yang et al. (2009) with some modifications. A total of 20 leaf discs (0.2 cm^2 per disc) per replicate from *A. spinosa* leaves were washed (3 times) to eliminate surface electrolytes and placed in stoppered vials containing 15 ml of distilled water. After 4 h under stirring at 25 °C, the initial conductivity was determined by a conductivity metre (Hach, model. sensION + EC7). The total conductivity calculations were determined after the samples had been autoclaved for 20 min at 120 °C. The conductivity values of leaves were calculated as the percentage of the initial conductivity of the total conductivity. There were 5 replicates per treatment (one plant per replicate).

Polyphenol enzymes: extraction and assay

Shikimate dehydrogenase (SKDH)

For the biochemical measurements, samples of fresh leaves from control and treated plants were immediately lyophilised and ground to a fine powder in the presence of liquid nitrogen.

For extraction of shikimate dehydrogenase (SKDH; EC 1.1.1.25), finely ground powder (100 mg) was extracted in 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0). The homogenates obtained were centrifuged at 15,000×*g* for 10 min at 4 °C.

SKDH activity was determined in 0.1 M Tris–HCl buffer (pH 9) as described by Ali et al. (2006). The assay

mixture consisted of 0.1 ml of enzyme extract, 1.45 ml of 0.5 mM NADP and 1.45 ml of 2 mM shikimic acid. Change of absorbance due to reduction of NADP at 340 nm was recorded over 1 min. SKDH activity was expressed in nmol mg^{-1} protein min^{-1}. There were 5 replicates per treatment (one plant per replicate).

Cinnamate 4-hydroxylase (C4H)

For extraction of cinnamate 4-hydroxylase (C4H; EC 1.14.13.11) activity, finely ground powder (100 mg) was extracted in 200 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing 2 mM of 2-mercaptoethanol. The homogenates obtained were centrifuged at 10,000×*g* for 10 min at 4 °C.

C4H activity was assayed by the method described by Sánchez-Rodríguez et al. (2011). The extract enzyme (0.1 ml) was homogenised with 4.8 ml of reaction buffer consisting of 50 mM K_2HPO_4/KH_2PO_4 buffer containing 2 mM trans-cinnamic acid, 0.5 mM NADPH and 2 mM of 2-mercaptoethanol. After incubation at 37 °C for 1 h, the reaction was stopped with 6 M of HCl and readjusted to pH 11. The absorbance of the reaction solution was recorded at 290 nm and C4H activity was presented as nmol mg^{-1} protein min^{-1}. There were 5 replicates per treatment (one plant per replicate).

L-Phenylalanine ammonia lyase (PAL)

For every sample, finely ground powder (100 mg) was extracted in 50 mM Tris–HCl buffer (pH 8.0), 14.4 mM 2-mercaptoethanol and 1 % (w/v) polyvinylpolypyrrolidone (PVPP), then centrifuged at 10,000×*g* for 10 min at 4 °C.

Activity of L-phenylalanine ammonia lyase (PAL, EC 4.3.1.5) in the crude enzyme extract was assayed by an adaptation of the method of Beaudoin-Eagan and Thorpe (1985). The assay mixture consisted of 1.9 ml of 50 mM Tris–HCl buffer (pH 8.0), 0.1 ml of enzyme extract and the reaction was activated by the addition of 1 ml of a solution of 15 mM L-phenylalanine. After 70 min at 37 °C, the reaction was stopped by the addition of 0.2 ml of 5 MHCl. The content of trans-cinnamic acid generated was determined by measuring absorbance at 290 in 1 cm quartz cuvettes. A molar extinction coefficient of 17.4 mM^{-1} cm^{-1} was adopted to quantify cinnamic acids formed during the enzymatic reaction. PAL activity was expressed in nmol cinnamic acid mg^{-1} protein min^{-1}. There were 5 replicates per treatment (one plant per replicate).

Polyphenol content

The finely ground powder was ground on ice in 80 % methanol (MeOH) and then sonicated for 10 min in ultrasonic bath (Elma, Germany) containing 700 ml ultrapure water. The homogenate was centrifuged. After centrifugation of the homogenate at 12,000×*g* for 10 min at 4 °C, the supernatants were used for a differential assay in the presence/absence of 1 % (w/v) polyvinylpolypyrrolidone (PVPP). For each sample, the supernatant was diluted in deionised water with and without PVPP. The homogenate (with PVPP) was well stirred (5 min) and centrifuged at 10,000×*g* for 5 min. New supernatants obtained were used subsequently to quantify the polyphenols.

The concentration of polyphenols was determined with Folin–Ciocalteu reagent using the colorimetric method adapted by Singleton and Rossi (1965) with some modifications. For each sample, the absorbance value read at 760 nm, from the supernatants without PVPP, was then corrected by subtracting the value derived from the serial absorbance in presence of PVPP. Measurements were carried out in five replicates per treatment and calculations were based on a calibration curve obtained with gallic acid. The levels of polyphenols were presented as mg GAE g^{-1} DW (mg of gallic acid equivalents/g of dry weight). There were 5 replicates per treatment (one plant per replicate).

Epicuticular wax loads

Leaf epicuticular wax loads (EWL) were assessed based on the principle of the method of Ebercon et al. (1977) with some modifications. Leaf-blade segments (15 pieces, each of 1 cm^2) were homogenised with 10 ml of chloroform for 10 s. After evaporation of the chloroform extract on a boiling water bath, 5 ml of acidic $K_2Cr_2O_7$ was added. The mixture was then heated for 30 min in boiling water bath and cooled quickly on an ice bath before adding 10 ml of distilled water to the mixture. The colour change was determined at 590 nm and the EWL values were presented as μg dm^{-2}. For EWL calculations, a calibration curve was established using known concentrations of polyethylene glycol-6000. There were 5 replicates per treatment (one plant per replicate).

Free proline content

Proline was measured as described by Bates et al. (1973). The finely ground powder (100 mg) was homogenised in 3 ml of 3 % sulfosalicylic acid solution. The assay mixture was centrifuged at 15,000×*g* for 15 min. The supernatant (2 ml) was treated with a mixture of 2 ml 2.5 % acid ninhydrin solution and 2 ml glacial acetic acid and boiled for 1 h. After addition of 2 ml toluene, the tubes were shaken and absorbance was determined at 520 nm using L-proline as a standard. The concentration of proline was presented as μmol g^{-1}DW. There were 5 replicates per treatment (one plant per replicate).

Soluble sugars content

Total soluble sugars concentration was assessed following the anthrone method (van Handel 1968). The finely ground powder was extracted with 1 ml of 80 % ethanol, centrifuged at 15,000×g for 10 min and the pellet was re-extracted twice with 90 % ethanol at 80 °C. The extract was then treated with freshly prepared anthrone reagent (2 ml). The absorbance was recorded at 625 nm by spectrophotometer and the concentration of soluble sugars was derived from a standard curve using glucose. The concentration of soluble sugar was presented as mmol g^{-1}DW. There were 5 replicates per treatment (one plant per replicate).

All reagents used were of analytical grade and were delivered from Sigma (St. Louis, USA), Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

Statistical analysis

Each data pointed the mean of five separate replicates, and mean values and standard deviations were calculated. Results were examined by the three-way analysis of variance (ANOVA) in order to examine the effect of ecotype, time, watering regime and their interactions in each of the physiological and biochemical study variables. Means were compared using the Tukey's Post hoc test after checking the normality and the homoscedasticity of data. Pearson correlation analysis was done for some variables for each ecotype after checking the assumptions of parametric tests. A canonical discriminant analysis (CDA) was performed on the four contrasting *A. spinosa* to identify the discriminating variables between them. Statistical analyses were performed using SPSS 10.0 for Microsoft Windows.

Results

Physiological parameters

Our results showed that drought stress with their characteristics (duration and intensity) has caused serious effects on the four contrasting ecotypes of *A. spinosa*, especially under severe drought stress. The primary physiological effects of drought stress are a reduction in Ψ_{pd}, g_s and leaf RWC (Fig. 1a–c). Highly significant differences ($P < 0.001$) between watering regime (WW, MS and SS) were noted and ecotype effect was also significant in the four *A. spinosa* ecotypes ($P < 0.001$). Compared to control, medium stress caused significant changes in leaf water status. At the end of the stress period (60 day), g_s decreased significantly ($P \leq 0.05$) by 71.5, 70.5, 80.3 and 79.6 % in Lks, Alz, Rab and Adm, respectively, under severe stress compared to control conditions. Similarly, Ψ_{pd} decreased significantly ($P \leq 0.05$) in all ecotypes during the water drought period. Ψ_{pd} reached a minimum value of −1.61, −1.31, −1.17 and −1.27 MPa in Lks, Alz, Rab and Adm, respectively, after 60 days of water drought treatment; however, we have not recorded significant changes in the control plants. The g_s values for Rab were the higher among ecotypes (Fig. 1b), whereas Ψ_{pd} was lower in Lakhssas (Fig. 1a) than in the other ecotypes. As compared with the control plants, a significant decrease ($P \leq 0.05$) 46.1, 46.4, 42.7 and 40.7 % in Lks, Alz, Rab and Adm, respectively, in leaf RWC occurred in the end of water drought period under severe treatment. Both inland ecotypes that exhibited low values of leaf RWC and low levels of g_s are those that indicated more negative Ψ_{pd} values. In addition, no significant changes occurred in the control plants for Ψ_{pd} and leaf RWC in each ecotype. Positive and significant correlations ($P < 0.01$) were recorded between g_s and Ψ_{pd} during the experiment ($r = 0.93$, $r = 0.85$, $r = 0.94$ and $r = 0.89$ in Lks, Alz, Rab and Adm, respectively). Also, positive and significant correlations ($P < 0.01$) were recorded between g_s and RWC ($r = 0.84$, $r = 0.84$, $r = 0.90$ and $r = 0.83$ in Lks, Alz, Rab and Adm, respectively). According to three-way ANOVA analysis, there were significant differences between ecotypes, watering regime, time and the interactions between them. Significant ecotype × watering regime and time × watering regime interactions were observed for these three parameters ($P \leq 0.05$) and ecotype × time and ecotype × watering regime × time interactions were significant only for g_s and Ψ_{pd} ($P \leq 0.05$).

The cell membrane integrity was evaluated by electrolyte leakage in leaves. Our result showed that EL increased significantly in leaves of plants under drought stress treatment compared to control plants (Fig. 2). This damage was observed 2 weeks after the beginning of treatment and we found that leaves of *A. spinosa* plants exposed to medium stress contained about two to 2.7-fold and those from plants exposed to severe stress about 3.4 to 4-fold higher foliar electrolyte concentrations than controls, in all ecotypes. Furthermore, a significant difference was found between ecotypes ($P < 0.001$). According to three-way ANOVA analysis, significant ecotype × watering regime, time × watering regime, ecotype × time and ecotype × watering regime × time interactions were observed for this parameter ($P < 0.05$).

For the four ecotypes, we noted significant differences ($P < 0.001$) in EWL and RTR in stressed plants of *A. spinosa* (medium and severe stress). We found that medium stress is able to induce some changes in EWL and RTR. Compared to control conditions, we recorded in stressed

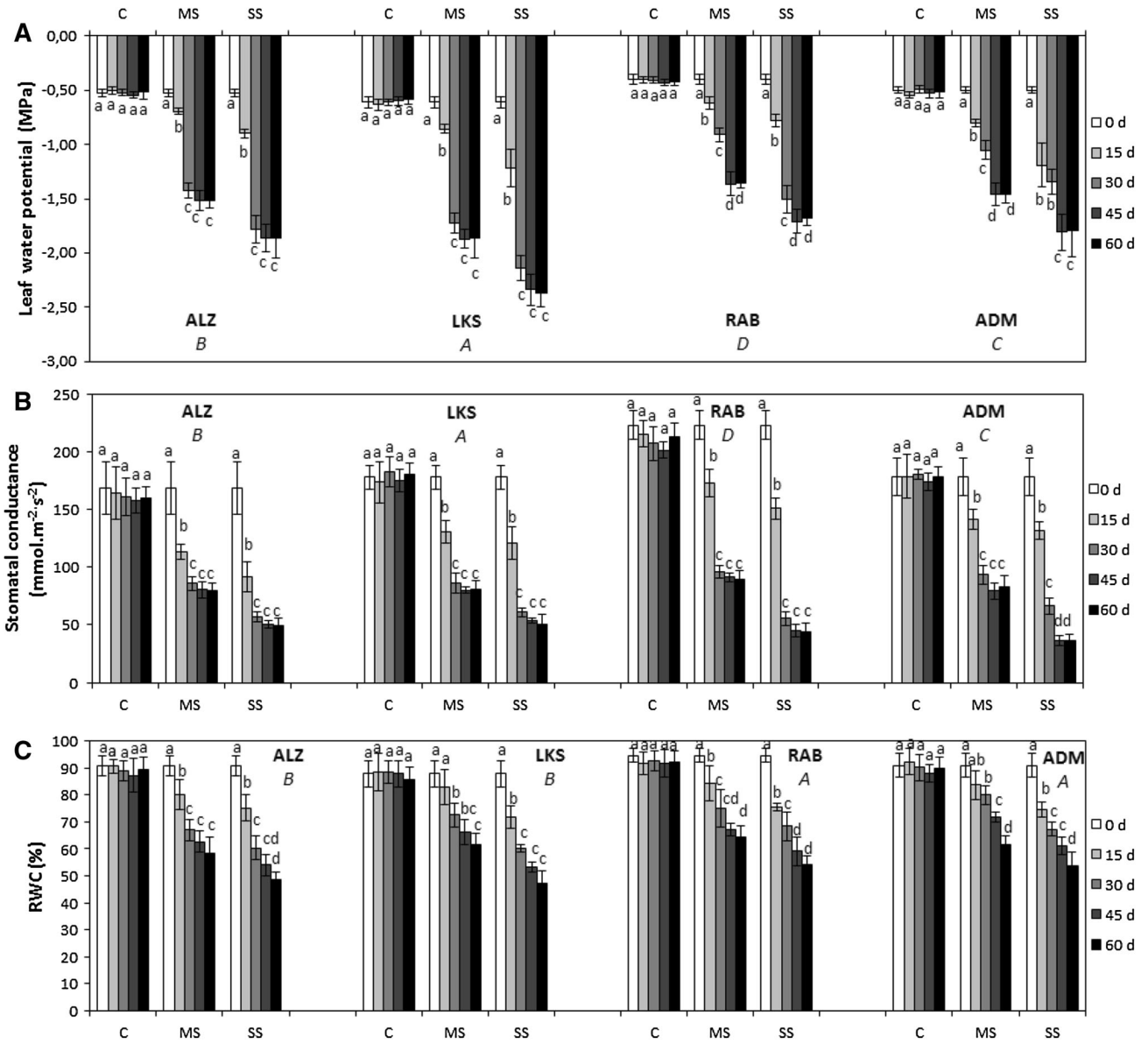

Fig. 1 Effect of drought stress on the predawn leaf water potential (Ψ_{pd}) (**a**), the stomatal conductance (g_s) (**b**) and RWC (**c**) of four *A. spinosa* ecotypes. Eighteen-month-old *A. spinosa* plants were exposed to three water regimes (100, 50 and 25 % of FC corresponding to *C* control, *MS* medium stress and *SS* severe stress, respectively) for 2 months. Values (means of five replicates ± SD) with distinct letters are significantly different at 5 % (Tukey's test). *Upper-case letters* (*A*, *B*, *C* and *D*) designate significant differences between ecotypes (*Alz* aoulouz, *Lks* lakhssas, *Rab* rabia and *Adm* admine)

plants higher values of EWL (Fig. 3a), however, those of RTR were lower (Fig. 3b). After 60 days of water drought treatment, Adm showed the smallest difference in RTR (51.8 %) and in EWL (21.2 %) compared to other ecotypes under severe stress. In contrast, Alz showed the greatest difference in RTR (36.5 % under severe stress), while both Rab and Alz again showed the greatest difference in EWL (32.6 and 30.4 % under severe stress, respectively). Nonetheless, we recorded higher levels of EWL in Lks. Significant differences ($P < 0.001$) in these two correlated parameters were reported between ecotypes and time effect was also significant ($P < 0.001$). EWL increase began from the first 15–30 days in all ecotypes, whereas the decrease in RTR began after 30 days in the coastal ecotypes and from the first 15 days in the inland ecotypes. A significant time × watering regime interaction was observed for these both parameters ($P \leq 0.05$), but ecotype x watering regime interaction was significant only for RTR. Negative and significant correlations ($P < 0.01$) were recorded between RTR and EWL during experiment ($r = -0.52$, $r = -0.76$, $r = -0.88$ and $r = -0.57$ in Lks, Alz, Rab and Adm, respectively).

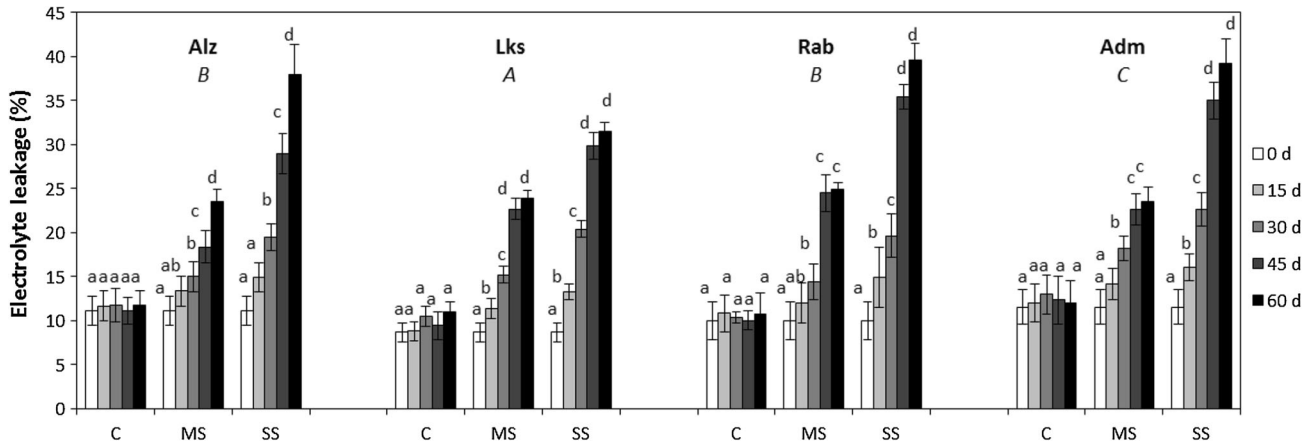

Fig. 2 Effect of drought stress on EL in four *A. spinosa* ecotypes. Eighteen-month-old *A. spinosa* plants were exposed to three water regimes (100, 50 and 25 % of FC corresponding to *C* control, *MS* medium stress, and *SS* severe stress, respectively) for 2 months. Values (means of five replicates ± SD) with distinct letters are significantly different at 5 % (Tukey's test). *Upper-case letters* (*A*, *B*, *C* and *D*) designate significant differences between ecotypes (*Alz* aoulouz, *Lks* lakhssas, *Rab* rabia and *Adm* admine)

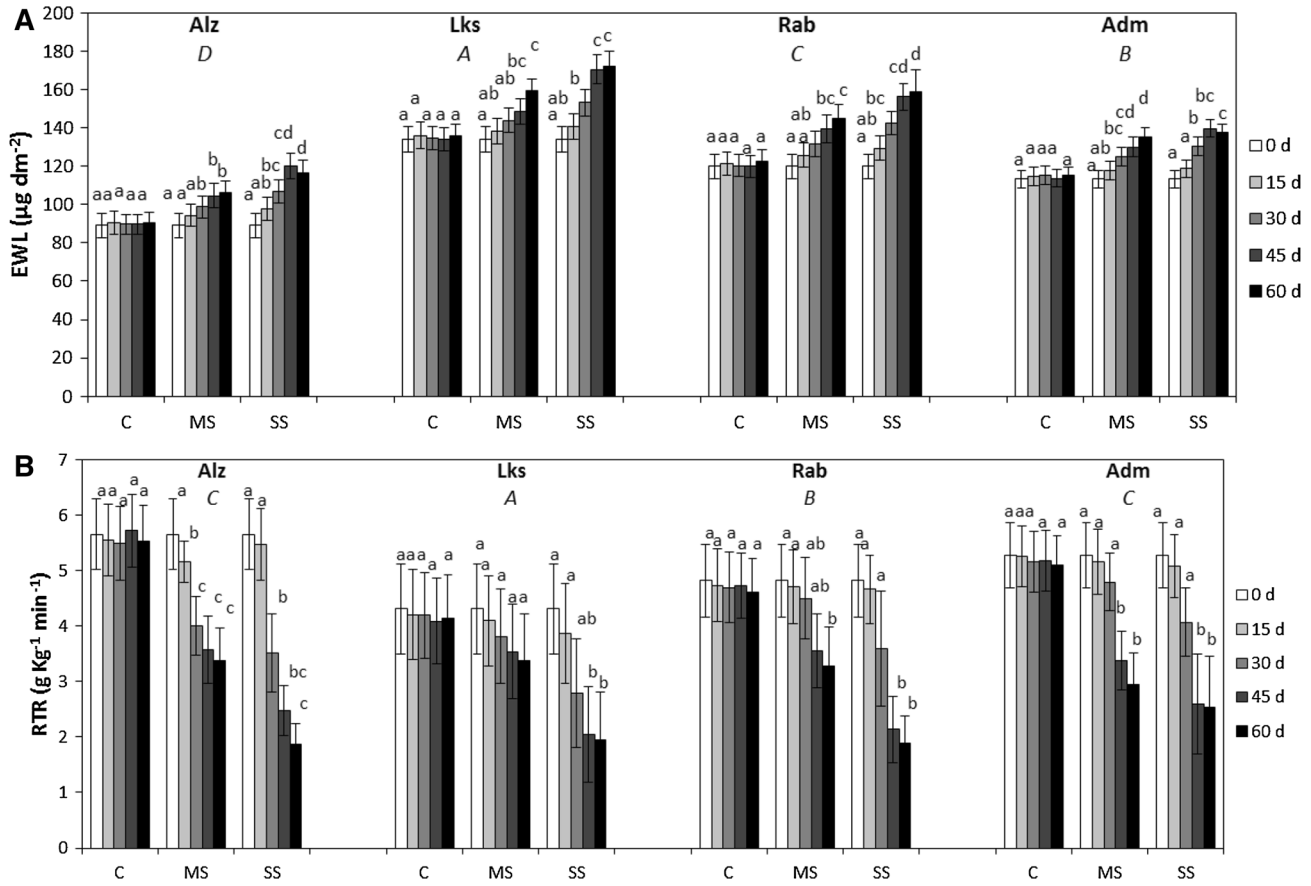

Fig. 3 Effect of drought stress on EWL (**a**) and RTR (**b**) in four *A. spinosa* ecotypes. Eighteen-month-old *A. spinosa* plants were exposed to three water regimes (100, 50 and 25 % of FC corresponding to *C* control, *MS* medium stress and *SS* severe stress, respectively) for 2 months. Values (means of five replicates ± SD) with distinct letters are significantly different at 5 % (Tukey's test). *Upper-case letters* (*A*, *B*, *C* and *D*) designate significant differences between ecotypes (*Alz* aoulouz, *Lks* lakhssas, *Rab* rabia and *Adm* admine)

Biochemical parameters

Changes in the polyphenol amount and SKDH, PAL and C4H activities of leaves from four contrasting ecotypes studied under drought stress are given in Fig. 4. The mean values for all ecotypes of total phenol content increased in parallel with the activity of the enzymes involved in flavonoid and phenylpropanoid synthesis in response to the increasing intensity of stress and prolongation of stress during drought period, presenting a significant difference compared to control ($P < 0.001$). Also, we recorded a significant ecotype effect for these biochemical parameters, and the stimulation of SKDH, PAL and C4H by drought stress was more pronounced in Lks (Fig. 4a–c). The polyphenol content in *A. spinosa* leaves was increased significantly in drought-stressed plants (Fig. 4d). We noted that medium stress can significantly stimulate the secondary metabolism in *A. spinosa*. During drought period and particularly under severe stress, Lks showed the greatest difference under severe drought stress in SKDH, PAL and C4H compared with well-watered conditions (29.2, 51.5 and 61.2 %, respectively), while Rab and Lks showed the greatest difference in polyphenols (19.9 and 19.3 %, respectively). Positive and significant correlations were recorded between SKDH, PAL and C4H activities and changes in polyphenol content during drought period. We found the following significant correlations at $P < 0.01$: between polyphenol content and SKDH activity ($r = 0.40$, $r = 0.80$, $r = 0.89$, and $r = 0.65$ in Lks, Alz, Rab and Adm, respectively), between polyphenol content and PAL activity ($r = 0.85$, $r = 0.63$, $r = 0.73$ and $r = 0.68$ in Lks, Alz, Rab and Adm, respectively) and between total phenol content and C4H ($r = 0.84$, $r = 0.96$, $r = 0.82$ and $r = 0.84$ in Lks, Alz, Rab and Adm, respectively). According to three-way ANOVA analysis, a significant time × watering regime interaction was observed for all activities of enzymes implied in shikimate and phenylpropanoid pathways in *A. spinosa* plants and polyphenol content ($P < 0.05$). Ecotype × watering regime interaction was only significant for activities of enzymes studied ($P < 0.05$), while ecotype x time interaction was only significant for SKDH and C4H activities ($P < 0.05$). Ecotype × watering regime × time interaction was not significant for all these parameters except for SKDH ($P < 0.05$).

Compared to control, the stressed plants showed a significant increase of proline and soluble sugars amounts in the leaves under both treatments (Fig. 5). We also recorded significant differences in terms of the content of these both parameters among ecotype, watering regime and time. During drought stress treatment period, the highest proline accumulation was registered in Lks ecotype (80 and 48.5 % under severe and medium drought stress, respectively) (Fig. 5a). Soluble sugars tend to significantly accumulate in the water-stressed *A. spinosa* plants, of which the highest accumulation was recorded in Rab and Lks again (44.3 % in both ecotypes under severe, and 32.1 and 30.3 % under medium drought stress, respectively) (Fig. 5b). Significant ecotype x watering regime and time × watering regime interactions were observed for these both parameters ($P \leq 0.05$), but ecotype x time and ecotype × watering regime × time interactions were significant only for proline.

Canonical discriminant analysis

A canonical discriminant analysis (CDA) was performed using on the one hand the physiological parameters (5 variables) (CDAp) and on the other hand the biochemical parameters (7 variables) (CDAb). Then, another CDA was established for all of the physiological and biochemical parameters (12 variables) (CDApb) as predictors of membership in a diagnostic group. This group corresponded to the four contrasting ecotypes of argan tree studied in our experiment. The results obtained from CDA affirmed the existence of differences in global characteristics of studied ecotypes. Wilks's lambda denoted a high significance of the model (Wilks's $\lambda = 0.44$ for CDAp, Wilks's $\lambda = 0.028$ for CDAb and Wilks's $\lambda = 0.013$ for CDApb) and calculated F value also indicates significance ($P < 0.0001$) for these analyses. Three discriminant functions (DF) were calculated, accounting for 71.2, 23.4 and 5.3 % of the total variance for CDAp, 75.1, 15.5 and 9.4 % for CDAb and 72.8, 16.8 and 10.4 % for CADpb, respectively. The null hypothesis of discriminant functions is tested using χ^2 test. Indeed, the χ^2 test showed for the three analyses a significant discriminatory power for the three functions ($P < 0.001$). For CDAp, the eigenvalues of the first two functions (0.74 and 0.24, respectively) showed them to explain most of variance (94.7 %) and their canonical correlations (correlation between the discriminant scores and the levels of the dependent variable) were $r_1 = 0.65$ and $r_2 = 0.44$. The high correlation indicates a function that discriminates well. Concerning CDAb, the eigenvalues of the first two functions (6.91 and 1.42, respectively) showed them to explain most of variance (85.8 %) and their canonical correlations were $r_1 = 0.94$ and $r_2 = 0.77$. The eigenvalues of the first two functions (9.22 and 2.13, respectively) for CDApb showed them to represent a total variance of about 89.6 % and their canonical correlations were $r_1 = 0.95$ and $r_2 = 0.83$.

The 2D scatter plots of discriminant space (Figs. 6, 7, 8) display the distribution of the samples spanned by the first two functions. We chose different shapes to distinguish between the four ecotypes. Based on the standardised coefficients of the canonical discriminant functions of CDAp (Fig. 6), Ψ_{pd} and g_s were highly weighted in the positive

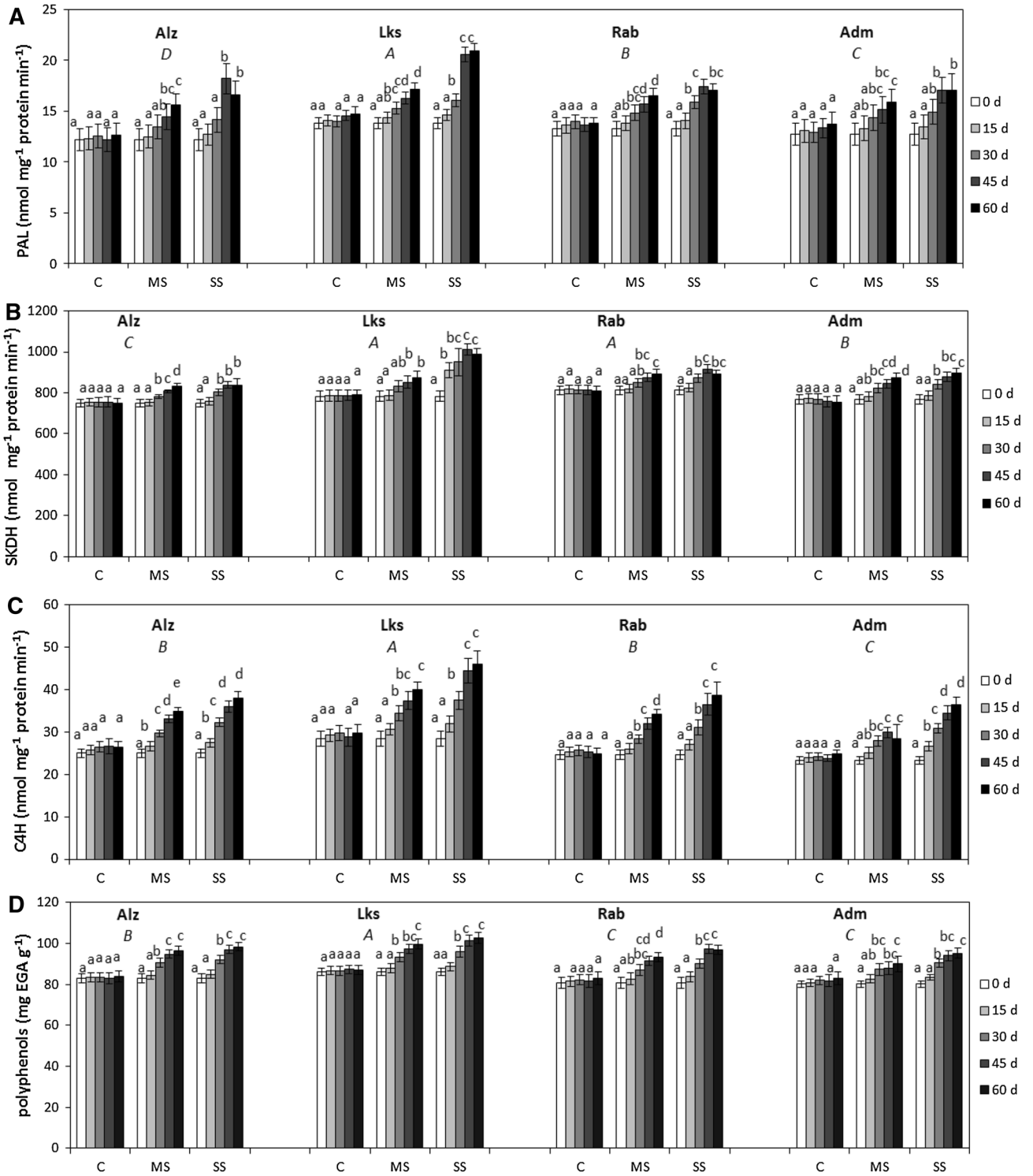

Fig. 4 Effect of drought stress on PAL activity (**a**), SKDH activity (**b**), C4H activity (**c**) and polyphenol content (**d**) in four *A. spinosa* ecotypes. Eighteen-month-old *A. spinosa* plants were exposed to three water regimes (100, 50 and 25 % of FC corresponding to *C* control, *MS* medium stress and *SS* severe stress, respectively) for 2 months. Values (means of five replicates ± SD) with distinct letters are significantly different at 5 % (Tukey's test). *Upper-case letters* (*A*, *B*, *C* and *D*) designate significant differences between ecotypes (*Alz* aoulouz, *Lks* lakhssas, *Rab* rabia and *Adm* admine)

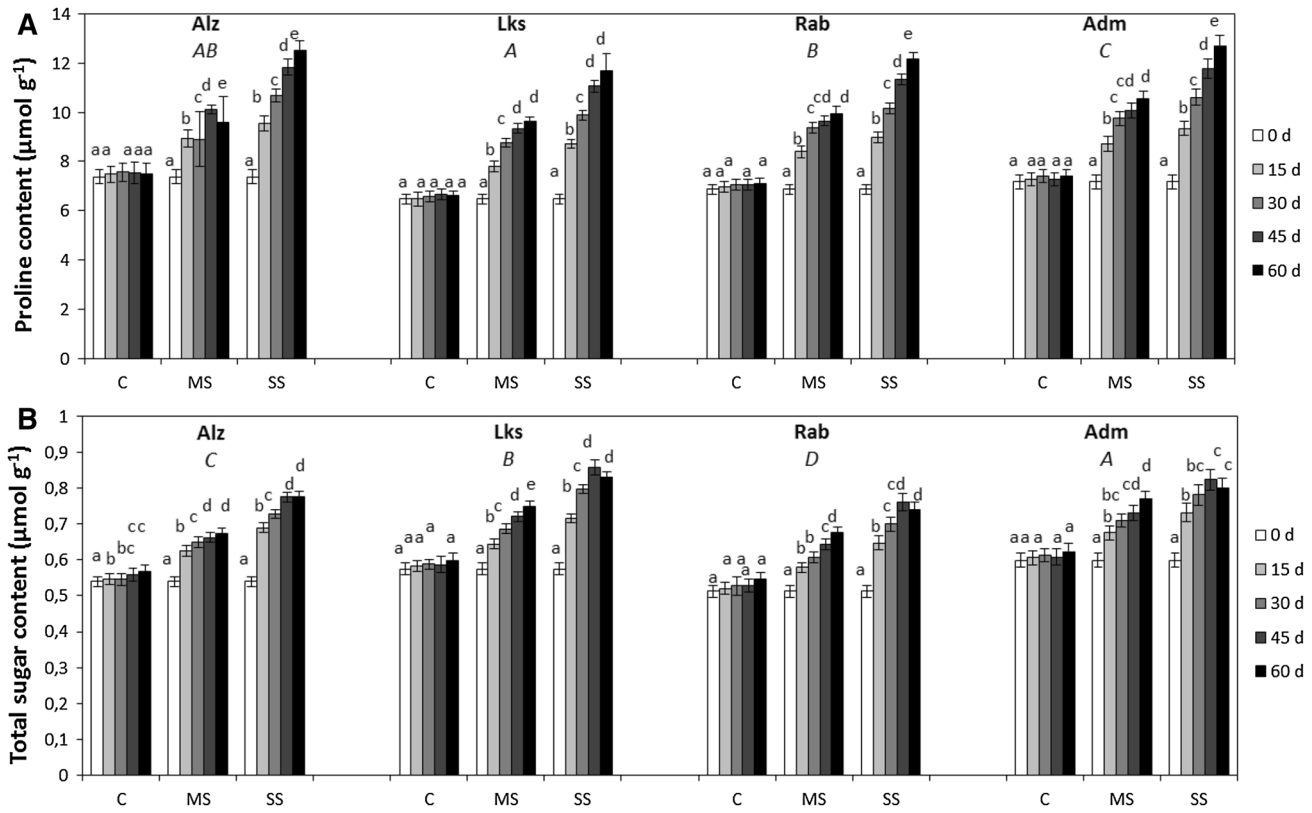

Fig. 5 Effect of drought stress on proline content (**a**) and total sugar content (**b**) in four *A. spinosa* ecotypes. Eighteen-month-old *A. spinosa* plants were exposed to three water regimes (100, 50 and 25 % of FC corresponding to *C* control, *MS* medium stress and *SS* severe stress, respectively) for 2 months. Values (means of five replicates ± SD) with distinct letters are significantly different at 5 % (Tukey's test). *Upper-case letters* (*A*, *B*, *C* and *D*) designate significant differences between ecotypes (*Alz* aoulouz, *Lks* lakhssas, *Rab* rabia and *Adm* admine)

part of CDAp-1 while EL and RTR in the negative part. g_s and EL were highly weighted in the positive part of CDAp-2 whereas RTR was again the physiological characteristic most strongly weighted to negative part of CDAp-2. Lks was clearly distinguished from other ecotypes by the first DF, while Rab was distinguished from other ecotypes by the second DF. For CDAb, a scatter plot (Fig. 7) relative to two discriminant functions shows a good separation among ecotypes. EWL and polyphenol content were highly weighted in the positive part of CDAb-1 and soluble sugars content and C4H activity in the negative part. C4H activity and total phenol content showed highest standardised coefficients on the second DF in the positive part, while soluble sugars content, PAL activity and EWL in the negative part. Indeed, the first DF contributed mostly to distinguish between (Lks–Rab) and (Alz–Adm). However, Adm was clearly distinguished from other ecotypes by the second DF. Equal numbers of plants were compared in each ecotype. The scatter plot of CDApb illustrated the separation of the four ecotypes taking into account all parameters studied (Fig. 8). As the CDAb, EWL and polyphenol content were highly weighted in the positive part of CDApb-1, however, EL and soluble sugars content exhibited highest standardised coefficients in the negative part. Soluble sugars content and EL were highly in the positive part of CDApb-2 whereas C4H activity and polyphenol content were highly weighted to negative part of CDApb-2. The CDAb, Lks and Rab ecotypes were clearly distinguished from the other ecotypes by the first DF, while the both inland ecotypes were little distinguished from the both coastal ecotypes by the second DF.

Discussion

Drought stress is one of the most important environmental factors limiting growth, development and yield in arid and semi-arid areas. As is generally known, drought is a complex physical–chemical–biological process. Water availability significantly influenced physiological and biochemical parameters studied. *Argania spinosa* plants' responses to water deficit were dependent on the amount of water lost and the duration of the stressed conditions. According the three-way ANOVA test for the factors fixed in our experimental design (time, watering regime and

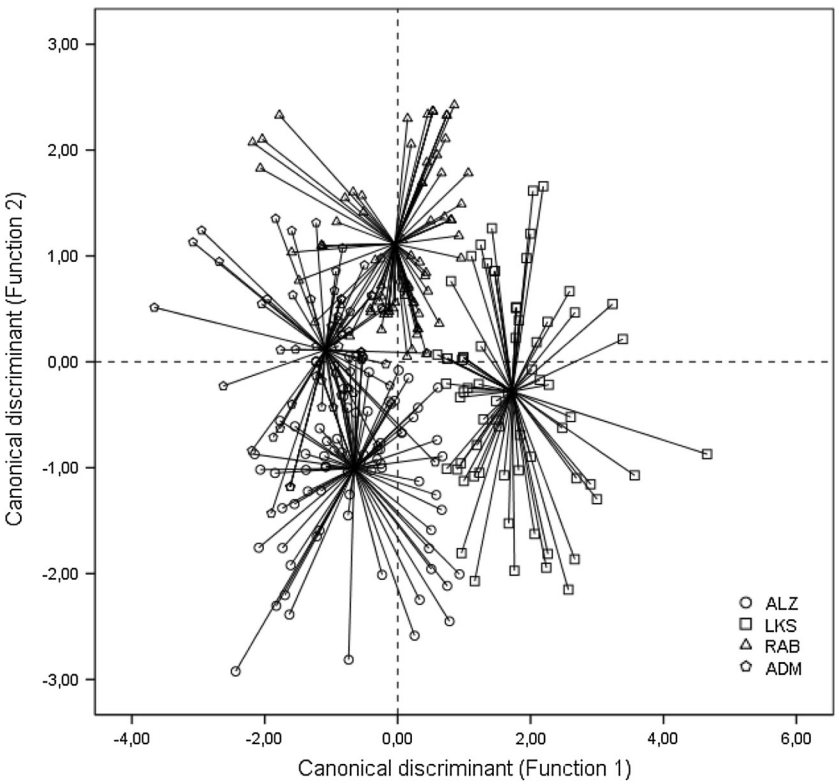

Fig. 6 2D *scatterplot* showing the separation of the study ecotypes according to the two discriminant function gradients obtained by CDA for physiological parameters

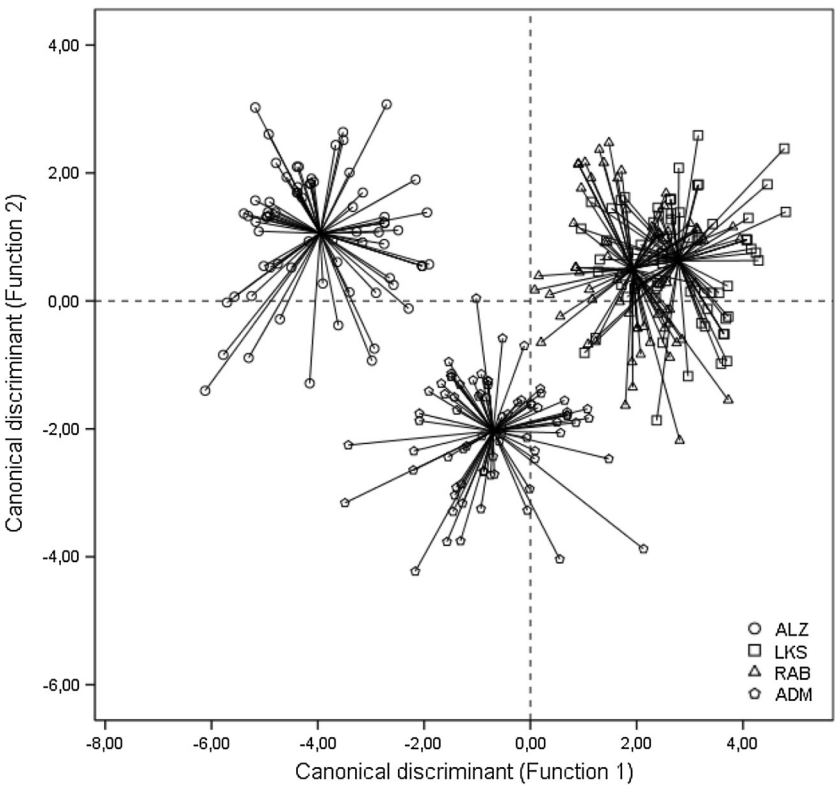

Fig. 7 2D *scatterplot* showing the separation of the study ecotypes according to the two discriminant function gradients obtained by CDA for biochemical parameters

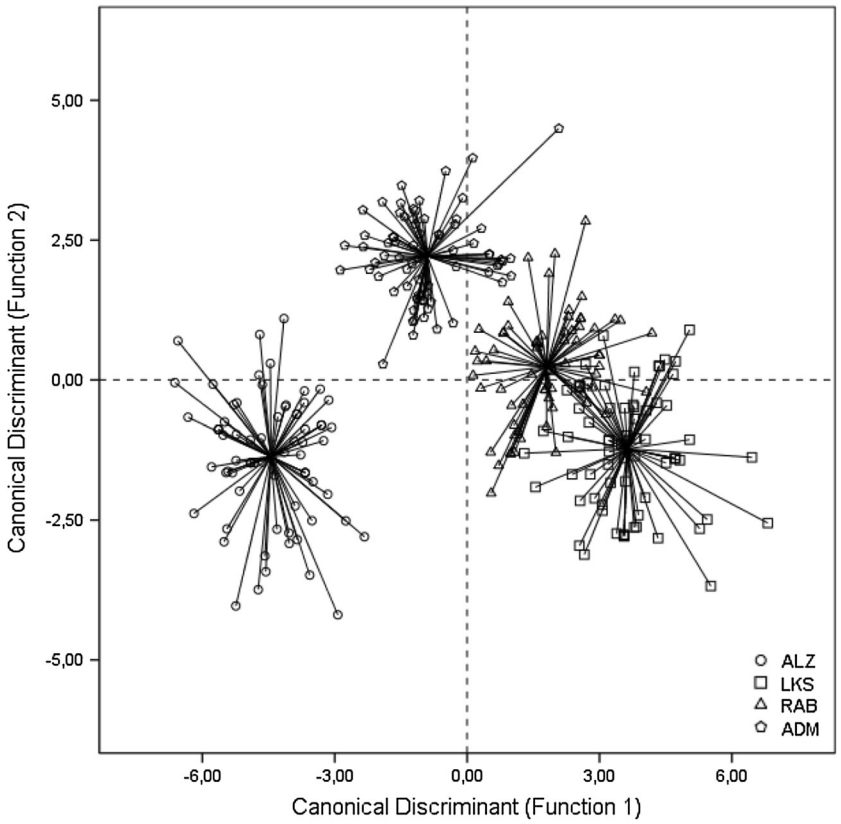

Fig. 8 2D *scatterplot* showing the separation of the study ecotypes according to the two discriminant function gradients obtained by CDA for physiological and biochemical parameters

ecotype) and their interactions, we noted differential responses of the parameters studied in *A. spinosa*. The reduction in Ψ_{pd}, g_s and leaf RWC (Fig. 1) has already been observed and has been considered a clear response to drought. In our study, we found a marked relationship between Ψ_{pd} and g_s and between g_s and RWC in which stomatal closure was very effective in preventing water loss in all ecotypes studied. In fact, the changes in water potential and/or soil water content were demonstrated to cause significant stomatal closure (Pita et al. 2005). As the stress becomes more severe, the Ψ_{pd} decreased in stressed *A. spinosa* plants and these plants showed the stomata more closed. This agrees with the results of several studies in which qualitative relationships were described between Ψ_{pd} and stomatal closure (Gomes et al. 2004; Wahbi et al. 2005). Furthermore, leaf RWC is considered as an indicator to evaluate plant water status and resource use measures (Teulat et al. 1997; Garnier et al. 2001). Under drought stress conditions, a decrease in RWC has been recorded in various varieties of plants (Nayyar and Gupta 2006). During moderate and severe stress, the effect of the drought stress revealed significant effects on the four ecotypes. In terms of comparison, the Ψ_{pd} of inland ecotypes decreased more under drought stress than did the coastal ecotypes. There were clear intraspecific differences in stomatal sensitivity, suggesting different adaptations to drought related to the ecotype effect. Rab ecotype had high g_s, whereas Lks and Alz seem to have the most conservative water-use characteristics, since both inland ecotypes were having lower maximum stomatal conductance (Fig. 1b).

Compared to control, we noted significant increase induced in electrolyte leakage in stressed plants as shown in (Fig. 2). The increases in electrolyte leakage reflecting membrane intactness in *A. spinosa* plants under drought stress suggested that water-stressed plants encountered cellular damage. Quan et al. (2004) also found higher EL in maize (*Zea mays* L.) plants under drought stress than in plants grown under well-watered conditions. The increase in cell membranes leakiness would be interpreted as an injury and loss of membrane integrity related to chain reactions induced by reactive oxygen species (ROS). Lipid peroxidation is one of these reactions, which causes membrane injury (Sairam et al. 2005; Lei et al. 2007) and this might accelerate the senescence processes (Thompson 1988). Our results showed that membrane integrity of Lks was more conserved compared to other ecotypes and drought stress-induced membrane injury, especially in Adm ecotype.

Significant differences were recorded in the EWL and the RTR of the studied *A. spinosa* ecotypes. For the whole range of ecotypes, the EWL values increase while those of RTR decrease under drought stress treatments. We obtained negative and significant correlations between RTR

and EWL suggesting a relationship between them. Similar results were reported between both parameters in sorghum (Jordan et al. 1984) and in wheat (Premachandra et al. 1992). Epicuticular waxes enhance the plant resistance to environmental stresses helping leaves in retention of water (Goodwin and Jenks 2005). This explains the lower values of RTR recorded in *A. spinosa* ecotypes studied. Previous studies had shown that the concentration of wax deposited on leaf surfaces increases significantly under drought stress in various plants, including rose (Jenks et al. 2001), peanut (Samdur et al. 2003), tree tobacco (Cameron et al. 2006), sesame (Kim et al. 2007a, b) and barley (González and Ayerbe 2010). Its synthesis stimulated by drought can present a mechanism of resistance and protection from damages, allowing plants to boost water retention and ensure its growth by developing water conservation measures in stressed conditions (Zhang et al. 2005; Goodwin and Jenks 2005; Seo and Park 2011). The expression of genes involved in regulating both pathways of biosynthesis, and transport of cuticular wax was detected in some transgenic plants under drought stress (Seo and Park 2011). Yang et al. (2011) showed that changes in the expression of these genes expect to influence the development of stomata and improve drought tolerance. Changes in the values of these two parameters that interfere with each other (increase of EWL and decrease of RTR) have revealed good tolerance to drought in barley plants (González and Ayerbe 2010). Furthermore, the concentration of cuticular wax deposition on leaves was often significantly related to the transpiration rate and the degree of drought tolerance (Zhang et al. 2005; Kim et al. 2007a, b; González and Ayerbe 2010).

Polyphenol contents were increased in parallel with the activities of enzymes implied in flavonoid and phenylpropanoid synthesis by drought stress in all ecotypes of *A. spinosa*. This result shows that drought stress can induce shikimate and phenylpropanoid pathways in *A. spinosa* plants. Generally, biotic and abiotic stress promote the synthesis of polyphenols, involved in plants protection mechanism (Rodziewicz et al. 2014). Many studies have reported that the polyphenol can increase in different organs and tissues of plants under drought stress (Boughalleb and Mhamdi 2011; Sánchez-Rodríguez et al. 2011; Bettaieb et al. 2010; Lee et al. 2007; Parida et al. 2007). In the present study, the ecotypes differed in these secondary compounds accumulation induced by water drought stress. The biosynthesis of these compounds would be key in ensuring protection and prevention against damage caused by drought stress. The enhanced level of polyphenols may be beneficial to enhance the degree of drought tolerance in *A. spinosa* ecotypes studied. The polyphenols have been considered pertinent in oxidative stress tolerance by neutralising ROS. Indeed, some phenolic compounds have the ability to reduce fluidity of the membranes by altering peroxidation kinetics in cell membrane (Arora et al. 2000). Steric effect of these phenolic compounds could limit diffusion of ROS and restrict peroxidation reaction. Indeed, the polyphenols have been demonstrated to ensure effective stress acclimatisation in plants (Parida et al. 2007; Rivero et al. 2001). Constitutive and drought-induced expression profiles of genes implied in polyphenol biosynthesis were coordinated and correlated with the polyphenol levels (André et al. 2009). We found positive and significant correlations between SKDH, PAL and C4H activities and changes in polyphenol contents during drought period. Indeed, in our experiment, an increase of polyphenols significantly correlated to the increase in activity of enzymes involved was recorded in the four contrasting ecotypes of *A. spinosa*, suggesting de novo synthesis of polyphenols under drought stress. Rivero et al. (2001) indicated a positive link between PAL activity and soluble phenolic concentration in tomato and watermelon species, while also suggesting a determining role played by activating the PAL enzyme in relation to the accumulation of phenolic compounds in response to heat and cold stressors. Supporting our results, previous studies have demonstrated that the stress adaptation involves an intensified activity of PAL in some plants submitted to drought stress including cherry tomato (Sánchez-Rodríguez et al. 2011) and lettuce (Oh et al. (2009). PAL and C4H are involved in the biosynthesis of various important secondary metabolites from phenylalanine as a carbon source (Singh et al. 2009). Under drought stress, *A. spinosa* plants showed a significant increase in SKDH activity as that recorded in PAL and C4H. SKDH catalyses the reversible reduction of dehydroshikimiate to shikimic acid, which was demonstrated to be activated in some cherry tomato cultivar under water-stress conditions (Sánchez-Rodríguez et al. 2011). The phenylpropanoid pathway is associated to the shikimate pathway via phenylalanine. Under drought stress, these enzymes are involved in the regulation of accumulation of polyphenols which are very abundant in *A. spinosa*. In drought-stressed plants of cotton (*Gossypium hirsutum*), Parida et al. (2007) observed a significant increase of polyphenols content in their leaves. The polyphenol level was considerably more elevated in the tolerant genotype than in its sensitive counterpart. Such results may indicate the involvement of polyphenols in osmotic potential maintenance, as well as in scavenging free radicals under drought stress.

The PRO and soluble sugars content in leaves of all ecotypes increased substantially in parallel with the increase of stress severity. Accumulation of PRO and soluble sugars in Lks leaves was higher as compared to others ecotypes. Proline was viewed to be an important inert compatible osmolyte protecting cellular structures under osmotic stress (Kavi-Kishor et al. 2005). Proline

accumulation constitutes a notable indicator of drought tolerance in higher plants. Numerous studies have reported that the proline content increases during drought in various plants including olive tree (Boughallleb and Mhamdi 2011) and populous (Yin et al. 2005). Proline's function as molecular chaperone protecting protein integrity has been demonstrated, as it has ability to enhance the activities of different enzymes. Various studies attribute to proline a significant antioxidant capacity, which in turn implies ROS scavenging activity, with proline serving as singlet oxygen quencher (Matysik et al. 2002; Szabados and Savoure 2009). Also, the accumulation of soluble sugar is strongly correlated to the acquisition of drought tolerance in plants. In our study, the highest accumulation was recorded in Lks which suggests to be associated with drought tolerance. Soluble sugar compounds have been reported to be both an osmolyte by balancing the osmotic adjustment and an osmoprotectant (Ashraf and Harris 2003). Accumulation of soluble sugar compounds protects membranes and proteins in cells exposed to stress that caused water deficit and reduces aggregation of denatured proteins (Ashraf and Harris 2003). Gibson (2005) indicated that the sugar flux may act as a signal for metabolic regulation. Also, sugars have been suggested to enhance oxidative burst, increasing lignification of cell walls and stimulate the synthesis of flavonoids (Morkunas and Ratajczak 2014).

The 2D scatter plots of discriminant space relative to two discriminant functions of physiological and biochemical parameters are presented in (Figs. 6, 7, 8). These scatter plots show a good separation among study ecotypes of *A. spinosa*. Concerning CDAp (Fig. 6), the horizontal separation was characterised in the first DF. Thus, first DF quantifies the degree to which all ecotypes differ in physiological parameters, which we argue to be the result of differences in their adaptation and tolerance under drought stress. Lks was mainly separated from all other ecotypes by a lower Ψ_{pd}. Taking account of the second DF, the both inland ecotypes: Lks and Alz were slightly distinguished from both coastal ecotypes by a lower electrolyte leakage. For CDAb (Fig. 7), differences in biochemical parameters studied were also evident between ecotypes under drought stress. The first DF revealed that Lks and Rab ecotypes were clearly separated from the other ecotypes by higher EWL accumulation, confirming the high values of EWL recorded in these both ecotypes. The second DF showed that Adm ecotype was mainly separated from all other ecotypes by its lower soluble sugar amount, suggesting that this ecotype has a low osmoregulation compared with the other ecotypes. By analysing all the parameters studied and their respective effects on the discrimination between the four ecotypes, we found that EWL, polyphenol content, EL, soluble sugars and C4H activity could be suggested as selective traits for drought tolerance in *A. spinosa*. These physiological and biochemical parameters could be used as indirect selection traits to select the tolerant ecotypes. According to the results of CDA for the four contrasting ecotypes, we proposed the following order in terms of drought tolerance: Lks > Alz > Rab > Adm.

Conclusion

In summary, an integrated approach reflecting a combination of physiological and biochemical aspects has been adopted in our work to investigate the drought stress responses of *A. spinosa*. Intraspecific differences were recorded in osmoregulation, secondary metabolism and leaf water status between *A. spinosa* ecotypes studied. The present results suggest that genetic influence is very obvious, because all were in the same environmental conditions within the growth chamber, thus, the observed variations in physiological and biochemical responses could be linked to genetic differences. Based on our research results from this preliminary pot-study, it is clear shown that the ability of drought tolerance in the four contrasting ecotypes studied is different, and we can suggest the following order according to the ability of drought tolerance: Lks > Alz > Rab > Adm. Other research with variations in photosynthetic performance and carbon metabolism may give a more exhaustive picture of the response of *A. spinosa* against drought stress. However, these results require confirmation and validation by field study in order to determine the effects of natural environment conditions.

Author contribution statement Abdelghani Chakhchar designed the study, performed the statistical analyses and drafted the manuscript. Said Wahbi and Mouna Lamoui helped in designing the study and writing the manuscript. Abderrahim Ferradous contributed in seed collection and preparation of plant material. Abdelhamid El Mousadik, Saad Ibnsouda-Koraichi, Abdelkarim Filali-Maltouf and Cherkaoui El Modafar coordinated the study and supervised the research project. All the authors read, corrected and approved the manuscript in its final form.

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