RESEARCH ARTICLE

DIFFERENTIAL DROUGHT TOLERANCE OF FOUR CONTRASTING *ARGANIA SPINOSA* ECOTYPES ASSESSED BY ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANTS

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ABSTRACT

Argan tree plays a great socio-economical and ecological role in the arid and semi-arid zones of Southwest Morocco. The objective of this study was to characterize biochemical tolerance mechanisms of *Argania spinosa* under drought stress. Changes of ascorbate-glutathione cycle enzymes and non-enzymatic antioxidants were investigated for the first time in four contrasting ecotypes (Lks, Alz, Rab and Adm). Eighteen-month-old *A. spinosa* plants were subjected to drought stress (50% and 25% field capacity) during two months. Intra-specific differences were observed in all parameters studied. Under drought stress, inland ecotypes showed a significant increase in activity of ascorbate-glutathione cycle enzymes: ascorbate peroxidase (APX), monodehydro-ascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) compared to coastal ecotypes. Non-enzymatic antioxidants, namely ascorbic acid (AA), -tocopherol (-toc) and reduced gluthatione (GSH) were increased significantly in drought-stressed plants. Under severe drought stress, we recorded a strong induction of enzymatic and non-enzymatic defense systems. The four study ecotypes were separated by canonical discriminant based mainly on the following biochemical parameters: DHAR, GSH and -toc. Lks ecotype was distinguished from the other ecotypes by a significant increase of antioxidant enzymes and metabolites, suggesting a better ability of drought tolerance.

INTRODUCTION

Drought stress is one of the main environmental factors that negatively affect plant growth and productivity in various regions of the world. It actively and continuously determines the natural distribution of plant species. 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. Plant responses to drought stress are species- and genotype-dependent characteristics. Trees species cannot escape from drought as annual plants and have developed appropriate drought-tolerance mechanisms to cope with temporary water limitations to ensure their survival and reproduction (Pita *et al*. 2005).Increasing crop tolerance to drought stress would be the most economical approach to improve agricultural productivity and to reduce agricultural use of freshwater resources. Understanding the mechanisms of drought tolerance has been a major goal of plant biologists and crop breeders (Xiong *et al*. 2006; Islam *et al*. 2011; Baloglu *et al*. 2012). Drought-stress tolerance involves subtle changes in the biochemical processes of plants. Biological molecules are

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susceptible to attack by reactive oxygen species (ROS), including several proteins, polyunsaturated fatty acids and nucleic acids (Pandhair and Sekhon 2006). Drought stress leads to an imbalance between antioxidant defenses and the amount of ROS resulting in oxidative stress such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxylradicals, which are toxic for plant cells (Noctor *et al.* 2014). To maintain homeostasis and prevent oxidative stress, the plants evolved a scavenging system composed of non-enzymatic radical scavengers such ascorbic acid (ASA), -tocopherol (-toc) and reduced gluthatione (GSH), and enzymatic antioxidants such Ascorbate-glutathione cycle enzymes (Foyer-Halliwell-Asada cycle) including ascorbate peroxidase (APX), monodehydro-ascorbate reductase (MDHAR), dehydro-ascorbatereductase (DHAR) and glutathione reductase (GR) (Noctor and Foyer 1998; Asada, 1999). ASA, and GSH and -toc serve as potent non-enzymatic antioxidants of defense system within cell. Thus, the Ascorbate-glutathione cycle is an important pathway of indirect and direct ROS scavenging in cells (Foyer and Noctor 2005*a*, 2005*b*). APX is an important enzyme participating in cell detoxification due to its presence in al cell compartments and its high affinity for hydrogen peroxide (H_2O_2). The electron donor is ASA, the pool of which is regenerated by MDHAR and DHAR with the participation of GSH and NADPH. GSH is oxidized o GSSG and reduced back to GSH by GR. Besides participating in the Ascorbate-glutathione cycle, ASA is able also to reduce the oxidized form of -toc, an important antioxidant associated with cell membranes (Noctor and Foyer, 1998; Asada, 1999). An increased antioxidantcapacity is a well-known adaptive mechanism for responding to drought conditions (Ratnayaka *et al*. 2012; Uzilday *et al*. 2012). Increased expression or activities of major antioxidative enzymes is often taken as an indicator of increases in ROS (Noctor *et al*. 2014).

Nevertheless, there is still insufficient knowledge on biochemical mechanisms describing the responses of *A. spinosa* to drought stress. The argan tree (*Argania 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). This tree has important socio-economical 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). About 1.3 million people are living in rural areas where traditional sylvo-pastoral systems are based on the argan tree (Chaussod *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. The understanding of the biochemical adaptive mechanisms controlling drought tolerance of *A. spinosa* is the major our aim to suggest criteria for early selection of the most tolerant ecotypes to be planted in areas exposed to drought risk and to improve productivity. In this study, our aims are to (i) characterize the impact of drought stress on the production of enzymatic and non-enzymatic antioxidants and examine if this antioxidative mechanism could be operational in the leaves of *A .spinosa* plants exposed for 2 months to drought stress.(ii)assess differences in biochemical parameters studied between four contrasting *A. spinosa* ecotypes and (iii)

determine indices that can used as biochemical markers of cell redox state in *A. spinosa* to select tolerant ecotypes.

MATERIALS AND METHODS

Study site and sampling procedures

Sampling of seeds of *A. spinosa* was conducted in four regions of the argan tree forest in the south-west of Morocco: Essaouira, Agadir, Taroudant and Tiznit. Climatic, geographical and hydrological conditions of these four regions are markedly different (Table 1 and Fig 1). We chose two 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 biochemical processes. After stratification in moist compost (3weeks), pre-germinated seeds are transferred into alveolate containers with rigid walls characterized 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 plastic pots filled with a mixture of soil and peat 1/1 (v/v) to which 10% 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 chamber during the experiment were maintained at 28 ± 1 °C temperatures during day and 25 ± 1°C during night in a16:8 photoperiod and the relative humidity ranged between 65 and 70 %. The average maximum photosynthetically active radiation (PAR) was 400µmol m^{-2} s^{-1} provided by a combination of fluorescent and incandescent lamps. Uniform young *A. spinosa* plants of similar height, aged eighteen months, were selected for the experiment for each ecotype. The experimental layout was completely randomized with three factors (ecotype, time and watering regime). The plants were selected randomly to three different watering regimes as follows: one well watered treatment (100% of field capacity (FC)) corresponding to the control and two water-stressed treatments (50 and 25% of FC) which correspond to medium and to severe stress, respectively. The treatments were applied for 2 months and each treatment included fifteen plants for each ecotype. The fully expanded young leaves of each replication were harvested from plants every fortnight during the experimental period to determine biochemical parameters.

Ascorbate-glutathione cycle. Enzymes extraction

For the enzymes activity determination, 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 by homogenizing on ice the powder (0.1 g for each enzyme x 5 replicates per treatment) in 50 mM K2HPO4 /KH2PO4 buffer (pH 7.8) 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 the 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.

Activity of ascorbate-glutathione cycle enzymes

The Ascorbate peroxidase (APX, EC 1.11.1.11) was measured by monitoring the initial ascorbate oxidation by H_2O_2 at 290 nm (Extinction coefficient (E) = 2.8 mM^{-1} cm^{-1}) (Nakano and Asada 1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1 mM sodium ascorbate, 0.1 mM EDTA and enzyme extract. The reaction was started by the addition of 10 mM H_2O_2. APX activity was expressed in nmol ascorbate (AsA) per min per mg of protein. There were 5 replicates per treatment (one plant per replicate).

The 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 (E=6.2 mM^{-1} cm^{-1}) (Edwards et al. 1990). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 0.2 mM NADPH, 1 mM oxidized glutathione (GSSG) and enzyme extract. GR activity was expressed in nmol oxidized GSSG per min per mg of protein. There were 5 replicates per treatment (one plant per replicate).

The monodehydroascorbate reductase (MDHAR; 1.6.5.4) was measured as ß-nicotinamide adenine dinucleotide (NADH) oxidation at 340 nm (E=6.2 mM^{-1} cm^{-1}) (Hossain et al. 1984). The reaction mixture contained 50 mM K_2HPO_4 /KH_2PO_4 buffer (pH 7.8), 1.5 mM ascorbate, 0.5 unit's ascorbate oxidase, 0.1 mM NADH and enzyme extract. Correction was made by subtracting values obtained in the absence of ascorbate oxidase. MDHAR activity was expressed in nmol ascorbate produced per min per mg of protein. There were 5 replicates per treatment (one plant per replicate).

The dehydroascorbate reductase (DHAR; EC 1.8.5.1) was determined by following the increase of ascorbate formation at 265 nm (E= 14 mM^{-1} cm^{-1}) (Nakano and Asada 1981; Hossain et al. 1984). The reaction mixture contained 50 mM K_2HPO_4 /KH_2PO_4 buffer (pH 7.0), 1 mM reduced glutathione (GSH), 0.5 mM dehydroascorbate acid (DHAsA) and the enzyme extract. The reaction rate was corrected for the non-enzymatic reduction of DHAsA by GSH. DHAR activity was expressed in nmol NADH per min per mg of protein. There were 5 replicates per treatment (one plant per replicate).

Non-enzymatic antioxidants: Ascorbic acid, reduced glutathione and -Tocopherol

The content of ascorbic acid (AA) was estimated as described by (Omaye et al. 1979; Jaleel et al. 2008) with some modifications. Extract was prepared by homogenizing the aliquots of frozen powder in 10% TCA and centrifuged at 15,000 x g for 15 min. The extract (0,5 ml) was treated with 1 ml DTC reagent (2,4-dinitrophenyl hydrazine-thiourea-$CuSO_4$ reagent), incubated at 30°C for 3 h and 0.5 ml of ice-cold 65% H_2SO_4 was added. The reaction mixture was allowed to stand at 30°C for 30 min. The absorbance at 520 nm was determined and the AA content was derived from a standard curve prepared with known amount of AA and the results were expressed in µmol g^{-1} DW. There were 5 replicates per treatment (one plant per replicate).

The content of reduced glutathione (GSH) was assayed as described by (Griffith and Meister 1979; Jaleel et al. 2008) with some modifications. Extract was prepared by homogenizing the aliquots of frozen powder in 2ml of 2% metaphosphoric acid and centrifuged at 15,000 x g for 15 min. 0.5 ml 10% sodium citrate was added to neutralize the supernatant. The reaction mixture contained 0,1 ml extract, 0,1 ml distilled water, 0,1 ml 5,5-dithio-bis-(2-nitrobenzoic acid) and 0,7 ml NADPH. The reaction was allowed for 4 min at 25 °C and then 10 µl of glutathione reductase was added. The absorbance at 412 nm was determined and the GSH content was derived from a standard curve prepared with known amount of GSH and the results were expressed in µmol g^{-1} DW. There were 5 replicates per treatment (one plant per replicate).

The content of -tocopherol (-toc) was estimated as described by (Backer et al. 1980; Jaleel et al. 2008) with some modifications. Extract was prepared by homogenizing the aliquots of frozen powder in a mixture of petroleum ether and ethanol (2:1.5 v/v) and centrifuged at 15,000 x g for 15 min. The reaction mixture contained 1 ml extract and 0.2 ml of 2% 2,2dipyridyl in ethanol. The reaction was mixed thoroughly and kept in dark for 6 min. The resulting red color was diluted with 5 ml of distilled water and mixed well. The absorbance at 520 nm was determined and the -toc content was derived from a standard curve prepared with known amount of -toc and the results were expressed in µmol g^{-1} DW. There were 5 replicates per treatment (one plant per replicate).

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

Statistical analysis

All data were subjected to a three-way analysis of variance (ANOVA) to test the effect of ecotype, watering regime and time in each the biochemical study variables. Means were separated using the Tukey's Post hoc test. A Pearson correlation analysis was done for some variables for each ecotype. In order to determine which variables discriminated between A. spinosa ecotypes, a canonical discriminant analysis (CDA) was carried out on biochemical parameters. Statistical tests were considered significant at $P < 0.05$. All statistical analyses were performed with SPSS 10.0 for Windows.

RESULTS

Enzymatic antioxidants

As shown in figure 2, activities of the enzymes related with ascorbate–glutathione cycle showed similar patterns of change. We found that drought stress significantly increased the activities of antioxidant enzymes and the content of non-

enzymatic antioxidants in *A. spinosa* plants, and highly significant differences between drought stress levels were observed ($P < 0.05$). The ascorbate-glutathione cycle enzymes APX, MDHAR, DHAR and GR showed significant differences in terms of activity among ecotypes ($P < 0.05$) (tab). The constitutive activities of these enzymes were higher in inland ecotypes than in coastal ecotypes during the experimental period. In all ecotypes, the ascorbate-glutathione cycle enzyme activities was significantly increased in the range of drought periods between 45 and 60 days with a slight decrease at 60th day, for some enzymes in some ecotypes, particularly under severe stress and a highest activity of these enzymes was registered in Lks (Fig 2). Under medium drought stress, we noted an obvious increase of the activities of these enzymes compared with control. At 15 days, we started to note a significant stimulation of the activity of ascorbate-glutathione cycle enzymes.

significant time x watering regime interaction was observed for all antioxidant enzyme activities studied ($P < 0.05$). Ecotype x watering regime interaction was only significant for DHAR ($P < 0.05$), while ecotype x time interaction was only significant for one parameter (APX) ($P < 0.05$). Ecotype x watering regime x time interaction was not significant for all these parameters ($P < 0.05$).

Non-enzymatic antioxidants

The non-enzymatic antioxidants content was found to increase significantly when drought stress intensified. For all ecotypes, also the highest accumulations of GSH, AA and -toc were recorded in 45-60 day drought period for both watering regimes (Fig 3). Drought stress induced an obvious production of GSH, AA and -toc compared to the control.

Table 1 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: average minimum temperature of the coldest month of the year and M: average maximum temperature of the coldest month of the year
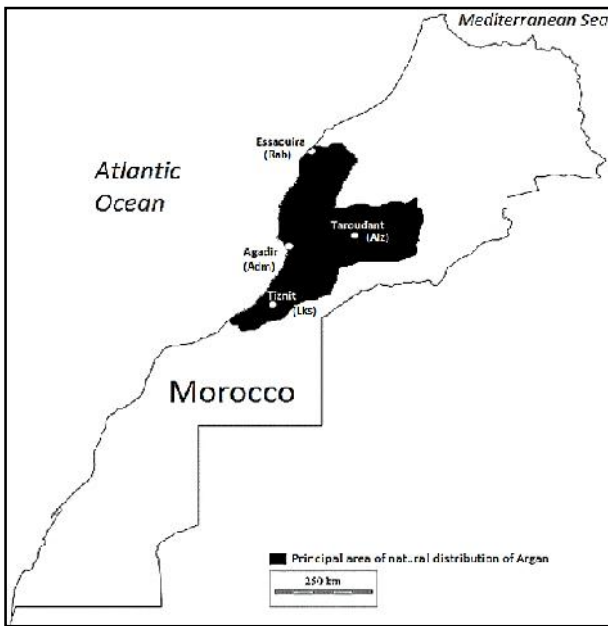

Figure 1 Geographical provenances of *A. spinosa* ecotypes sampled and studied.Rab, Adm, Alz and Lks ecotypes from Essaouira, Agadir, Taroudant and Tiznit provenances, respectively.

Figure 2 Effect of drought stress on APX activity (A), GR activity (B), MDHAR activity (C) and DHAR activity (D) in leaves 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 Control: C, Medium Stress: MS and Severe Stress: SS, respectively) for 2 months. 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).

Leaf APX, MDHAR, DHAR and GR activities were affected by both watering regimes. At 45th day, APX activity was increased 2-3 fold compared with the control in the ecotype studied under severe drought stress, while GR activity was increased 42.2, 45.0, 42.7 and 33.8% in Alz, Lks, Rab and Adm, respectively. An increase of approximately 19-20 % was noted in MDHAR activity, whereas DHAR activity was increased 40.6, 45.8, 48.4 and 44.0% in Alz, Lks, Rab and Adm, respectively, compared to the control. At the same day, we detected an increase (1.4-2 fold) in APX activity and an increase of about 10 to 28% in GR, MDHAR and DHAR activities, under medium drought stress, compared to the control. According to Three-way ANOVA analysis, a
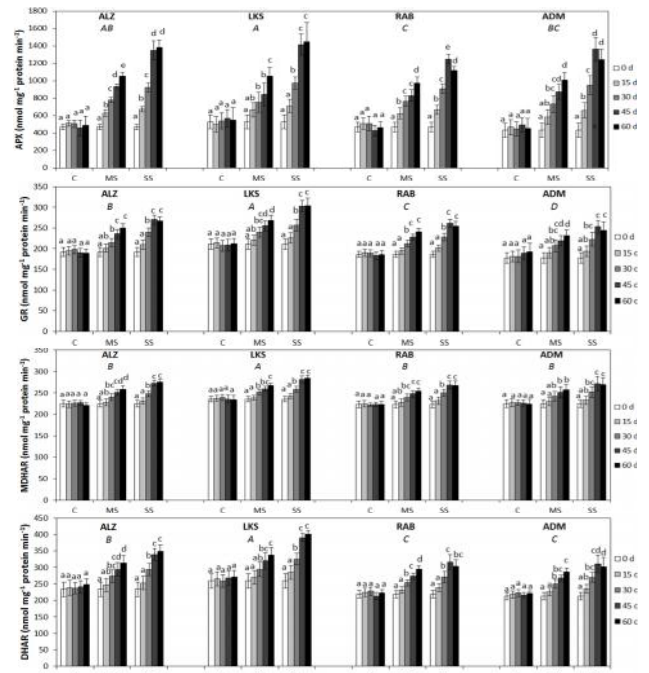
As well as the results obtained of enzymatic antioxidants, we detected significant changes in the non-enzymatic antioxidants content from the 15th day. At 45th day, GSH and -toc contents were increased 1.6-2.5 fold under severe drought stress and 1.3-1.8 fold under medium drought stress, compared with to the control. AA content was increased 11.5, 19.4, 22.5 and 17.5%

in Alz, Lks, Rab and Adm, respectively, under severe drought stress while we noted an accumulation of about 8 to 11% in inland ecotypes and an increase of approximately 13 to 14% in coastal ecotypes under medium drought stress, compared to the control. According to three-way ANOVA analysis, there were significant differences between ecotypes, watering regime, time and the interactions between them. A significant time x watering regime interaction was recorded for the three non-enzymatic antioxidants studied ($P < 0.05$). Ecotype x watering regime interaction was only significant for GSH and -toc ($P < 0.05$), while ecotype x time and ecotype x watering regime x time interactions were not significant for these parameters ($P < 0.05$).
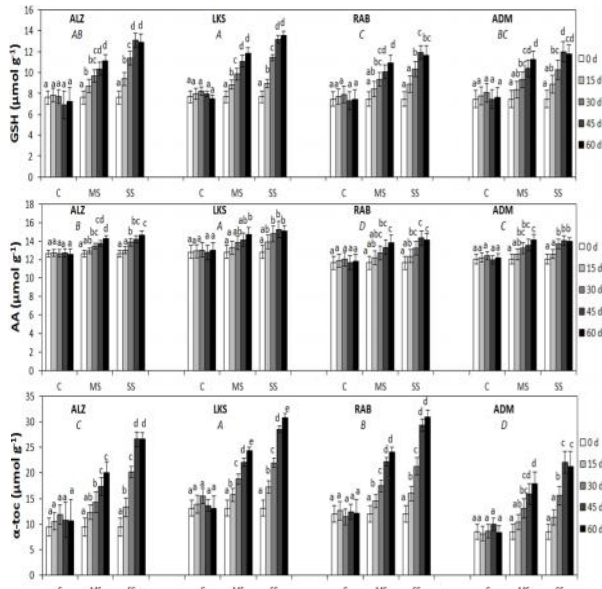

Figure 3 Effect of drought stress on concentration of GSH (A), AA (B) and -toc (C) in leaves of four *A. spinosa* ecotypes. Eighteen-month-old *A. spinosa* plants were exposed to three water regimes (100, 50 and 25% of FC corresponding to Control: C, Medium Stress: MS and Severe Stress: SS, respectively) for 2 months. 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).
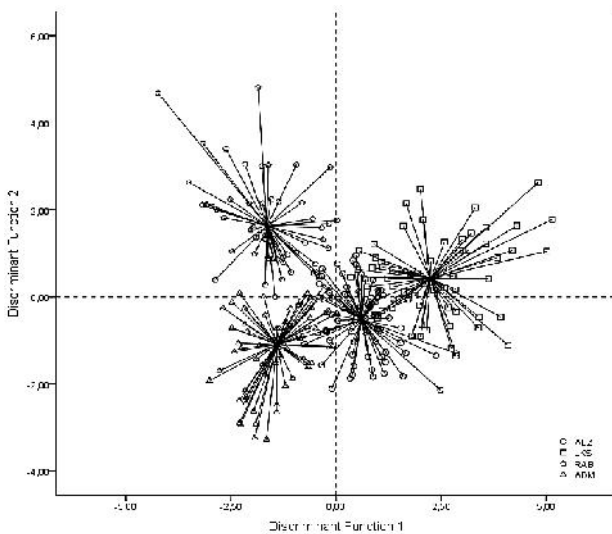

Figure 4 2D scatterplot showing the distribution of the study ecotypes along the two discriminant function gradients obtained by canonical discriminant analysis (CDA) for biochemical parameters.

Canonical Discriminant Analysis

A Canonical Discriminant Analysis (CDA) was performed using these biochemical parameters (7 variables) (CDAb) 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 of CDA confirmed the existence of differences in global characteristics of ecotypes. Wilk's lambda denoted a high significance of the model (Wilks's = 0.15) and calculated *F*-value also indicates significance ($P < 0.0001$) for this analysis. Three discriminant functions (DF) were calculated, accounting for 70.8%, 25.2% and 4.0% of the total variance. The ^{2}-test showed for both analyses a significant discriminatory power for the three functions ($P < 0.001$). The eigenvalues of the first two functions (2.27 and 0.81, respectively) showed them to explain most of variance (96.0 %) and their canonical correlations were $r_1 = 0.83$ and $r_2 = 0.67$. The 2D scatterplot of discriminant space (Fig 4) display the distribution of the samples spanned by the first two functions. Based on the standardized coefficients of the canonical discriminant functions of CDA, DHAR activity and AA contentwere highly weighted in the positive part of CDA-1 while GSH content and MDHAR activity in the negative part. -toc was the biochemical characteristic most strongly weighted to positive part of CDA-2, whereas APX activity and GSH content were highly weighted in the negative part. The inland ecotypes (Lks and Alz) were clearly distinguished from the coastal ecotypes by the first DF, while Rab and Lks were distinguished from other ecotypes by the second DF. Equal numbers of plants was compared in each ecotype.

DISCUSSION

Drought stress is considered as the main environmental factor of growth, development and yield limitations in arid and semi-arid areas. Drought stress promotes the production of reactive oxygen species. They are highly active molecules that can easily damage membrane and other cellular components (Mittler 2002; Apel and Hirt 2004; Pandhair and Sekhon 2006; Møller *et al*. 2007). Plants have developed its own machinery to overcome drought stress through production of enzymatic and non-enzymatic antioxidants as a defense system for scavenging and detoxifying ROS. The ascorbate-glutathione cycle shall be considered an efficient antioxidant system in the detoxification of H_2O_2, involving four enzymes: APX, GR, MDHAR and DHAR (Noctor and Foyer 1998; Asada 1999). The cycle maintains a ratio of a reduced per oxidized ascorbic acid and glutathione for proper scavenging ROS in plant cells (Mittler 2002). The increased activity of these four enzymes in *A. spinosa* leaves of the four ecotypes, under moderate and severe drought stress (Fig 2), could maintain H_2O_2 detoxification. Previous researchs had shown that drought stress can cause an increase in the ascorbate-glutathione cycle enzymes in many plants including apple (Ma *et al*. 2011), sunflower and sorghum (Zhang and Kirkham 1996a, 1996b) and *Prunus* hybrids (Sofo *et al*. 2005). Also, in a recent study, (Zhang *et al*. 2014) reported, in two apple rootstocks, that the drought stress induced markedly increases in the activity of these four enzymes. Our studies showed that the different responses of these enzyme activities to drought stress may depend on ecotype, drought stress period, stress severity and intensity of ROS production. The constitutive and inducible

activities of these enzymes were higher in inland ecotypes, especially in Lks, than in coastal ecotypes. The result suggests that inland ecotypes have a high scavenging capacity of ROS compared to other ecotypes. Furthermore, transcriptional changes in ascorbate-glutathione cycle enzymes were studied by detection of their gene expression level under drought stress and a significant gene expression was registered indifferent species, including apple (Ma *et al.* 2011) and *Triticum aestivum* (Secenji *et al.* 2008) and. GR plays a very important role in maintaining high ratio of NADPH/NADP$^+$ in the cell and a crucial role in the generation of GSH from GSSG allowing GSH to be used by DHAR to reduce DHA to AsA, while APX is the major enzyme responsible for the elimination of H_2O_2. The oxidized ascorbate forms (monodehydroascorbate and dehydroascorbate) are recycled to reduced ascorbate via two pathways catalyzed by MDHAR and DHAR, respectively, consuming NAD(P)H and reduced glutathione as electron donors (Noctor and Foyer 1998; Asada 1999; Mittler 2002; Møller *et al.* 2007). (Cia *et al.* 2012) reported that the RG and APX activities changed in sugarcane varieties according to variety and drought stress intensity.

In addition of enzymatic system, drought stress has led to an arsenal non enzymatic antioxidant defense to counter the phenomenon of oxidative stress in *A. spinosa* plants. A significant increase in AA, GSH and -toc in all ecotypes was observed in response to drought stress. These non-enzymatic antioxidants have been reported to increase under drought stress in sunflower and sorghum (Zhang and Kirkham 1996b) and apple (Ma *et al.* 2011). AA is an important antioxidant, which reacts not only with H_2O_2 but also with O^{2-}, OH and lipid hydroperoxidases (Foyer and Noctor 2005a, 2005b). Therefore, AA influences many enzyme activities and minimizes the damage caused by oxidative process through synergic function with other antioxidants (Foyer and Noctor 2005a, 2005b; Shao *et al.* 2008). -toc is major lipophilic antioxidant synthesized by all plants. -toc interact with the polyunsaturated acyl groups of lipids, stabilize membranes, and scavenge and quench various ROS and lipid soluble byproducts of oxidative stress and in this case the reduced scavenging form may be regenerated by ascorbate or other antioxidants (Foyer and Noctor 2005a, 2005b; Shao *et al.* 2008). GSH takes part in the control of H_2O_2 levels. GSH is involved directly in the reduction of most active oxygen radicals generated due to stress (Foyer and Noctor 2005a, 2005b; Shao *et al.* 2008). Mutants with decreased non-enzymatic antioxidant contents have been shown to be hypersensitive to stress (Sharma *et al.* 2012).

The increase of enzymatic and non-enzymatic antioxidants during the progression of stress suggests a strict relationship of these antioxidants with drought stress conditions in the four *A. spinosa* ecotypes studied. Comparative study of the antioxidant responses in drought tolerant and drought sensitive genotypes revealed higher antioxidant capacity in tolerant genotypes (Sharma *et al.* 2012). Hence, these both mechanisms that reduce oxidative stress may play an important role in drought tolerance of *A. spinosa*. Our results showed that the ascorbate-glutathione cycle enzymes and non-enzymatic metabolites were obviously upregulated in *A. spinosa* leaves under severe drought stress, suggesting an overexpression of genes related

to antioxidant system defense. The improved tolerance to drought in *A. spinosa* might be correlated to the increased capacity of antioxidative defense system.

The 2D scatterplot of discriminant space relative to two discriminant functions of biochemical parameters is presented in (Fig 4). This scatterplot shows a good separation among study ecotypes of *A. spinosa*. The horizontal separation was characterized in the first DF. Thus, first DF quantifies the degree to which all ecotypes differ in biochemical parameters, which we argue to be the result of differences in their adaptation and tolerance under drought stress. The inland ecotypes were mainly separated from the coastal ecotypes by higher GSH content and higher DHAR activity, confirming the high values recorded in experimental period (Fig 2 and Fig 3). Taking account the second DF, the Lks and Rab were slightly from the other ecotypes by a high accumulation of - toc, which Rab ecotype was mainly distinguished from the other ecotypes by a lower APX activity and lower AA content. Our results suggest that the inland ecotype have a good drought tolerance and are equipped with a very effective antioxidant system, especially the Lks ecotype.

CONCLUSION

In summary, a biochemical approach has been adopted here to demonstrate changes in antioxidative mechanism in the leaves of *A .spinosa* plants exposed for 2 months. Intra-specific differences were observed in enzymatic and non-enzymatic defense systems between *A. spinosa* ecotypes. The present results suggest that genetic influence is very obvious, because it was the same environmental conditions within the growth chamber. Drought stress induced in *A. spinosa* a significant increase in the non-enzymatic antioxidants that act as ROS scavengers either in conjunction with the antioxidative enzymes or independently. Based on our research results from this preliminary pot-study, it is clear shown that the ability of drought tolerance of the four contrasting ecotypes studied is different. The inland ecotypes seem more drought-tolerant than coastal ecotypes. They showed a great response in terms of antioxidative capacity, especially for Lks ecotype. DHAR, GSH and -toc could be considered as biochemical markers to select the tolerant ecotypes of *A. spinosa* for any project of regeneration and/or domestication in arid and semi-arid areas.

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