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Electrolyte ions and glutathione enzymes as stress markers in *Argania spinosa* subjected to drought stress and recovery

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Understanding the mechanisms underlying *Argania spinosa* responses to drought stress is essential for its regeneration and domestication. Toward that end, an integrative study of tolerance responses to drought stress in four *A. spinosa* ecotypes (2 contrasting coastal ecotypes (Adm and Rab) and 2 contrasting inland ecotypes (Alz and Lks)) have been conducted. Responses to soil drying and re-watering were measured at physiological and biochemical levels. Soil drying resulted in significant increase in leaf concentrations of potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) with differential responses between ecotypes. The glutathione-related enzymes: glutathione peroxidase (GP), glutathione reductase (GR) and glutathione S-transferase (GST) showed a significant increase in their enzymatic activity in *A. spinosa* plants subjected to drought stress. Additionally, a significant increase in thiol protein content in the four ecotypes was recorded, during drought stress. These antioxidant traits responded differently depending on ecotype. However, rapid and significant changes in the studied physiological and biochemical traits were observed during recovery from drought, only after four days. According to the traits having the most discriminating power, the both inland ecotypes, especially Lks ecotype, seem to be potential candidates for regeneration of argan forest and their domestication in arid and semi-arid environments.

Key words: *Argania spinosa*, drought stress, glutathione enzymes, thiol compounds, recovery.

INTRODUCTION

Drought caused by water scarcity and/or the uneven distribution of rainfall is the main abiotic factor limiting

crop productivity worldwide. Currently, drought becomes a global challenge to ensure the survival of agricultural crops and sustainable food production. It is related to almost all aspects of biology. Study of the drought stress has been one of the main directions in global plant biology (Somerville and Dangl, 2000). In the Mediterranean ecosystem, plants are subjected to severe and permanent drought stress, particularly during summer months (Nogués and Baker, 2000). Some plants as argan tree [*Argania spinosa* (L.) Skeels], endemic species to Southwestern part of Morocco, have developed a variety of strategies and mechanisms in response to changes in the environment, especially drought stress (Diaz-Barradas et al., 2010, 2013; Chakhchar et al., 2015a, b or c). Water restriction can lead to morphological, physiological, biochemical and molecular changes. Nonetheless, the drought tolerance is present in almost all plants, but its magnitude varies from one species to another and even within the same species. It causes subtle changes in physiological and biochemical processes of plants. Understanding the metabolic and physiological aspects of drought stress responses in plants is therefore of critical importance.

Drought stress often disturbs the balance of nutrients and electrolyte ions in the plants such as potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}). However, high concentrations of these elements in the leaves can mitigate the negative effects of drought stress (Cakmak, 2005; Wu et al., 2013). In addition, the drought stress can alter cell homeostasis through the production of reactive oxygen species (ROS), potentially damaging agents (Parent et al., 2008). When the reduction of oxygen in the chloroplasts is incomplete, the majority of these ROS are generated and oxidative damage may occur (Mittler, 2002; Miller et al., 2010; Gill and Tuteja, 2010). In order to maintain homeostasis and prevent oxidative stress, plants have evolved a defense system included, among others enzymes, the glutathione antioxidant enzymes such as glutathione reductase (GR, EC 1.6.4.2), the glutathione S-transferase (GST, EC 2.5.1.18) and glutathione peroxidase (GP, EC 1.11.1.9) (Noctor et al., 2002; Gill and Tuteja, 2010). Besides these enzymatic antioxidants, other non-enzymatic compounds are considered to be relevant markers of oxidative stress, such as the thiol compounds (Deneke, 2000).

Despite recent studies and our understanding of physiological and biochemical response of argan tree to drought stress (Diaz-Barradas et al., 2010, 2013; Chakhchar et al., 2015a, b or c), information regarding the adaptive mechanisms underlying the regulation of *A. spinosa* metabolism during recovery from drought is scarce. This tree has important socio-economic and ecological roles in South-West Morocco, where it grows in over 800,000 hectares, and in which it also plays a great role in the biodiversity of the forest's ecosystem (Msanda et al., 2005). *A. spinosa* is a potential very important tree species for vegetable oil, which could generate a great interest from the horticultural industry. Also, the argan trees can be exploited for firewood, timber, as a forage for cattle, especially in drought years, and as a shade tree for cereal crops, thereby, supporting the economy of the indigenous population. About 1.3 million people of local population are living in rural areas where traditional sylvo-pastoral systems are based on the argan tree (Chaussod et al., 2005).

For a better understanding of how *A. spinosa* ecotypes differ in their tolerance to drought stress and recovery, this study was planned to better characterize some drought tolerance traits through an integrative analysis of physiological and biochemical responses to drought in *A. spinosa*. Therefore, these questions were asked: (1) how physiological and biochemical mechanisms vary under drought and recovery conditions; (2) how these responses to drought and recovery conditions vary between the studied ecotypes; and (3) how could discriminateion be made between these ecotypes in terms of tolerance degree using the studied adaptive traits.

MATERIALS AND METHODS

Plant and experimental design

Sampling of seeds of *A. spinosa* was conducted in four regions of the argan tree forest in South-West Morocco. Climatic, geographical and hydrological conditions of these four regions are markedly different (Chakhchar et al., 2015a, b). Two contrasting coastal ecotypes (site: Rabia (Rab) and Admine (Adm)) and two contrasting inland ecotypes (site: Aoulouz (Alz) and Lakhssas (Lks)) were chosen for a better interpretation of the mechanisms regulating biochemical and physiological processes. The protocol of cultivation and experimental layout was the same used previously (Chakhchar et al., 2015a, b, c). Uniform young *A. spinosa* plants of similar height, aged 29 months, were selected for the experiment for each ecotype. The effect of prolonged drought stress by cessation of irrigation for 15 and 30 days followed by rehydration during 4 days were simulated. The environmental conditions in 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 varied between 65 and 70%. The average maximum photosynthetically active radiation (PAR) was approximately 400 µmol $m^{-2}s^{-1}$ ensured by a combination of incandescent and fluorescent lamps.

Soil moisture content

Pots were randomized to each treatment to determine the soil moisture content. Soil samples were taken 5 cm deep without plant residues, weighed (fresh weight) and dried in an oven at 80°C for

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72 h before measuring the dry weight. Moisture content of the soil sample was expressed in percent according to the formula:

Soil moisture content (%) = [(wet weight - dry weight) / dry weight] × 100.

Physiological traits

Endogenous content of ions (Mg^{2+}, K^{+} and Ca^{2+})

Leaf material collected was carefully rinsed with deionized water and the fresh weight of each sample was determined. Then, the leaf material was calcined at 600°C for 6 h and the dry weight of each sample was measured. Each sample was ground into a fine powder and digested with concentrated nitric acid (HNO_3) overnight at 120°C. Samples were then dissolved in (1:1, v/v) HNO_3/$HClO_4$ (perchloric acid) to 220°C, resuspended in 5% (v/v) HNO_3 and analyzed for the determination of Mg^{2+}, K^{+} and Ca^{2+} content using inductively coupled argon plasma emission spectrometry. Ion contents were expressed in mmol/g DM. Three independent measurements per treatment (one repetition per plant) were opted for.

Biochemical traits

Glutathione enzymes extraction

Fresh leaves samples from control and treated plants were immediately ground to a fine powder in a mortar in the presence of liquid nitrogen. Enzymes were extracted on ice by homogenizing the powder (0.1 g for each enzyme × 5 replicates per treatment) in 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing 0.1 mM EDTA, 1% (w/v) polyvinyl pyrrolidone (PVP), 0.1 mM phenylmethanesulfonyl fluoride solution (PMSF) and 0.2% (v/v) Triton X100, 1 mM dithiothreitol and 20 mM ascorbate. There were 5 replicates per treatment (one plant per replicate).

Total soluble protein concentration for determination of the specific activities of the enzymes was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard. All spectrophotometric analyses were conducted on a Jenway (6305 UV/Vis. England) spectrophotometer.

Glutathione reductase activity (GR)

Glutathione reductase (GR; EC 1.6.4.2) was assayed by monitoring the ß-nicotinamide adenine dinucleotide 2'- phosphate (NADPH) oxidation coupled to the reduction of GSH at 340 nm using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ (Edwards et al., 1990). Reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.2 mM NADPH, 1 mM oxidized glutathione (GSSG) and 0.1 ml of enzyme extract. GR activity was expressed in nmol oxidized GSSG per min per mg of proteins. There were 5 replicates per treatment (one plant per replicate).

Glutathione-S-transferase activity (GST)

GST activity (EC 2.5.1.18) was measured in a reaction mixture containing 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5), 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 0.1 ml of enzyme extract. The reaction was initiated by the addition of 1 mM GSH and the formation of 2, 4-dinitrophenyl-S-glutathione (DNP-SG) was followed at 340 nm according the method described by Habig et al. (1974) and Habig and Jacoby (1981). GST activity was expressed in nmol GSH per min per mg of proteins using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ for the compound formed. There were 5 replicates per treatment (one plant per replicate).

Glutathione peroxidase activity (GP)

GPX activity (EC 1.11.1.9) was determined by adopting a coupled assay with GR to follow the GSH oxidation according the method described by Nagalakshmi and Prasad (2001). Reaction mixture contained 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.5), 10 mM Na_2-EDTA, 1 M NaCl, 10 mM GSH, 2 mM NADPH and 2.5 mM H_2O_2. Reaction was initiated by addition of 5 µl of GR (500 units/2.8 ml) and 0.1 ml of the enzyme extract. The consumption of NADPH was measured at 340 nm for 5 min. GPX activity was expressed in nmol NADPH oxidized per min per mg of proteins using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ for NADPH. There were 5 replicates per treatment (one plant per replicate).

Thiol compounds content

Aliquots of the fine powder plant material were homogenized in 50 mM Tris-HCl (pH 8.0) containing 20 mM EDTA. Homogenates were centrifuged for 20 min at 15,000 × g and supernatants were used for thiol assays. Thiol compounds content was determined by the method described by Nagalakshmi and Prasad (2001).

For determination of total thiols content, 0.2 ml of supernatant were mixed with 0.8 ml of 0.2 M Tris-HCl (pH 8.2) and 0.05 ml of 0.01 M dithionitrobenzoic acid (DTNB, dissolved in methanol). Mixture was brought to 3 ml by addition of absolute methanol. After incubation for 15 min at room temperature, the absorbance was measured at 412 nm. Total thiols content was estimated using an extinction coefficient of 13.1 $mM^{-1}cm^{-1}$. For non-protein thiols content, 0.5 ml of supernatant was mixed with 0.8 ml of distilled water and 0.2 ml of 50% (w/v) trichloroacetic acid. After incubation for 15 min incubation under stirring, the homogenates were centrifuged at 15,000 × g for 15 min. Supernatant (0.25 ml) was mixed with 1 ml of 0.4 ml M Tris-HCl (pH 8.9) and 0.025 ml of 0.01 M DTNB. After a second incubation for 15 min, the absorbance was measured at 412 nm against a reagent blank. Thus, protein thiols content was calculated by subtracting the non-protein thiols content from the total thiols content. The results were expressed in µmol/g. There were 5 replicates per treatment (one plant per replicate).

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 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 the 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 version 17.

RESULTS

Soil moisture content was affected by the cessation irrigation of *A. spinosa* plants. Significant differences were recorded in the level of soil moisture content in the four contrasting ecotypes (Figure 1). After 30 days of

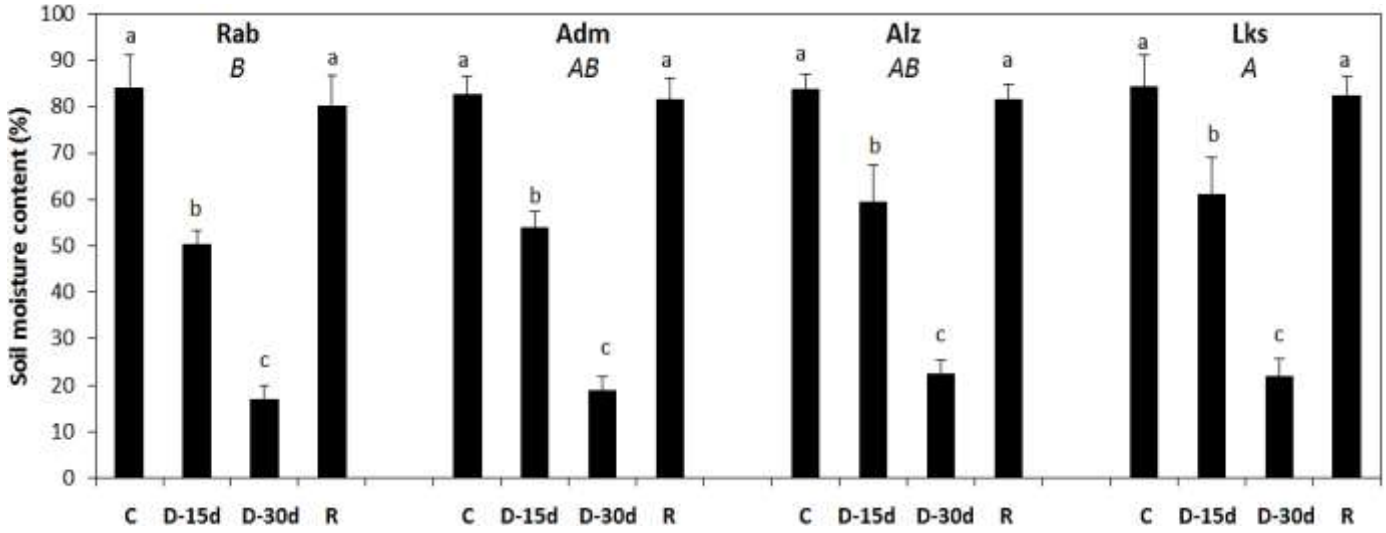

Figure 1. Soil moisture content recorded under drought stress and rehydration conditions. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C: Control, D-15d: 15-days period of drought stress; D-30d: 30-days period of drought stress; R: Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at 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).

withholding watering, soil moisture content decreased very significantly compared to the control (80.0, 77.3, 73.3 and 74.1% in Rab, Adm, Alz and Lks, respectively). This physiological-edaphic trait evolved similarly in the four ecotypes during the drought and rehydration periods. Indeed, significant differences among these four ecotypes have not been registered. The rehydration of pots containing argan plants quickly restored the level of the soil moisture content only after four days (Figure 1). According to multivariate analysis of variance, the ecotype × watering regime interaction was not considered statistically significant for this trait ($P = 0.014$).

Withholding of water to *A. spinosa* plants induced a significant accumulation in leaf concentrations of inorganic ions K^+, Ca^{2+} and Mg^{2+} ($P < 0.001$) (Figure 2a, b and c). After 30 days of withholding watering, the percentage of K^+ accumulation varied between 45 and 61%, while Mg^{2+} varied between 47 and 91% in the four contrasting ecotypes. Adm and Rab ecotypes showed the highest accumulation of K^+ (61.0%) and Mg^{2+} (91.9%), respectively. However, Ca^{2+} concentration has been doubled even tripled in the leaves of stressed plants in some ecotypes compared to control plants. The highest accumulation of Ca^{2+} was noted in Alz ecotype. In addition, significant differences in the concentrations of these three inorganic ions in leaves of the control plants were recorded ($P < 0.001$), which Lks ecotype showed the greatest constitutive concentration of K^+ and Ca^{2+}. Thus, both inland ecotypes (Alz and Lks) showed the highest constitutive concentration of Mg^{2+}. During rehydration period, the leaf concentrations of K^+, Ca^{2+} and Mg^{2+} quickly decreased significantly, by referring to the levels noted under drought conditions, to achieve concentrations close to those recorded in control conditions (Figure 2a, b and c). The kinetics of recovery was different for the three inorganic ions depending on the ecotype. According to multivariate analysis of variance, the ecotype × watering regime interaction was statistically significant for all these three ions ($P < 0.001$).

The activity of glutathione enzymes GP, GR and GST was significantly increased in the leaves of plants subjected to drought stress ($P < 0.001$) (Figure 3a, b and c). In the four contrasting ecotypes studied, a significant stimulation of these enzymes depending on the stress prolongation was found. Induced activities of these enzymes glutathione revealed ecotype-dependent differences. However, ecotypic differences regarding the constitutive activity except for GST have not been recorded. After 15 days of withholding watering, the activities of these three enzymes have been shown to be higher in both inland ecotypes paralittoraux than both coastal ecotypes. Whereas after 30 days of withholding watering, GP activity increased significantly by approximately 12.8, 13.7, 20.9 and 20.3% in Rab, Adm, Alz and Lks, respectively (Figure 3a), the activity of GR increased by approximately 36.6, 33.0, 37.8 and 33.2% in Rab, Adm, Alz and Lks, respectively (Figure 3b) and the significant increase of GST activity was estimated to be 65.6, 79.6, 70.5 and 70.0% in Rab, Adm, Alz and Lks, respectively (Figure 3c). However, after 4 days of rehydration, the specific activity of these enzymes decreased significantly in the four ecotypes suggesting significant recovery kinetics of glutathione enzymes in *A. spinosa*. Using the multivariate analysis of variance, the ecotype × watering regime interaction was judged statistically significant only for the GR ($P < 0.001$) and GP

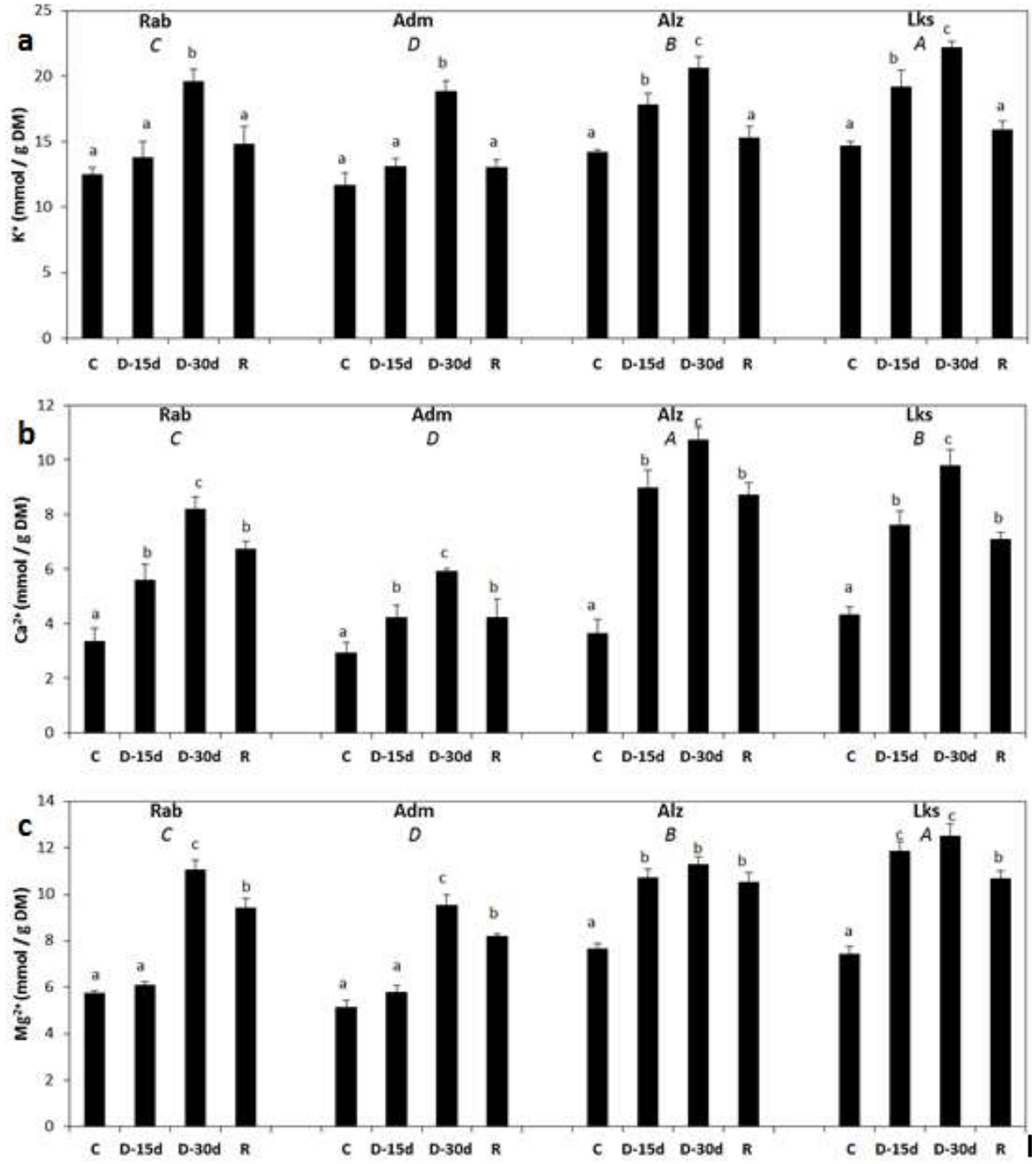

Figure 2. Effect of drought stress and rehydration conditions on leaf concentrations of K^+ (a), Ca^{2+} (b) and Mg^{2+} (c) in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C: Control, D-15d: 15-days period of drought stress; D-30d: 30-days period of drought stress; R: Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at 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).

(P = 0.007).

Drought stress caused significant increase in total thiols content in the leaves of argan tree plants (P < 0.001) (Figure 4). After 30 days of withholding watering, this significant increase was estimated at 31.5, 41.2, 13.5 and 18.2% in Rab, Adm, Alz and Lks, respectively. The increase of total thiols content in the four contrasting ecotypes was mainly due to the significant increase in

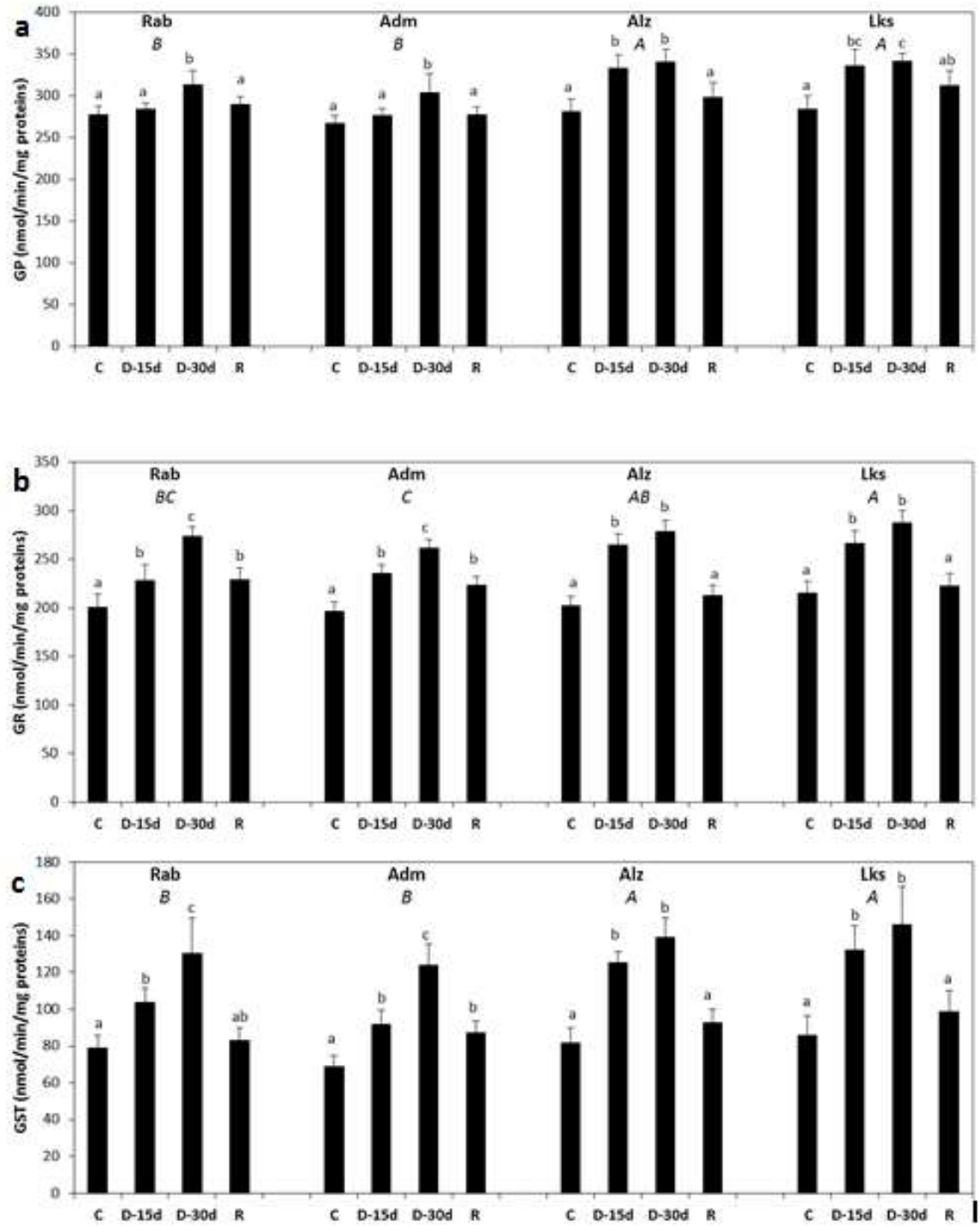

Figure 3. Effect of drought stress and rehydration conditions on enzymatic activity of GP (a), GR (b) and GST (c) in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C: Control, D-15d: 15-days period of drought stress; D-30d: 30-days period of drought stress; R: Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at 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).

protein thiols under drought conditions (Figure 4). The rate of these proteins is one of the relevant markers of oxidative stress. This increase recorded in stressed plants was 2 to 4 times higher compared to control plants.

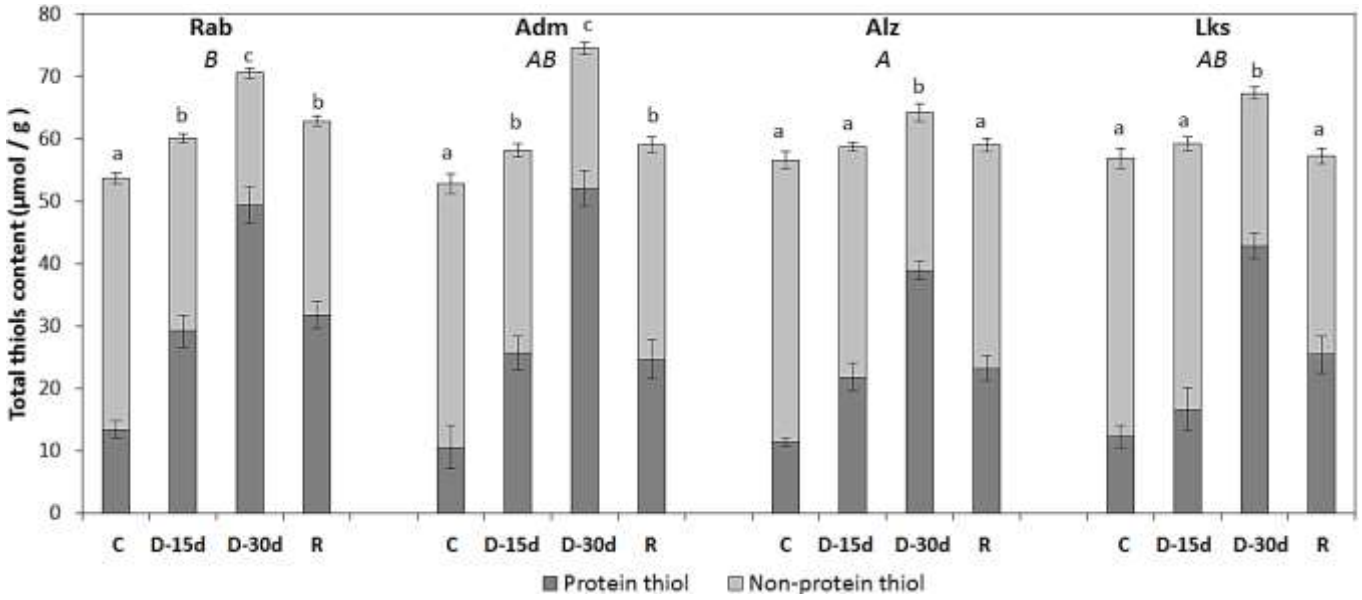

Figure 4. Effect of drought stress and rehydration conditions on thiol compounds content in four *A. spinosa* ecotypes. *A. spinosa* plants of 29 months age were exposed to the following water treatments: C: Control, D-15d: 15-days period of drought stress; D-30d: 30-days period of drought stress; R: Rehydration. Values (means of five replicates ± SD) with different letters are significantly different at 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. Statistical characteristics of the discriminant functions extracted from CDA.

Parameter	Discriminant function	Eigen value	Variance (%)	Cumulative (%)	Canonical correlation
CDA_1	1	2098.64	98.6	98.6	1.00
	2	19.49	0.9	99.5	0.98
	3	11.12	0.5	100.0	0.96
CDA_2	1	490.34	96.4	96.4	0.99
	2	16.12	3.2	99.6	0.97
	3	2.26	0.4	100.0	0.83

However, the non-protein thiols content decreased significantly during the drought stress period ($P < 0.001$). This decrease was estimated at 47.3, 46.8, 43.8 and 45.4% in Rab, Adm, Alz and Lks, respectively. Under control conditions, significant differences were noted among ecotypes especially for the non-protein thiols content ($P < 0.001$) and total thiols ($P < 0.002$). After the rehydration phase, levels converging to those noted in the control plants were recorded (Figure 4). The recovery kinetics of these thiol compounds were significant depending on the ecotype. Following the multivariate analysis of variance, ecotype × watering regime interaction revealed a statistically significant difference in these three compounds ($P < 0.001$).

Canonical discriminant analysis

The results obtained of the CDA confirmed the existence of differences in global characteristics of ecotypes (Table 1). Wilk's lambda denoted a high significance of the both selected models (CDA_1 for drought stress after 30 days (Figure 5) and CDA_2 for rehydration conditions (Figure 6)) ($P \leq 0.001$). The first two discriminant functions (DF) accounted for approximately 99.5 and 99.6% for CAD_1 and CDA_2, respectively. The null hypothesis of discriminant functions is tested using χ^2-test. The canonical correlations for the first two functions in each model were highly significant.

The canonical plot of CDA_1 (Figure 5) showed a clear separation of the four contrasting ecotypes taking into account both first functions. Based on the standardized coefficients of the canonical discriminant functions, the non-protein thiols content and the concentration of Ca^{2+} were highly weighted in the positive part of DF1 whereas the GST activity and the concentration of Mg^{2+} were strongly weighted to negative part. The non-protein thiols

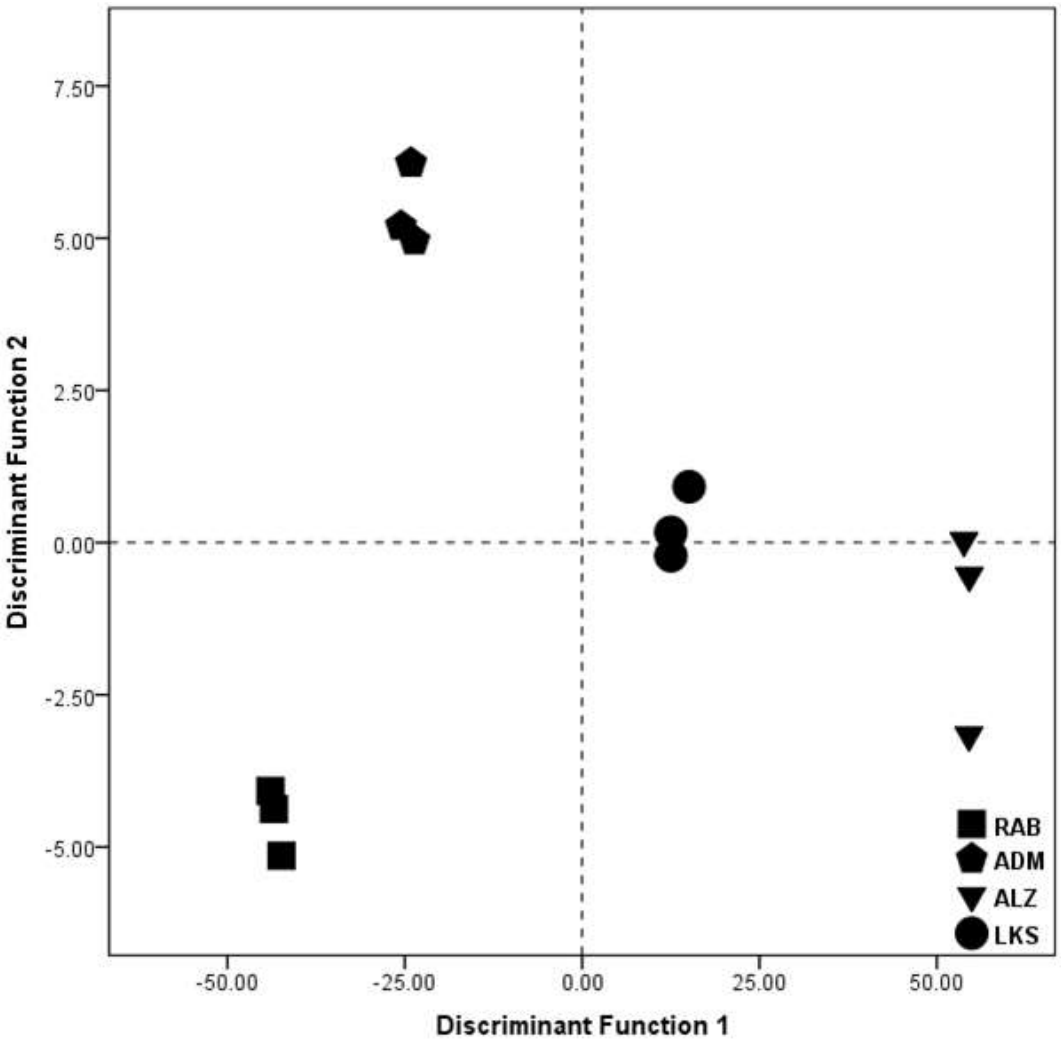

Figure 5. 2D scatterplot showing the distribution of the four ecotypes studied according to the two DF gradients obtained by CDA for physiological and biochemical traits under drought stress conditions.

content and the GR activity were highly weighted in the positive part of DF2, while the both GST and GP activities were greatly weighted in the negative part. Concerning CDA_2 (Figure 6), the concentration of Ca^{2+} and the GR activity were highly weighted in the positive part of the first DF, while the protein thiols content and the concentration of K^+ were highly weighted in negative part. The both protein thiols and non-protein thiols contents were strongly weighted in the positive part of DF2; however, both GP and GST activities were highly weighted in negative part.

The first DF in both analyses (drought stress and rehydration) contributed mostly to distinguish between coastal ecotypes (Rab and Adm) and inland ecotypes (Lks and Alz). However, the second DF clearly separated between both coastal ecotypes on the one hand and secondly between both inland ecotypes, according the CDA_1 and CDA_2, respectively.

DISCUSSION

Behavioral responses of plants to drought stress are complex and different mechanisms are adopted when they are subjected to this water constraint. Drought stress induces a number of physiological, biochemical and molecular reactions that regulate plant growth and productivity.

The results of the soil moisture content revealed a similar soil water status for all ecotypes. However, the drought prolongation significantly affected this trait. The reduction of soil moisture content is an obvious consequence of withholding watering and existence of the plant in the pot. This trait plays an important role in the hydrological and biological processes. In arid and semi-arid areas, soil water is a critical factor that affects plant growth and can thus determine plant distribution models (Engelbrecht et al., 2007). At the same time,

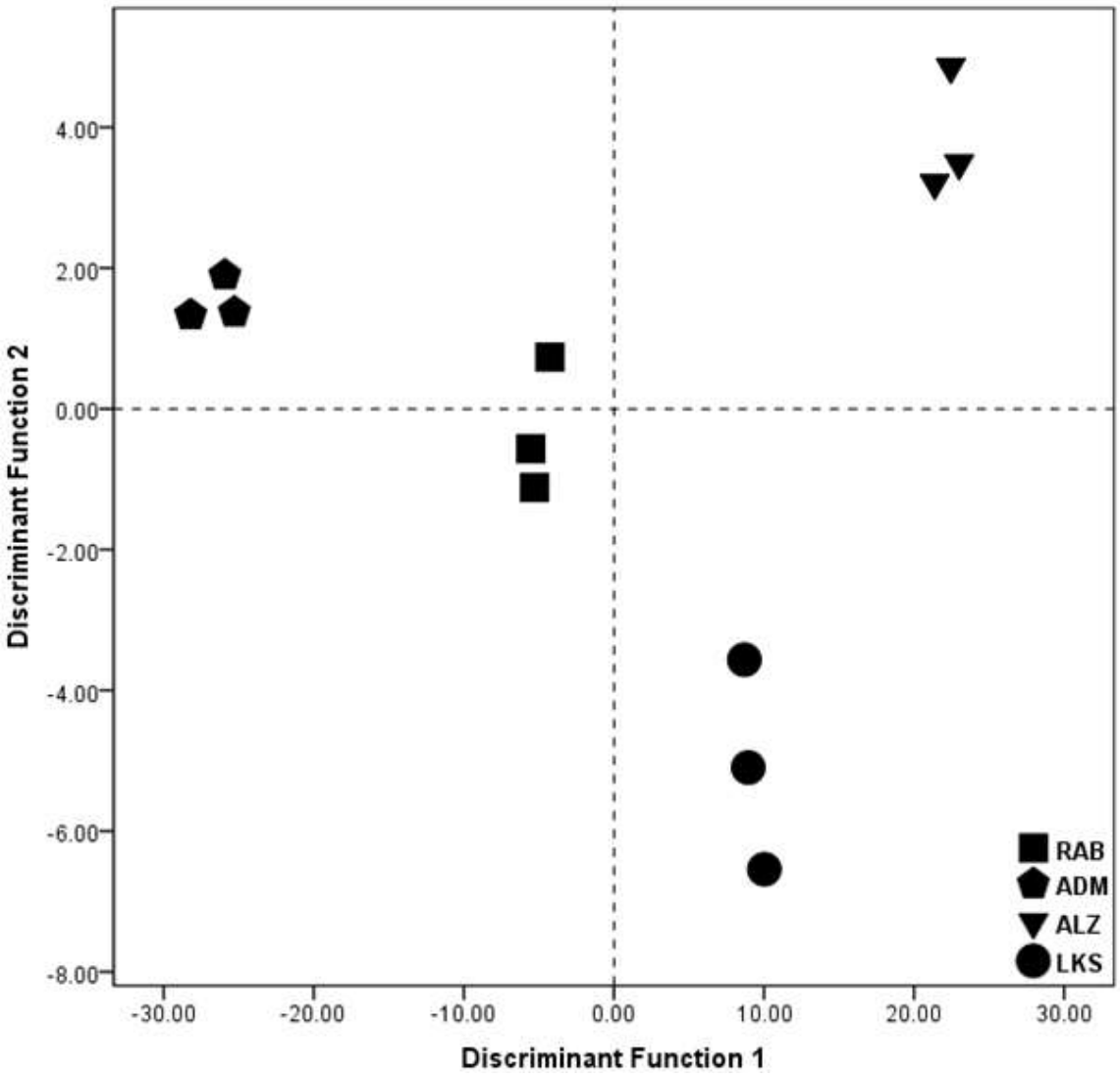

Figure 6. 2D scatterplot showing the distribution of the four ecotypes studied according to the two DF gradients obtained by CDA for physiological and biochemical traits under rehydration conditions.

plants affect the soil moisture by forming a biological pathway for the water transport from the soil to the atmosphere by their root system (Wang et al., 2010). When the seasonal or interannual drought occurs, the photosynthesis and transpiration responses in the ecosystem strongly depends on the total amount of water stored in the soil, such that the plants have the ability to extract water and push it towards the aerial parts, hence the ability to withstand stress-induced by depletion of moisture and on the decrease of soil water potential (Jipp et al., 1998).

The accumulation of various ions during drought stress period is of great interest. The *A. spinosa* plants showed significant accumulation in leaf concentrations of inorganic ions K^+, Ca^{2+} and Mg^{2+} after withholding watering. As a necessary element for the physiological and molecular processes, Ca^{2+} plays an important role in the regulation of plant metabolism, growth and development. Yuan-Yuan et al. (2009) reported that Ca^{2+} can improve the hydrophobicity of the cell membrane, while reducing its permeability by direct effects as a structural basis to ensure a plant resistance to drought. K^+, the famous osmoticum, was strongly accumulated in stressed *A. spinosa* plants. Patakas et al. (2002) also reported a significant accumulation of K^+ in vine plants subjected to drought conditions and showed the contribution of this ion in the adjustment of osmotic potential. In addition to its role as an osmotic substance in maintaining osmotic balance and osmoregulatory strategy of plants, the increase in K^+ concentration can also lead to increased stomatal conductance since it plays an important role in the regulation of stomatal oscillations (Mahajan and

Tuteja, 2005; Nasri et al., 2008). A significant accumulation of the foliar concentration of Mg^{2+} in stressed plants during drought stress period was also recorded. Mg^{2+} is an essential macronutrient for plant growth, because it allows the activation of over 300 enzymes (as cofactor) and the synthesis of organic molecules necessary for the growth of plants (Wilkinson et al., 1990).

In fact, the high foliar concentrations of K^+, Ca^{2+} and Mg^{2+} recorded in stressed argan plants can be suggested as a physiological mechanism of tolerance that their role is to mitigate the negative effects of drought. In terms of comparison, both inland ecotypes seem be more tolerant considering their constitutive high concentrations of these ions. After rehydration, leaf concentrations of K^+, Ca^{2+} and Mg^{2+} were significantly reduced, by referring to the concentrations noted in drought stress period, before reaching levels converging to those noted in the control plants. The argan plants showed an alternative and quicker strategy to promote drought tolerance by accumulating sufficiently high levels of inorganic ions. Indeed, the energy cost of the osmotic adjustment using inorganic ions is much lower than that of the use of organic molecules synthesized in cells (Hu and Schmidhalter, 1998; Patakas et al., 2002). High concentrations of these elements in the leaves can help *A. spinosa* plants to overcome the negative effects of drought.

The glutathione-related enzymes GP, GR and GST showed a significant increase in their activity in *A. spinosa* plants subjected to drought stress. GR is a key enzyme in the ascorbate-glutathione cycle that protects cells against oxidative damage by maintaining a high ratio of GSH/GSSG. This enzyme has been shown to be activated in response to abiotic stress (Anderson and Davis, 2004). In our study, drought stress induced a significant increase in GR activity reflecting its important role in H_2O_2 scavenging according to Asada-Halliwell pathway in plant cells (Noctor et al., 2002). This response is intended to maintain a high ratio of $NADP^+$/NADPH, thus ensuring $NADP^+$ availability to accept electrons of photosynthetic electron transport chain and facilitate the regeneration of ascorbate oxidized (Noctor et al., 2002; Yang et al, 2008). Sofo et al. (2005) reported a significant increase in GR activity proportional to the severity of drought stress in plant leaves of four interspecific hybrids of *Prunus*.

GST isoenzymes in plants are known to function in the detoxification of herbicides, hormonal homeostasis, phytohormones transport, vacuolar sequestration of anthocyanins, tyrosine metabolism, detoxification of hydroperoxides, the regulation of apoptosis as well as in plant responses to biotic and abiotic stress (Dixon et al., 2002, 2010; Gill and Tuteja, 2010). Some GST isoforms exhibit activity of glutathione peroxidase, which suggests that their primary function may be to reduce toxic lipid peroxidation products and the maintenance of the membrane integrity under stress conditions, for example: osmotic stress (Dixon et al., 2003). In this study, a significant increase was detected in GST in plants of four contrasting ecotypes during drought stress confirming the protective role of GST in the argan tree.

Furthermore, the glutathione peroxidase activity increased significantly under drought stress conditions in plant leaves of all ecotypes. As GR and GST, the GP is a large family of various isoenzymes that use glutathione to reduce H_2O_2 and organic and lipid hydroperoxides, and therefore, protect plant cells against oxidative stress (Noctor et al., 2002). The overexpression of GP was found to improve abiotic stress tolerance in transgenic plants (Gill and Tuteja, 2010). The activity levels of GR, GST and GP appear to link directly to the degree of stress. Their activities increased in parallel with the increase of drought intensity and subsequently they reduced during rehydration. This response could limit cellular damage caused by ROS during drought stress period. Down regulation of these enzymes observed in rehydrated plants is probably due to a reduced need for the elimination of ROS.

During drought stress period, the content of total thiols in the leaves of *A. spinosa* plants increased significantly in the four ecotypes. This increase is mainly due to the significant increase in thiol protein content. The rate of protein thiols is a relevant marker of oxidative stress. However, the non-protein thiol content decreased significantly under drought stress conditions. The thiols are antioxidants that act through various mechanisms (Deneke, 2000). High levels of glutathione and other thiols have been associated with an increased tolerance to oxidative stress under abiotic stress conditions (Szalai et al., 2009; Nazar al., 2011). According to our results, the increase of the total thiols content could have signed the occurrence of oxidative stress and reflected the antioxidant capacity of thiols in the argan tree. These thiols seem to efficiently allocate antioxidant defense system in the argan tree under drought stress conditions. After rehydration, the decrease of the protein thiols can be explained by the fact that the thiol groups are oxidized by reducing free radicals and these denatured proteins are removed.

A good separation between the four contrasting ecotypes of *A. spinosa* was obtained taking into account the canonical plots (Figures 5 and 6). The vertical separation in the four analyses was established by the first DF which it quantifies the greatest 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. According to the canonical plot, the first discriminant function has separated the inland ecotypes from the coastal ecotypes, while the second discriminant function has been attributed to distinguish between both coastal ecotypes on one hand and another hand between both the inland

ecotypes under drought and rehydration conditions, respectively. Both inland ecotypes (Lks and Alz) were clearly separate from both coastal ecotypes (Adm and Rab), under drought conditions, especially by high concentration of non-proteins thiol and Ca^{2+} and high GST activity. These traits reflected a significant antioxidant capacity and good membrane stability, justified by the high Ca^{2+} content, in both inland ecotypes. This permits us to suggest that the inland ecotypes studied are more tolerant to drought stress than coastal ecotypes. During rehydration conditions, both inland ecotypes have shown good recovery, compared to other ecotypes, but in different way. In fact, these both ecotypes have reacted differentially under rehydration condition. Taking into account the second discriminant function and the traits having the most discriminating power: GR and GST; Lks ecotype seems to possess a very effective antioxidant defense system ensuring a good and quick recovery.

Conclusion

The results obtained in this study in pots showed significant differences in physiological and biochemical responses of the four contrasting ecotypes of *A. spinosa*. These ecotypes experimentally exposed to edaphic drought induced by cessation of irrigation followed by rehydration in order to evaluate the potentialities and the flexibility of tolerance in the argan tree. Intra-specific differences were observed in the traits referring to the ionic state, antioxidant system and oxidative damage. Indeed, the drought stress induced significant changes in the studied traits. However, the kinetics of recovery in *A. spinosa* plants after rehydration was rapid; it was manifested by significant levels close to those recorded in the control plants only after four days. According to the canonical discriminant analysis, the inland ecotypes, especially Lks, were clearly distinguished from others ecotypes by high antioxidant capacity switch connected with the high activity of glutathione system enzymes. In terms of degree of drought tolerance, Lks ecotype showed a better upregulation of its protective mechanisms compared to other ecotypes. The research results are constructive for contribution to select tolerant ecotypes in order to develop the Argan sector.

Conflict of Interests

The authors have not declared any conflict of interests.

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