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Differential physiological and antioxidative responses to drought stress and recovery among four contrasting *Argania spinosa* ecotypes

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ABSTRACT

Our study was undertaken to ascertain whether the change of the water status and the activation of superoxide dismutase and their isoenzymes in Argan tree can support edaphic drought tolerance and its recovery under rehydration. An experiment was conducted on four contrasting ecotypes of *Argania spinosa* plants: two contrasting coastal ecotypes (Admine (Adm) and Rabia (Rab)) and two contrasting inland ecotypes (Aoulouz (Alz) and Lakhssas (Lks)). Drought stress significantly decreased the leaf water potential and stomatal conductance in the four contrasted ecotypes. In terms of biochemical responses, significant accumulation of carbonyl groups, hydrogen peroxide and superoxide radical has been recorded in the leaves of stressed plants reflecting oxidative stress. In parallel, the activities of total superoxide dismutase (SOD) and their isoenzymes Cu/Zn-SOD, Cu/Zn-SOD and Fe-SOD were also found to have increased to scavenging ROS and protecting the cell against induced oxidative stress. The recovery kinetics of *A. spinosa*, as a response to rehydration, were significant and rapid. According to the traits having the most discriminating power, both inland ecotypes (Lks and Alz) showed a better upregulation of its protective mechanisms compared to coastal ecotypes (Rab and Adm). All these adaptive traits make the inland ecotypes as an elite resource of drought tolerance and might become the new focus of domestication research of argan tree in arid and semi-arid environments.

ARTICLE HISTORY

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KEYWORDS

Argan tree; drought stress; oxidative stress; rehydration; tolerance

Introduction

Drought is the most important limiting factor for agricultural production. It becomes an increasingly serious problem in many parts of the world and urgent for the international community (Passioura 2007). Despite an extensive literature on the topic, there is controversy regarding the mechanisms of tolerance to drought in plants. Responses to drought are multiple and interconnected. It has been established that drought stress induces a certain number of physiological, biochemical and molecular reactions that regulate plant growth and productivity (Yoon et al. 2014). However, this abiotic stress impairs numerous metabolic and physiological processes in plants. Drought stress can cause increased generation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-), the hydroxyl radical (OH) and the hydrogen peroxide (H_2O_2) (Mittler 2002; Gill & Tuteja 2010). These ROS generate the oxidation of photosynthetic pigments, membrane lipids, proteins and nucleic acids and thereby cause damage to cell structures and metabolism particularly that associated with photosynthesis (Smirnov 1993; Yordanov et al. 2000; Møller et al. 2007). Therefore, the plants have developed several protection mechanisms in order to prevent the damaging effect of ROS by a stimulation of an effective antioxidant defense system. Our earlier studies have shown that drought stress induces in argan tree,

[*Argania spinosa* (L.)] Skeels, a strong induction of both enzymatic and non-enzymatic defense systems that act as ROS scavengers either in conjunction or independently, suggesting a better ability of drought tolerance (Chakhchar et al. 2015a). Plant enzymatic antioxidant mechanisms include superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione peroxidase, guaiacol peroxidase and glutathione S-transferase. The metalloenzyme superoxide dismutase (SOD) is the most effective antioxidant intracellular enzyme which is ubiquitous in all aerobic organisms and in all the subcellular compartments subject to the ROS generated by the oxidative stress. It is well established that various environmental stresses often lead to increased generation of ROS, where SOD has been proposed to play an important role in stress tolerance in plants. It is the first line of defense against the toxic effects of high levels of ROS (Mittler 2002; Gill & Tuteja 2010). Its structure allows distinguishing three isoenzymes: Mn-SOD, Cu/Zn-SOD and Fe-SOD. In addition to that, the indices characterizing the water status in plants, especially the leaf water potential and stomatal conductance are considered the most important adaptive traits involved in physiological tolerance to drought (Chaves et al. 2003). *A. spinosa* proved to tolerate drought stress by effectively controlling water loss mainly through stomatal closure and

improving water use efficiency (Diaz Barradas et al. 2010, 2013; Chakhchar et al. 2015b, 2015c). Diaz Barradas et al. (2013) also reported that the leaf conductance response of *A. spinosa* to drought environment seems to depend on the variation of vapour pressure deficit (VPD). In fact, plants of *A. spinosa* adapt to drought by down-regulation of several physiological/biochemical processes, thereby maintaining growth even under severe drought stress (Chakhchar et al. 2015a, 2015b, 2015c).

Drought treatment reveals different physiological and biochemical responses in *A. spinosa* with regard to intensity and duration of stress duration, as well as ecotype effect (Chakhchar et al. 2015a, 2015b, 2015c). The argan tree is endemic to the Southwestern part of Morocco, where it grows in over 800,000 hectares (Msanda et al. 2005). *A. spinosa* species belongs to the Mediterranean–Saharan transition zone (McGregor et al. 2009). This tree has important socio-economic and ecological roles in this area, in which it also plays a great role in the biodiversity of the forest's ecosystem (Msanda et al. 2005). It is potentially a very important tree species for vegetable oil (Ait Aabd et al. 2014), extracted from the kernels of argan seeds, which could generate a great interest from the horticultural industry. Therefore, for a better understanding of how *A. spinosa* ecotypes differ in their tolerance to drought stress, measurements at physiological and biochemical levels are required. Moreover, analyses carried out during a period of rehydration could better elicit the tolerance mechanisms of *A. spinosa* plants suffering a drought stress and highlight subtle differences among the studied ecotypes. The quick recovery of some physiological and biochemical functions under rehydration seems to be

an important factor of ecotype classification on the basis of drought tolerance.

The present study was planned (i) to identify the effect of both drought stress and rehydration on some physiological and biochemical mechanisms by some adaptive traits associated with better drought tolerance among *A. spinosa* ecotypes, (ii) to characterize the oxidative stress in the argan by assessing oxidative damage and (iii) to determine indices that can be used as markers for discriminating tolerant ecotypes.

Materials and methods

Plant material and experimental design

Sampling of seeds of *A. spinosa* was conducted in four regions of the argan tree forest in South-West Morocco (Figure 1). We chose two contrasting coastal ecotypes (site: Rabia (Rab) and Admine (Adm)) and two contrasting inland ecotypes (site: Aoulouz (Alz) and Lakhssas (Lks)) for a better interpretation of the mechanisms regulating biochemical and physiological processes. Climatic, geographical and hydrological conditions of these four regions are markedly different, where the average annual rainfalls (mm) in Rab, Adm, Alz and Lks sites are around 295, 225, 232 and 189, respectively. The mean minimum temperature (°C) is about 9.6, 7.2, 5.6 and 7.3 in Rab, Adm, Alz and Lks sites, respectively, while the mean maximum temperature (°C) is about 22.2, 27.1, 35.7 and 31.2, respectively (Chakhchar et al. 2015a, 2015b).

The protocol of cultivation and experimental layout was the same as that used previously (Chakhchar et al. 2015a,

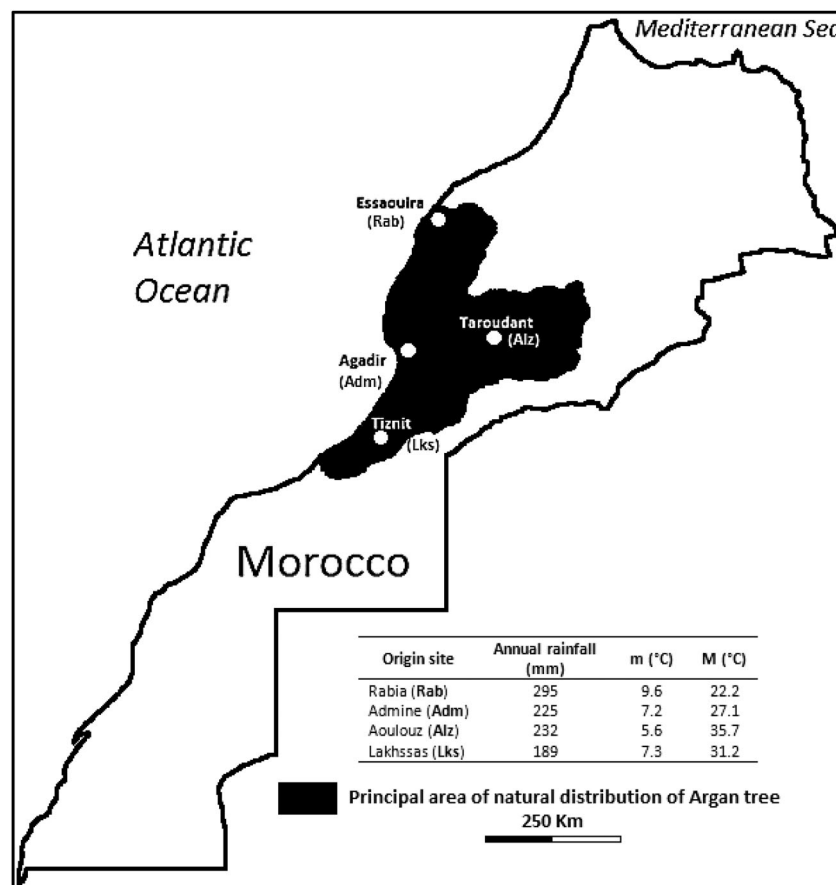


Figure 1. Biogeographical distribution of the four studied *A. spinosa* ecotypes. Rab, Adm, Alz and Lks ecotypes from Essaouira, Agadir, Taroudant and Tiznit provenances, respectively.

2015b, 2015c). Uniform young *A. spinosa* plants of similar height, aged 29 months, were selected for the experiment for each ecotype. We simulated the effect of prolonged drought stress by cessation of irrigation for 15 and 30 days followed by rehydration for 4 days. For the control and rehydrated plants, the soil humidity was maintained at the level of 75% field water capacity. 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 rate of relative humidity was varied between 65% and 70%. The average maximum photosynthetically active radiation (PAR) was approximately $400 \mu\text{mol m}^{-2}\text{s}^{-1}$ ensured by a combination of incandescent and fluorescent lamps.

Physiological traits

Leaf water potential (Ψ_{pd})

The predawn leaf water potential (Ψ_{pd}) was measured with a pressure chamber (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 (g_s)

g_s was measured using a portable steady-state diffusion porometer (Model Sc-1, Decagon Devices, Pullman, USA). 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).

Biochemical traits

For the biochemical measurements, samples of fully expanded young leaves (upper portion of the stem) from control and stressed plants were immediately lyophilized and ground to a fine powder in the presence of liquid nitrogen. According to Bradford (1976), total soluble protein content for the determination of the specific activities of the enzymes was determined using bovine serum albumin (BSA) as a standard.

Activity of total superoxide dismutase and its isoenzymes

Total superoxide dismutase activity was evaluated by monitoring its ability to inhibit the photochemical reduction of p-nitrobluetetrazolium chloride (NBT) at 560 nm according to the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.8), 0.1 mM Ethylenediaminetetraacetic acid (EDTA), 33 μM NBT, 10 mM L-Methionine, 4 μM riboflavin and enzyme extract. Reactions were carried out for 8 min at a light intensity of $50 \text{ mmol photons m}^{-2}\text{s}^{-1}$. To distinguish SOD isoforms the sensitivity of Cu/Zn-SOD and Fe-SOD to H_2O_2 (5 mM) and Cu/Zn-SOD to potassium cyanide (KCN) (3 mM) was used, whereas Mn-SOD is unaffected (Sairam et al. 2009). A complete reaction mixture without enzyme served as a blank. Reaction was stopped by switching off the light and the absorbance. One unit of SOD activity was defined as the amount of enzyme, which causes 50% inhibition of NBT reduction under the assay condition, and the results were expressed as enzyme units (nmol/min) per mg

of protein. There were five replicates per treatment (one plant per replicate).

Superoxide radical content ($\text{O}_2^{\cdot-}$)

The assay was based on the capacity of the extracts to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of the riboflavin–light–NBT system (Beauchamp & Fridovich 1971). The method used by Chaitanya and Naithani (1994) was followed after some modifications. The finely ground powder (100 mg) was homogenized in 50 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.8) containing 10 μM diphenyleneiodonium chloride (DPI; to inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent $\text{O}_2^{\cdot-}$ generation) and 1 mM diethyldithiocarbamate (DEDC; to inhibit SOD activity). The homogenate was centrifuged at $10,000\times g$ for 15 min and supernatant was immediately used for the estimation of $\text{O}_2^{\cdot-}$. The assay mixture contained 50 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.8), 10 mM L-methionine, 4 μM de riboflavin, 0.1 mM EDTA, 60 μl de NBT and 0.25 ml of recovered extract. After incubation under fluorescent lamps ($50 \text{ mmol photons m}^{-2}\text{s}^{-1}$) for 10 min, the absorbance was recorded at 540 nm and the results were expressed in $\mu\text{mol/g}$ using an extinction coefficient of $12.8 \text{ mM}^{-1}\text{cm}^{-1}$. There were five replicates per treatment (one plant per replicate).

Hydrogen peroxide

H_2O_2 was measured spectrophotometrically according to Nag et al. (2000) based on hydrogen peroxide-titanium (Ti) complex formation. Leaf material was ground with liquid nitrogen and the fine powdered material (0.1 g) was mixed with 3 mL 0.5% (w/v) TCA in an ice bath. The extracted solution (0.8 mL) was reacted by 0.1 mL of 15% (w/v) titanium sulfate (TiSO_4) in 25% (v/v) sulfuric acid (H_2SO_4). The reaction mixture was centrifuged for 10 min at $10,000\times g$ and then the absorbance was recorded at 410 nm. The results of H_2O_2 content were expressed in nmol $\text{H}_2\text{O}_2/\text{g}$.

Protein-bound carbonyls content

The carbonyl group content of leaf extracts was measured by reaction with 2,4-dinitrophenyl hydrazine (DNPH) as described by Levine et al. (1994) with some modifications. Extracts (100 mg) were incubated with 1% (v/v) Triton X-100 and 1% (w/v) streptomycin sulfate for 20 min to remove the nucleic acids. After centrifugation at $10,000\times g$, the supernatants (200 μL) were reacted with 300 μL of 10 mM DNPH in 2 M hydrochloric acid (HCl). After 1 h incubation at room temperature, proteins were precipitated with 10% (w/v) trichloroacetic acid and the pellets washed three times with 500 μl of ethanol:ethylacetate (1:1). The pellets were finally dissolved in 0.5 of 6 M guanidine hydrochloride solution (containing 133 mM Tris, 13mM EDTA, pH 7.4) in 20 mM potassium phosphate at pH 2.3, and the absorption at 370 nm was measured. Protein recovery was estimated for each sample by measuring the absorption at 280 nm. The results were expressed in nmol/mg of protein using a molar absorption coefficient for aliphatic hydrazones of $22 \text{ mM}^{-1}\text{cm}^{-1}$. There were five replicates per treatment (one plant per replicate).

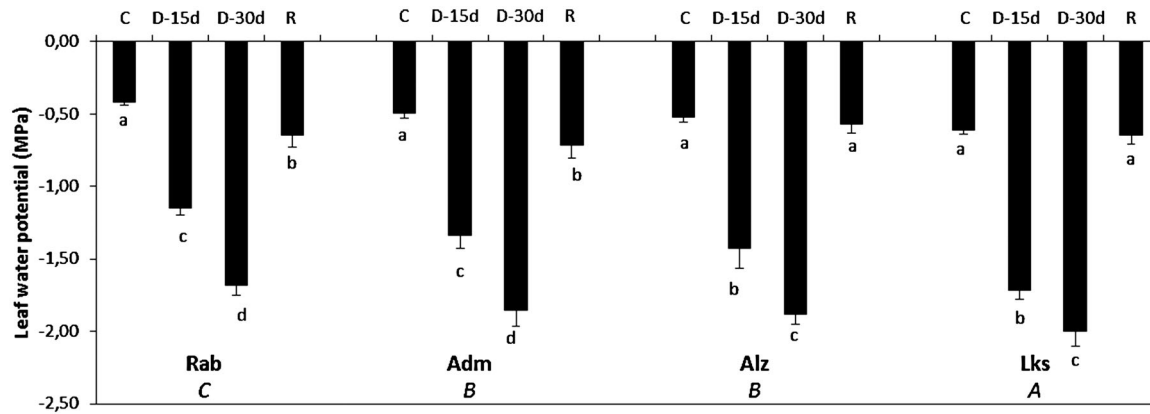


Figure 2. Effect of drought stress and rehydration on the predawn leaf water potential (Ψ_{pd}) in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C – Control, D-15d – 15-days period of drought, D-30d – 30-days period of drought and R – Rehydration. Values (means of five replicates \pm SD) with different letters are significantly different at the 5% level Tukey's test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

Statistical analysis

Each measure of each trait corresponds to the mean of five separate replicates and mean values and standard deviations were calculated. After checking the normality as well as homoscedasticity of data, the results were examined by the three-way analysis of variance (ANOVA) in order to test the effect of ecotype, time, watering regime and their interactions in each of the physiological and biochemical study variables (traits). Means were compared using Tukey's Post hoc test. A Pearson correlation analysis was done for some variables for each ecotype. A canonical discriminant analysis (CDA), by entering all independent variables into the equation at once, was performed on the four contrasting *A. spinosa* to determine which variables discriminated between them. Statistical analyses were conducted using SPSS 10.0 for Microsoft Windows.

Results

Physiological traits

Drought stress significantly reduced leaf water potential and stomatal conductance in plants of *A. spinosa* ($P < .001$) (Figures 2 and 3). After 30 days of withholding watering, we recorded very negative values of water potential in plants of four ecotypes compared to control plants (time 0). Thus,

stomatal resistance gradually increased according to the prolongation of drought stress. A significant ecotype effect was noted for these both physiological traits ($P < .001$). The leaf water potential decreased approximately 3- to 4-fold in the leaves of plants subjected to drought stress, and the Lks ecotype showed more negative values than other ecotypes. However, stomatal conductance was reduced by approximately 72.6%, 71.2%, 60.1% and 59.1% in Rab, Adm, Alz and Lks, respectively. During rehydration, leaf water potential and stomatal conductance increased progressively to reach levels close to those recorded in control conditions (Figures 2 and 3). The recovery kinetics of both physiological traits proved rapid in *A. spinosa* and different among ecotypes. According to multivariate ANOVA, the interaction ecotype \times watering regime has been significant for both these physiological traits ($P < .001$). During drought period, positive and significant correlations at $P < .01$ were established between negative values of leaf water potential and stomatal conductance values ($r = .98$, $r = .98$, $r = .96$ and $r = .92$ in Rab, Adm, Alz and Lks, respectively).

Biochemical traits

H_2O_2 , a product of superoxide dismutase reaction, significantly increased in drought-stressed plants ($P < .001$). This

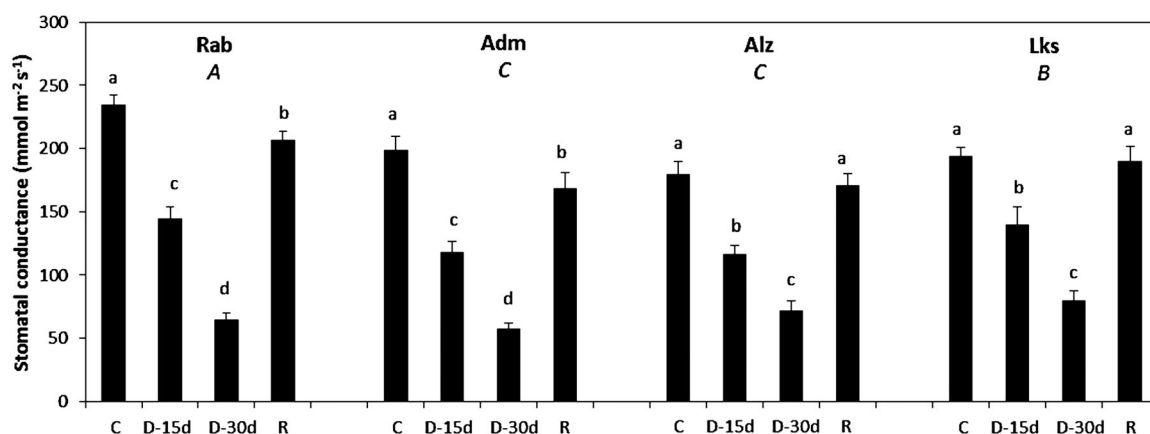


Figure 3. Effect of drought stress and rehydration on stomatal conductance in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C – Control, D-15d – 15-days period of drought, D-30d – 30-days period of drought and R – Rehydration. Values (means of five replicates \pm SD) with different letters are significantly different at the 5% level Tukey's test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

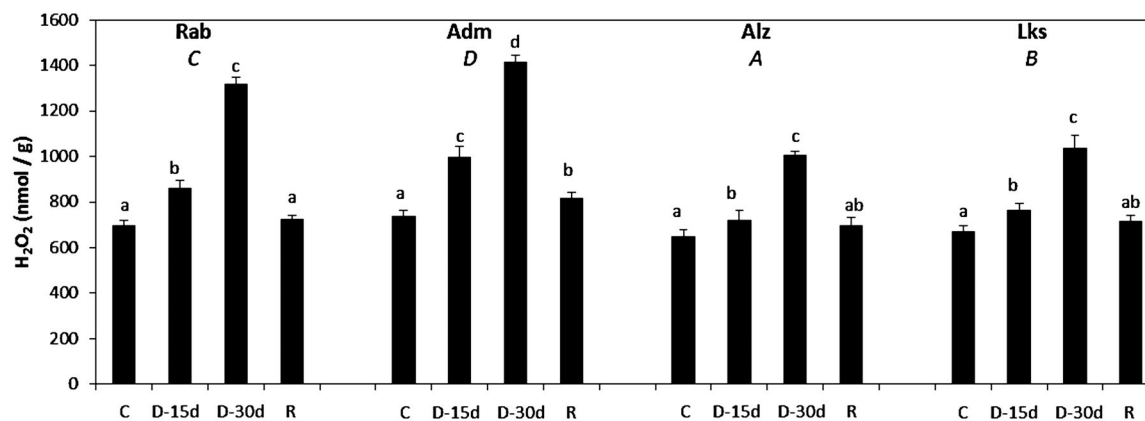


Figure 4. Effect of drought stress and rehydration on hydrogen peroxide content (H_2O_2) in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months' age were exposed to the following water treatments: C – Control, D-15d – 15-days period of drought, D-30d – 30-days period of drought and R – Rehydration. Values (means of five replicates \pm SD) with different letters are significantly different at the 5% level Tukey's test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

increase in hydrogen peroxide was detected in the four contrasting ecotypes of *A. spinosa* (Figure 4). After 30 days of withholding watering, we recorded a significant accumulation of endogenous H_2O_2 of about 89.3%, 91.3%, 54.9% and 54.4% in Rab, Adm, Alz and Lks, respectively. Indeed, it has been shown that the accumulation of this reduced metabolite was very pronounced in coastal ecotypes compared to inland ecotypes. In addition, a highly significant difference ($P < .001$) of the H_2O_2 concentration among ecotypes was recorded before the occurrence of stress. However, during the rehydration phase, H_2O_2 rapidly decreased significantly in the leaves of drought-stressed plants, such as the kinetics of recovery has been revealed slightly different for the four ecotypes (Figure 4). The concentrations of H_2O_2 noted after rehydration have been showed statistically very close to the levels recorded in the control plants. According to multivariate ANOVA, the ecotype \times watering regime interaction has been significant for this biochemical trait ($P < .001$).

The superoxide radical is a highly toxic species that is produced by many biological and photochemical reactions. Our results showed that during the period of drought stress, significant and greater production of superoxide radical was detected in the four *A. spinosa* ecotypes ($P < .001$) (Figure 5). After 30 days of drought treatment, the production of superoxide radical increased substantially, by approximately 71.7%, 76.6%, 66.2% and 59.8% in Rab, Adm, Alz and Lks,

respectively; and a significant difference depending on ecotype was noted ($P < .001$). Indeed, both inland ecotypes showed lower concentrations of superoxide radical compared to the both coastal ecotypes, even in control conditions. After 4 days of rehydration, the accumulation of this ROS in leaf tissue decreased significantly by referring to the levels noted under drought treatment (Figure 5). According to multivariate ANOVA, the ecotype \times watering regime interaction was considered statistically significant for this trait ($P = .003$).

When proteins are oxidized, the carbonyl groups occur endogenously in cells. Therefore, oxidative damage to proteins can be estimated by the content of carbonyl groups. According to our results, the content of carbonyl groups showed similar changes to that of superoxide radical and hydrogen peroxide. Under drought treatment, the carbonyl group content increased significantly (Figure 6), and this increase was estimated to be 21.3%, 37.8% and 23.2% in Rab, Adm, Alz and Lks, respectively. A significant Ecotype effect was recorded ($P < .001$) during drought and control conditions. Indeed, the lower content of carbonyl groups was noted in Lks and the highest was recorded in Rab. During the period of rehydration, the carbonyl groups began to decline significantly in order to reach similar levels of those recorded under control conditions in the four ecotypes.

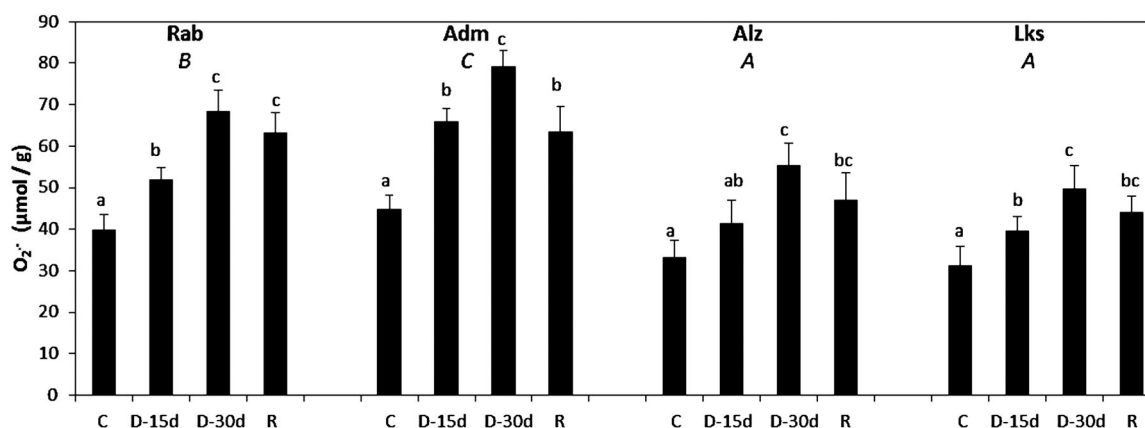


Figure 5. Effect of drought stress and rehydration on superoxide radical content (O_2^-) in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months' age were exposed to the following water treatments: C – Control, D-15d – 15-days period of drought, D-30d – 30-days period of drought and R – Rehydration. Values (means of five replicates \pm SD) with different letters are significantly different at the 5% level Tukey's test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

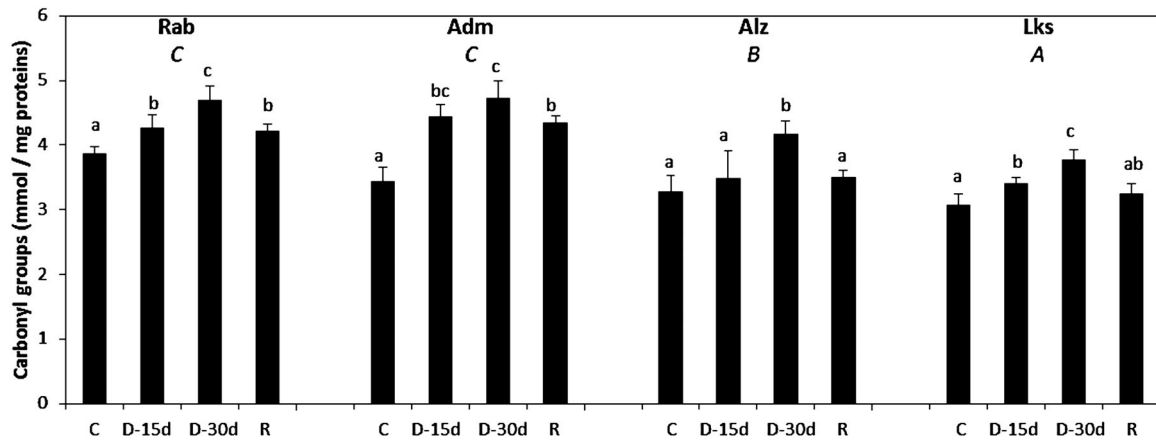


Figure 6. Effect of drought stress and rehydration on carbonyl groups content in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C – Control, D-15d – 15-days period of drought, D-30d – 30-days period of drought and R – Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at the 5% level Tukey’s test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

During the drought stress period, significant and positive correlations were established between the endogenous concentration of H₂O₂ and the content of carbonyl groups ($r = .86, P < .01$; $r = .84, P < .01$; $r = .76, P < .01$ and $r = .89, P < .01$ in Rab, Adm, Alz and Lks, respectively). Using the multivariate ANOVA, the ecotype x watering regime interaction was judged to be statistically significant for carbonyl groups ($P = .002$).

Drought stress treatment induced a significant increase in the activity of SOD through its isoenzymes in *A. spinosa* leaves ($P < .001$) (Figure 7). This increase proved to be different depending on the ecotype. Indeed, we recorded significant intraspecific differences in the constitutive and induced enzyme activities of Cu/Zn-SOD and Mn-SOD isoforms. However, only the induced activity of Fe-SOD showed significant differences among the studied ecotypes. The recorded values of enzymatic activity of these three isoenzymes are higher in the both inland ecotypes compared to both coastal ecotypes. After 30 days of withholding watering, the activity of Cu/Zn-SOD was stimulated by approximately 42.2%, 90.3%, 36.0% and 84.4% of Rab, Adm, Alz and Lks, respectively, the activity of Mn-SOD significantly increased by about 32.6%, 53.3%, 53.8% and 45.5% in Rab, Adm, Alz and Lks, respectively; and Fe-SOD activity has also increased

significantly by approximately 49.1%, 43.2%, 51.3% and 40.6% in Rab, Adm, Alz and Lks respectively. Nevertheless, during the rehydration phase, we observed a decrease in the activity of SOD and its isoenzymes by referring to the activities recorded after 30 days of drought (Figure 7). According the significant and rapid kinetics of recovery in *A. spinosa*, the enzyme activities of SOD and its isoforms have reached levels similar to those recorded in control conditions and distinct among the four ecotypes studied.

Under drought treatment, significant and positive correlations were recorded between the concentration of both ROS:O₂⁻ and H₂O₂ and the total activity of SOD and the activity of its isoenzymes (Tables 1 and 2). According to multivariate ANOVA, ecotype x watering regime interaction was judged statistically significant for all of these biochemical traits except for Fe-SOD (SOD and Cu/Zn-SOD at $P < .001$, and Mn-SOD at $P = .003$).

Canonical discriminant analysis

All physiological and biochemical traits studied were analyzed by CDA for each watering regime. The results obtained of the CDA confirmed the existence of differences in global characteristics of ecotypes (Figures 8–11). Wilk’s lambda

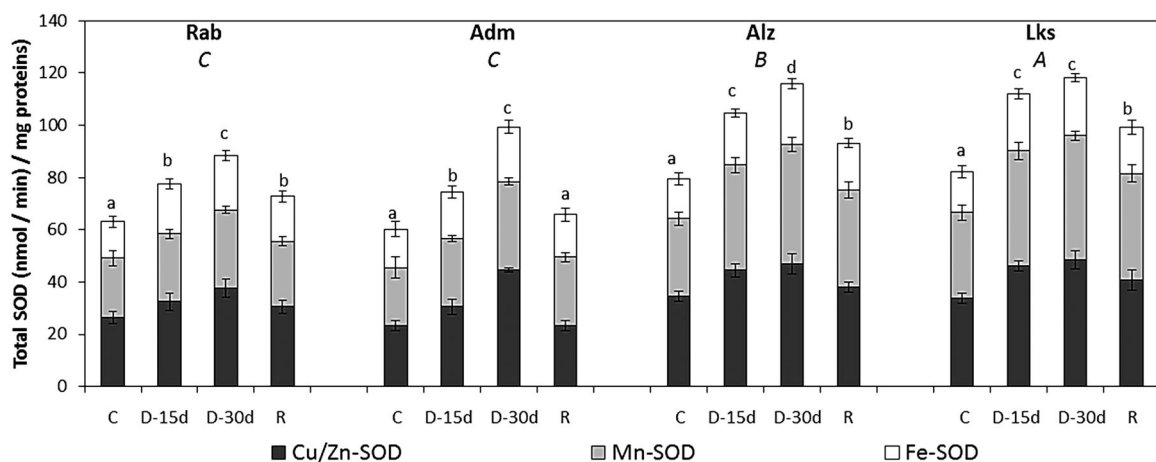


Figure 7. Effect of drought stress and rehydration on the activity of superoxide dismutase enzyme (SOD) and its isoenzymes in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months’ age were exposed to the following water treatments: C – Control, D-15d – 15-days period of drought, D-30d – 30-days period of drought and R – Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at the 5% level Tukey’s test. Upper case letters (A, B, C and D) indicate significant differences between ecotypes (Alz: Aoulouz, Lks: Lakhssas, Rab: Rabia and Adm: Admine).

Table 1. Correlation coefficients recorded at $P < .05$ among SOD and its isoenzymes and O_2^- under drought conditions in the four *A. spinosa* ecotypes.

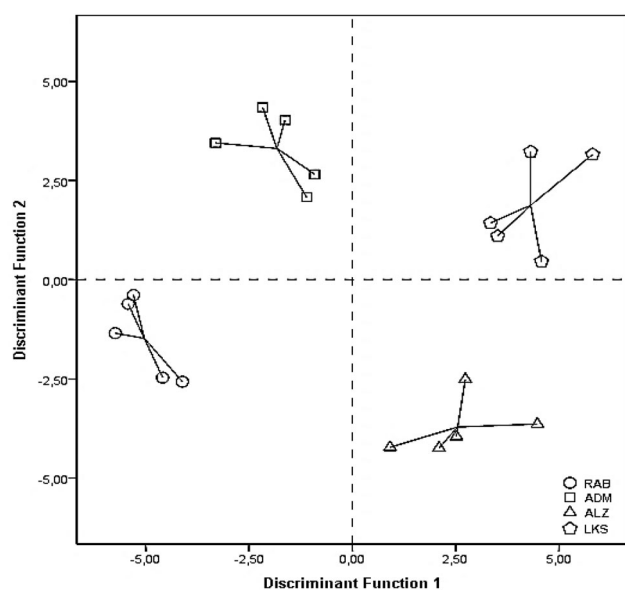
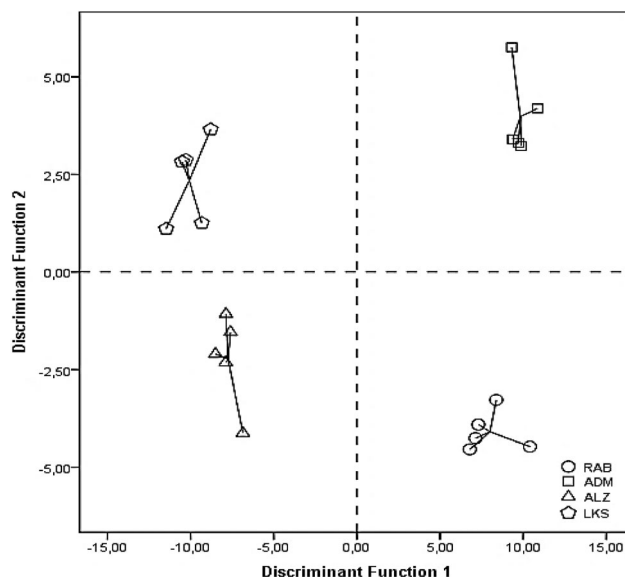
Ecotype	$O_2^- \times$ Cu/Zn-SOD	$O_2^- \times$ Mn-SOD	$O_2^- \times$ Fe-SOD	$O_2^- \times$ SOD
	Correlation coefficient (r)			
Rab	.82	.80	.83	.89
Adm	.92	.85	.66	.90
Alz	.69	.81	.86	.83
Lks	.75	.78	.64	.77

Table 2. Correlation coefficients recorded at $P < .05$ among SOD and its isoenzymes and H_2O_2 under drought conditions in the four *A. spinosa* ecotypes.

Ecotype	$H_2O_2 \times$ Cu/Zn-SOD	$H_2O_2 \times$ Mn-SOD	$H_2O_2 \times$ Fe-SOD	$H_2O_2 \times$ SOD
	Correlation coefficient (r)			
Rab	.80	.81	.75	.86
Adm	.96	.88	.72	.95
Alz	.68	.76	.79	.78
Lks	.76	.77	.60	.76

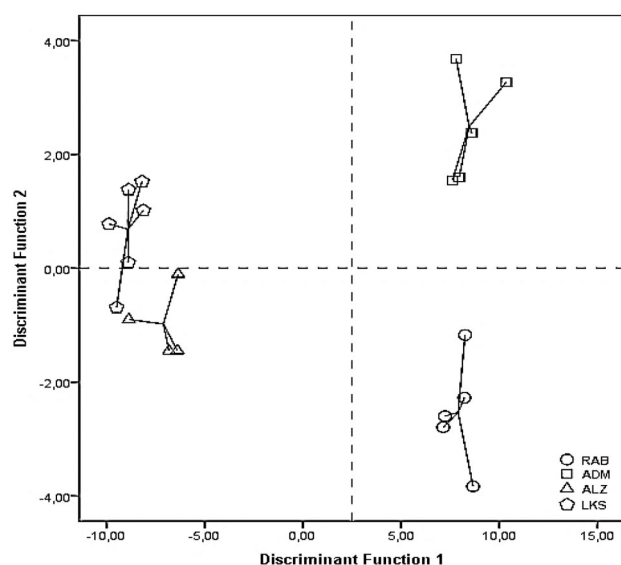
denoted a high significance of the four selected models (CDA₁ for control conditions (Figure 8), CDA₂ for drought stress (15 days) (Figure 9), CDA₃ for drought stress (30 days) (Figure 10) and CDA₄ for rehydration conditions (Figure 11)). The first two discriminant functions accounted for approximately 88.4%, 98.2%, 99.0% and 99.4% for CDA₁, CDA₂, CDA₃ and CDA₄, respectively. The null hypothesis of discriminant functions is tested using χ^2 test. Indeed, the χ^2 test showed for the four analyses a significant discriminatory power for all the functions ($P < .001$). The canonical correlations for the first two functions in each model were highly significant.

The canonical plot (Figures 8–11) showed a clear separation of the four contrasting ecotypes taking into account the first two functions. Based on the standardized coefficients of the canonical discriminant functions, Ψ_{pd} and total SOD were highly weighted in the positive part of both discriminant functions of CDA₁ (Figure 8), while carbonyl groups and g_s were highly weighted in the negative part of DF₁ and Cu/Zn-SOD and carbonyl groups were highly weighted in the negative part of DF₂. Concerning CDA₂ (Figure 9), total SOD and superoxide radical were highly weighted in the

**Figure 8.** 2D scatterplot showing the distribution of the four ecotypes studied according to the two discriminant function gradients obtained by CDA for physiological and biochemical traits under control conditions.**Figure 9.** 2D scatterplot showing the distribution of the four ecotypes studied according to the two DF gradients obtained by CDA for physiological and biochemical traits after a 15-day period of drought.

positive part of DF1 whereas both the isoenzymes Mn-SOD and Cu/Zn-SOD were strongly weighted to the negative part. H_2O_2 and Ψ_{pd} were highly weighted in the positive part of DF2 and carbonyl groups and g_s in the negative part. Both ROS: H_2O_2 and superoxide radical showed highest standardized coefficients on the first DF of CDA₃ (Figure 10) in the positive part, while Mn-SOD and Cu/Zn-SOD were highly weighted in the negative part. However, Mn-SOD and Cu/Zn-SOD were highly weighted in the positive part of DF1 of CDA₄ (Figure 11), whereas total SOD and carbonyl groups were greatly weighted in the negative part. Nonetheless, Mn-SOD and H_2O_2 were highly weighted in the positive part of DF2, while total SOD and g_s were greatly weighted in the negative part of both CDA₃ and CDA₄.

The first DF in the four analyses (control, both watering regime and rehydration) contributed mostly to distinguish among inland ecotypes (Lks and Alz) and the coastal ecotypes (Rab and Adm). However, both ecotypes Alz and Rab were

**Figure 10.** 2D scatterplot showing the distribution of the four ecotypes studied according to the two DF gradients obtained by CDA for physiological and biochemical traits after a 30-day period of drought.

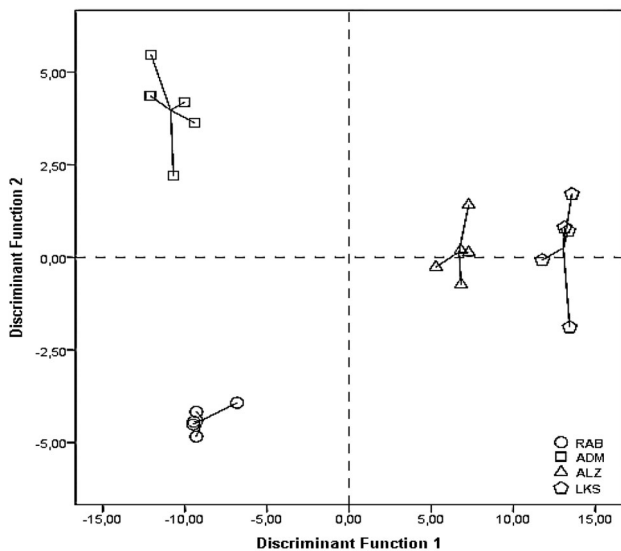


Figure 11. 2D scatterplot showing the distribution of the four ecotypes studied according to the two DF gradients obtained by CDA for physiological and biochemical traits after 4 days of rehydration.

clearly distinguished from other both ecotypes Lks and Adm by the second DF, except for CDA₄ (rehydration phase). Equal numbers of plants were compared in each ecotype.

Discussion

Under extreme drought conditions, the statistical analysis of our data revealed significant differences in all traits studied according to fixed factors (ecotype and watering regime). Drought stress has strongly influenced the physiological and biochemical traits studied in *A. spinosa* ecotypes. A significant decrease in leaf water potential and stomatal conductance was observed in the four contrasted ecotypes. Thus, we recorded a positive and significant correlation between both these traits reflecting a considerable change in water status during the experimental period. During drought stress phase, leaf water potential was significantly reduced in the stressed plants accompanied by a highly significant stomatal closure (Chakhchar et al. 2015b, 2015c). These qualitative relationships are in accord with the results reported between Ψ_b and g_s in other species (Gomes et al. 2004; Wahbi et al. 2005; Grzesiak et al. 2013). Furthermore, Diaz Barradas et al. (2013) have highlighted a significant relationship between VPD and stomatal conductance in *A. spinosa* in a summer day, suggesting that the stomata are responsive to VPD. In fact, the sensitivity of stomata to reduced leaf water potential varies widely depending on the species. The best information on the water status of the plants are provided by physiological indicators such as the leaf water potential, due to their dynamic nature and their direct relation to climatic and soil conditions as well as plant productivity (Remorini & Massai 2003; De Swaef et al. 2009). Also, because of the drought stress, the stomata close proportionally to the degree of stress and progressively limit the availability of CO₂ in chloroplasts (Medrano et al. 2002). These both physiological traits are likely to be associated with better adaptation and tolerance in the argan tree. We reported in previous studies that *A. spinosa* plants overcome the effect of drought stress through a gradual closing of the stomata providing partial stomatal regulation (Chakhchar et al. 2015b, 2015c).

Our results showed rapid recovery kinetics of the physiological parameters in *A. spinosa* plants. The rapid resumption of leaf water potential and stomatal conductance values similar to those recorded in control conditions in *A. spinosa* plants, after rehydration, has been observed in other plant species subject to severe stress (Ortuño et al. 2006; Hura et al. 2009; Rodríguez et al. 2012). Concerning the g_s , the significant kinetics of recovery, during rehydration phase, may be due to the fact that the opening of the stomata is controlled only by a hydro-active mechanism in response to stimulation of the environment (Torrecillas et al. 1995). Nevertheless, the stomata open and close in response to various environmental and endogenous stimuli. A more severe drought stress would probably have been necessary to trigger hormonal changes in the leaves, as an increase in abscisic acid and/or a decrease of cytokinins, the mechanisms that have delayed the opening of the stomata after hydration (Ruiz-Sánchez et al. 1997; Chaves et al. 2003). Pérez-López et al. (2008) reported in the olive tree very fast recovery kinetics of these both physiological traits, especially water potential. Besides changes in water status, the drought stress is responsible for several metabolic processes of plants. Indeed, significant accumulation of H₂O₂ and O₂⁻ has been recorded in the leaves of stressed plants suggesting that the most serious oxidative damages are produced under drought conditions. The accumulation of H₂O₂ in cells of *A. spinosa* can cause the formation of OH⁻ by the Fenton reaction, which may act as a second messenger to induce expression of genes or other reactions (Miller et al. 2010). This increase of hydrogen peroxide in plants of *A. spinosa* may be due to the dismutation of O₂⁻ which was produced in the electron transport chain of mitochondrial respiration or during photosynthetic electron transport in chloroplast or during the photorespiratory flow (Uzilday et al. 2012). Thus, the production of O₂⁻ can trigger the formation of the most reactive ROS as OH⁻, and eventually the singlet oxygen ¹O₂, which each of them can cause lipid peroxidation and consequently the weakening of the membrane cell (Halliwell 2006; Gill & Tuteja 2010). Corroborating our results, several authors have also reported a significant increase in production of O₂⁻ and H₂O₂, under drought stress conditions, in the leaves of various species, including apple (Wang et al. 2012), cucumber (Liu et al. 2009) and Kentucky bluegrass (*Poa pratensis* L.) (Bian & Jiang 2009). After rehydration, the concentrations of ROS studied decreased slightly by referring to contents noted under drought stress conditions. This suggests that oxidative stress is potentially less in the leaves of *A. spinosa* during recovery. This may be explained by a probable activation of elimination processes of ROS. In fact, the role of ROS in oxidative signaling is not clear or well understood when the plants are rehydrated.

Our results showed that the proteins oxidation was enhanced in the leaves of *A. spinosa* plants subjected to drought stress. The increase of carbonyl groups content, as an indicator of oxidative stress, is closely linked to the production of ROS, highlighting an accumulation of H₂O₂ which suggests the installation of oxidative stress in the argan tree under drought stress conditions. This explains the significant correlations established between these both biochemical traits. The values of carbonyl groups content observed in control plants could be the result of the generation of ROS as byproducts of normal physiological processes which produce a low level but detectable of proteins modified by oxidation into the tissues (Shi et al. 2005). Previous studies

have reported that environmental stresses induce an increase in the content of carbonyl groups in different species (Prasad 1996; Kingston-Smith & Foyer 2000; Junqua et al. 2000; Vanacker et al. 2006). After the rehydration phase, the proteins' oxidation decreased and the content of carbonyl groups, as oxidative stress indicator, reached a level close to the values recorded in control plants. This suggests that the *A. spinosa* plants rehydrated quickly activated degradation pathway to remove oxidized proteins.

Drought stress induced a significant increase in the activity of superoxide dismutase enzyme and its isoenzymes in the leaves of the argan tree. According to our results, the significant and positive correlations recorded among both ROS (O_2^- and H_2O_2) and SOD explain the existence of a strong antioxidant performance of SOD in terms of dismutation of superoxide to hydrogen peroxide and oxygen in *A. spinosa*. Similarly, a significant increase in SOD activity under drought stress was observed in *A. spinosa* plants aged 14 months (Chakhchar et al. 2015c) and in others, various plants: *Olea europea* (Ben Ahmed et al. 2009; Boughalleg & Mhamdi 2011), *Saccharum officinarum* (Patade et al. 2011), *Populus cathayana* (Zhang et al. 2012) and *Jatropha curcas* (Pompelli et al. 2010). In addition, a significant increase in the activity of the isoenzymes Cu/Zn-SOD, Mn-SOD and Fe-SOD has been reported in drought stress conditions (Uzilday et al. 2012). The upregulation of SOD and its isoenzymes are involved in the defense systems against oxidative stress due to biotic and abiotic stress and have a crucial role in the survival of plants under environmental stresses. In fact, an overexpression of Cu/Zn-SOD in transgenic tobacco plants showed tolerance to multiple stress (water and saline) (Badawi et al. 2004). Also, an overexpression of the Mn-SOD in tomato plants transformed revealed an increased tolerance to saline stress (Wang et al. 2007). However, Asensio et al. (2012) reported a greater complexity in the genomic analysis of Fe-SOD in stress conditions. This significant induction of the activity of SOD and its isoenzymes indicates that the ability to eliminate ROS in the argan tree has considerably increased. However, Bian and Jiang (2009) found that the expressions of Fe-SOD, Cu/Zn-SOD and Mn-SOD were down-regulated in the leaves of *Kentucky bluegrass* under drought stress, but they are recovered after rehydration. Indeed, we recorded a significant recovery of the enzymatic activities of SOD and its isoforms to reach levels similar to those recorded in the control plants. This suggests that there was a reduction of ROS as well as the high speed of the SOD regulatory mechanism in transcription to protect *A. spinosa* plants against oxidative damage.

The canonical plots reflect good separation among the four contrasting ecotypes of *A. spinosa*. The vertical separation in the four analyses was established by the first FD which quantifies the degree to which all ecotypes differ in their physiological and biochemical traits. This has allowed us to make a connection between the studied traits and the registered differences among ecotypes in their tolerance to drought stress. Both the inland ecotypes (Lks and Alz) were clearly separate from the both coastal ecotypes (Adm and Rab) by high activity of SOD, especially both the isoenzymes Mn-SOD and Cu/Zn-SOD. During rehydration (CDA₄), both the inland ecotypes showed a very similar response rate in terms of recovery. However, both the coastal ecotypes are mainly distinguished from other ecotypes by their high degree of oxidative stress related to high accumulation of

ROS (H_2O_2 , O_2^- and carbonyl groups). According the second DF, the stomatal conductance as physiological trait separated these both ecotypes under drought stress and rehydration conditions. Based on these results, it was revealed that both inland ecotypes (Alz and Lks) have a very effective antioxidant defense system compared to the other ecotypes. The traits having the most discriminating power were selected as the best adaptive traits for predicting drought tolerance, which could be also relevant for genetic improvement of *A. spinosa* in drought soil conditions. Both these inland ecotypes appear to be potential candidates for domestication, having regard to the importance of the ecological, social and economic role of *A. spinosa* in a drought environment.

The argan tree possesses physiological and biochemical characteristics, enabling it to adapt to certain climatic regimes characterized by pronounced aridity. The results obtained in this study showed significant differences in physiological and biochemical responses of the four contrasting *A. spinosa* ecotypes. Indeed, the induced edaphic drought induced significant changes in the studied traits. However, the kinetics of recovery of the argan tree as a result of rehydration was rapid. It was manifested in levels similar to those recorded in the control plants after only 4 days. According to the CDA, both the inland ecotypes (Lks and Alz) were clearly separated from the coastal ecotypes (Rab and Adm). Both physiological and biochemical traits having the most discriminating power appear to be considered as adaptive traits and can be interpreted as an internal mechanism of drought tolerance in *A. spinosa*. The drought tolerance of Alz and Lks ecotypes may enhance their potential for climatic adaptations under drier conditions with ongoing climatic change.

Future research on the drought adaptation and recovery strategies of the argan tree will be necessary to fully appreciate the development of the Argan sector.

Disclosure statement

No potential conflict of interest was reported by the authors.

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