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EFFECT OF WATER DEFICIENCY ON THE CELLULAR STATUS AND ANTIOXIDANT DEFENCES IN *ANTHYLLIS SERICEA*, A SAHARIAN PLANT

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Abstract

Drought is known as an important restricting factor of plant productivity in arid and semi arid areas of the world. The intended increase of temperature in many areas will intensify this problem. In this study the effect of drought stress was studied in a Saharan plant, *Anthyllissericea*, by Poly-ethylene glycol (PEG-6000) in three different treatments (-0.2 MPa (control), -1.2 MPa (moderate stress) and -2.1 MPa (severe stress)) after 14 days. Nitric oxide (NO) content, Hydrogen peroxide (H_2O_2), RWC, lipid peroxidation and enzymatic antioxidant levels from the leaves were analyzed. Initially, plant growth, RWC and the water potentiel (Ψw) were decreased with increase of osmotic stress. Drought induces the increase of NO and hydrogen peroxide levels reaching maximum in severe stress period. MDA, proline content and soluble sugars were found to be higher under moderate and severe stress conditions. Plant employs enzymatic antioxidant system to avoid the subproduction of (ROS) resulting by drought. The analysis of CAT, APX and POD activities showed a significant increase during drought stress. Under moderate and severe stress treatments, the higher activities of H_2O_2, NO, CAT and POD showed a stronger system of antioxidant defences in the metabolic regulation during the applied stress. These results propose that *A. sericea* has the capacity to activate important adaptative mechanisms under dry conditions involving activation of enzymatic antioxidative defense system and higher osmoprotectants accumulation.

Key words: *Anthyllis sericea*, Drought stress, Osmotic adjustment, Antioxidants, Nitric oxide and hydrogen peroxide.

Abbreviations: ASC, ascorbic acid; APX, ascorbate peroxidase, CAT, catalase; DW, Dry weight; FW, fresh weight; H_2O_2, hydrogen peroxide; MDA, malondialdehyded; NO, nitric oxided; POD, peroxidase; ROS, reactive oxygen species; RWC, relative water content; TW, turgid weight,

Introduction

A number of abiotic stress factors, such as, salt, temperature and drought negatively effect the over all plant growth (Waheed *et al*., 2016).Plants can tolerate, avoid or escape water stress according to the type of strategy adopted (Harb & Sarnarah, 2015) to survive for long periods. Drought stress affects strongly all metabolic aspects of the plant involving changes in morphological, physiological and biochemical levels. The ability of the plants to adopt the drought stress has become very interesting. The marked mechanisms developped by desert plant species in extreme environnemental conditions are, either the osmotic pressure on cytoplasm allowing cell turgescence (Chaves *et al.,* 2003; Bartels & Sunkar, 2005) or the transforming of their phenology choosing the short life cycle and reducing leaf surface. The stomatal closure causes under stress the higher level of leaf water potential and hence leaf water content; however this ones reduces leaf photosynthetis and limits the internal assimilation of CO_2 (Hare *et al.,* 1998).

One of the known effects of drought in different plant species is the increasing production of the reactive oxygen species (ROS) which may cause several cellular damages (Sharma & Dubey, 2005). ROS are inevitable products of biological mechanisms and can promote a direct and indirect role in the metabolism. Acclimation of desert plant species to extreme dry environments is associated with the accumulation of ROS species, which can initiate the damage of cellular membranes and nucleic acids (Halliwell & Gutteridge, 2007) and can be a regulator of biological processes in plant (Miller *et al.*, 2010).

Drought leads to activate the antioxidant systems (Zhu, 2002) which include the enzymatic antioxidants such as, superoxidismutase (SOD), APX and POD, and the non enzymatic compounds such as the ascorbic acid (AA) and the gluthanione (GSH). Superoxidismutase (SOD) is the first enzyme of cell defence against the ROS that being catalizes into H_2O_2 which than be scavenged by CAT and peroxidases (APX, POD) to H_2O (Noctor& Foyer, 1998). Under drought conditions, the enzymatic antioxidants can be increased or decreased or remain unchanged, depending on, species, duration of the stress and rapidity of the application.

Nitric oxide (NO) is an important molecule in a large number of physiological mechanisms under biotic and abiotic stress conditions (Siddiqui *et al.,* 2010), recent studies showed the significant if role of NO in plant's adaptation under water deficit. Some reports demonstrated that drought stress result a considerable NO production in the cucumber roots (Arasimowicz-Jelonek *et al.,* 2009) and in grapevine leaves (Patakas *et al.,* 2010). Talbi *et al.* (2015) reported the similar findings in *Oudenya* leaves. NO can have a protective role against the environmental stress factors as it defends the toxic damages of ROS (Qiao & Fan, 2008) by rising the production of antioxidant enzymes (Neill *et al.*, 2008). The feedback between the NO and the reaction oxygen species has been documented and ROS/NO rate can produce tolerance against drought stress, Tanou *et al.* (2009).

Another known stress defense processes of plants to cope with the extreme dry conditions is the production of proline, betaine and glycine, sugars and phenols, are the typically drought response so as to save there turgidity and the metabolic processes. The proline had been suggested an important osmoprotectant that stabilize protein structure and defend membranes from lipid peroxidation during drought periods. Proline provides the cellular turgescence and protective effects (Szabados & Savouré, 2010) against the ROS damages.

Biochemical and physiological mechanisms in desert plant species adapted to extreme dry conditions needed to be more highlighted. This study attempts to evaluate the enzymatic antioxidants, ROS production and physiological responses under three treatments of deficit water in *Anthyllis sericea*. Hence, it is the first report aims to bring to light the physiological and biochemical mechanisms associated with drought tolerance in this specie. *Anthyllis sericea* is an endemic Saharan plant, belongs to the leguminoseae family, distributed in the Mediterranean regions and indigenous to Libyan, Algerian and Tunisian desert where it has a various ecological and geographical distribution. It grows under severe environmental conditions. It is 5-40 cm tall. The stem is simple and much branched. The leaves are imparipinnate. The red flowers heads are spherical. In Tunisia, it is found almost exclusively on limonestone crusts eventually topped with a loamy or sandy soil often very thin (Chaieb & Boukhris, 1998). The range of this species is essentially included in the Saharan bioclimate superior, where the annual rainfall is between 50 and 100 mm. This spiny shrub is used in the folk medicine for inflammation treatment (Nartowska*et al.*, 2001) and also used for cattle and sheep feeding.

In this paper we aimed not only to evaluate the adaptative processes involved in plant survival under severe drought conditions, such as, growth parameters, water relations, MDA level, hydrogen peroxide and nitric oxide, osmotic adjustment content and antioxidant activities of CAT, APX and POD, but also to highlighting some new approaches for further genetic studies to enhance drought tolerance in some sensitive cultivated species. This study can be very useful in creating new methods for durable agriculture in arid regions using endemic species.

Materials and Methods

Plant material and drought stress application: Seeds of *A. sericea* were collected in June 2012 from Tunisian Sahara region «Dhahar» (32°30' 00"N, 9°50' 00"W) (Fig. 1). Seeds were disinfected in calcium hypochlorite solution (5%) for 5 min, sown in alveolar plates full of with loam and placed at controlled room (Maraghni *et al.*, 2010). The seedlings were transferred in PVC pots, placed in controlled growth room with temperature adjusted at 25/30°C, humidity was between 60 and 70% and the phytosynthetically radiation was adjusted at 250 µmol $m^{-2}s^{-1}$. The seedlings were grown in a nutrient solution that containing the macronutrients: $MgSO_4$, KH_2PO_4, K_2HPO_4, KNO_3, NH_4NO_3, Ca $(NO_3)_2$, and micronutrients: $MnCl_2$, $CuSO_4$ $5H_2O$, $ZnSO_4$ $7H_2O$, MO_7O_{24} $(NH_4)_6$, H_3BO_3, and 45 µM EDTA-Fe (Arnon & Hoagland, 1940). The pH was kept at 6. The experiment was placed in a growth chamber with three levels of drought treatment and three replicate plants in each treatment. After one month, the seedlings were irrigated with PEG solutions at different water potentials of -0.2 MPa (control), -1.2 MPa (moderate stress) and -2.1 MPa (severe stress) for 14 days. The concentration of PEG-6000 solution was determined following to Michel & Kaufmann (1973). After 14 days of culture under different drought treatments, leaves and roots were harvested. Number of leaves and fresh weights of shoots and roots were determined. Leaves and roots were frozen, powdered and at -80°C and stored until analysis.

Plant water relations: Plant water status was determined by measuring water potential (Ψw) with Scholander pressure chamber Instrument (USA) and relative water content with RWC = (FW - DW)/(TW - DW) ×100, where TW is the turgid weight and FW, DW are the fresh and the dry weights respectively. *Anthyllis sericea* leaves were harvested and weighed immediately to determine the fresh mass. Leaves were immersed over night in distilled water to calculate the turgid mass. At 80°C, leaves were dried for 48 h and DW was determined.

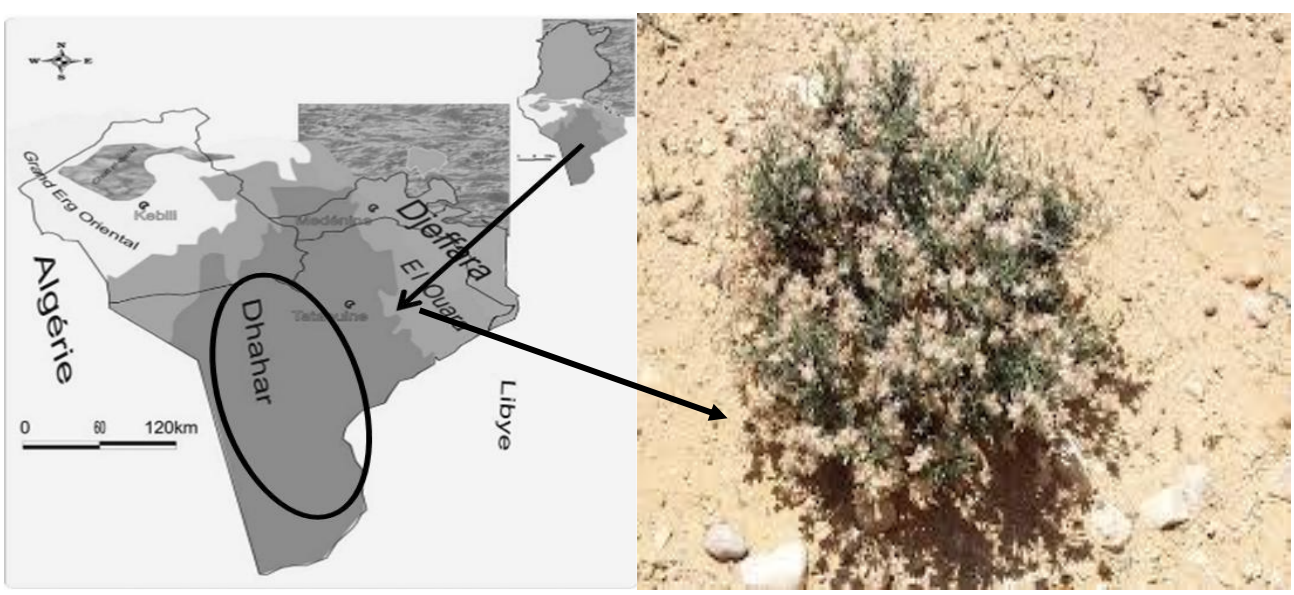

Fig. 1.Georaphic location of *Anthyllissericea* in the south of Tunisia:collecting of *Anthyllissericea* seeds from the zone of "Dhahar".

Determination of proline contents and soluble carbohydrates: Amount of 0.5 g of leaf samples from each group were extracted in sulphosalicylic acid aqueous 3% (w/v). 400 µl of ninhydrin reagent and glacial acetic acid was mixtured with the homogenate. After filtering the reaction was stopped by placing in ice. The extraction with the organic toluene started: at 520 nm, when the red colour was developed, the absorbance was measured. Proline concentration was calculated using a calibration curve and expressed as µmolproline g^{-1} FW (Bates *et al.,* 1973).

Dry leaves (100 mg) were extracted in methanol solution 80%. The supernatant was filtered and mixtured with 1ml of phenol (5%) and 5 ml of sulphuric acid (98%) reagents. After cooling, the absorbance was estimated at 490 nm and using D-glucose as standard (Robyt & White, 1987).

Lipid peroxidation, nitric oxide and hydrogen peroxide analyses: Malondialdehyde (MDA) content was measured, 500 mg of frozen powder was added to 1 ml of trichloroacetic acid (TCA) (0.1%). After 5 min of centrifugation, 1 ml of filtered supernatant was homogenized with 4 ml of 0.5% thiobarbituric acid solution in 20% trichloroacetic acid. The homogenate was centrifugated for 10 min and its absorbance was read at 600, 532 and 440 nm (Hudges*et al.,* 1999). The content of MDA was determined using the absorption coefficient, e = 157 mM^{-1} cm^{-1} and was expressed as nmol MDA g^{-1} FW.

Nitric oxide was determined according to Nakatsubo *et al.* (1998) by fluorimetry method using 4.5 diamino-fluoresceindiacetate (DAF-2). Hydrogen peroxide was measured (Pazmiño *et al.,* 2011) using homovallinic acid.

Enzymatic analysis: Guaiacol peroxidase (POD) activity (EC 1.11.1.7) was assayed with a final volume 1 ml of reaction mixture containing 700 µl of 50 mM phosphate buffer (pH 7.8), 100 µl of crude extract, 200 µl of guaiacol (25 mM) and 100 µl of H_2O_2. POD absorbance was read at 436 nm. Enzyme activity was determined using the extinction coefficient, e = 25.5 mM^{-1} $cm^{-1}$for the oxidized tetraguaiacol. The activity was expressed as µmolguaiacol oxidized min^{-1} mg^{-1} proteins (Chance & Maehly, 1955).

Total CAT activity (EC 1.11.1.6) was evaluated at 240 nm (Aebi, 1984). An aliquot of 3 ml contained 1,900 µl of 50 mM phosphate buffer, pH 7, with 100 µl of sample added to 1ml of H_2O_2 (30 mM). Enzyme activity was calculated as µmol H_2O_2 decomposed min^{-1} mg^{-1} proteins.

APX activity (EC.1.11.1.11) was estimated following the method described by Nakano & Asada (1981). The reaction mixture containing 4µl ascorbate (1mM), 50 mM of potassium phosphate buffer, pH=7, and 10µl EDTA. The reaction started by adding H_2O_2 and the measurements of the absorbance was obtained at 290 nm. APX activity is determined as µmol oxidized ascorbate min^{-1} mg^{-1} proteins and was calculated using the extinction coefficient, e = 25.5 mM^{-1} cm^{-1}.

Statical analysis: The experiments were repeated for three replicates. The data was examined by the ANOVA test, and the differences between the treatments were compared using Tukey's Test. The levels of significance were $p=0.05$. The data shown are mean values ± SD.

Results

Growth and water relations: During drought stress, induced by PEG, growth of *A. sericea* seedlings was significantly decreased at the highest PEG concentrations (-2.1 MPa) (Table 1). The number of leaves decreased significantly by 42 and 75% as compared to the controls for moderate and severe stress, respectively. Fresh weight on both shoots and roots was considerably reduced under severe drought stress.

Table 1. Number of leaves, roots and shoot fresh weight (mg FW $plant^{-1}$) one-month-old plants of *Anthyllis sericea*, subjected for 14 days by PEG at varying potentials solution (-0.2, -1.2 or -2.1 MPa).

Water potentiel (MPa)	Number of leaves	Shoot FW (mg FW $plant^{-1}$)	Root FW (mg FW $plant^{-1}$)
-0,2	63,29 ± 3,25a	2,29 ± 0,1a	0,53 ± 0,01a
-1,2	21,37 ± 3,12b	0,72 ± 0,2b	0,42 ± 0,1ab
-2,1	10,00 ± 1,6b	0,25 ± 0,4b	0,17 ± 0,2b

Mean ± SE (n = 9)

As indicated in Figure 2(a**)**; drought stress significantly affected the water potential of shoot of *A. sericea* by the increasing of the growing solutions. At the highest PEG concentration, shoot Ψw dropped to –3 MPa. RWC had a decreasing trend as Ψw of growth solutions dropped. This parameter decreased from 85% for control leaves to 58 and 40% for stressed leaves under moderate and severe stress, respectively. These two parameters decreased significantly with stressed organs compared to control plants (Fig. 2b).

Solutes accumulation: In the presence of PEG nutrient solution, the stressed leaves accumulated more proline under moderate and severe water deficit stress than controls (Fig. 3). Stressed plants accumulated higher soluble sugars content in leaves (Fig. 3). This was changed significantly in 14-day drought-stressed plants at Ψw = -2.1 MPa of nutrient solution.

Lipid peroxidation, hydrogen peroxide and NO accumulation: As shown in Figure 4, MDA content increased significantly in stressed leaves compared to controls. At 14 days of severe drought stress, MDA content was increased by six and seven fold in roots and leaves, respectively, as compared to the controls.

NO content and H_2O_2 followed a similar trend with that was observed in the previous findings, leaves subjected for the water stress showed an increase reaching maximum at -2,1MPa treatment (severe stress) (Fig. 4).

Enzymatic activities: In comparison with the well watered plant, drought stress strongly increased the activities of the cytosolic and peroxisome enzymes. The activity of catalase was changed by the stress. This activity was pronounced higher during severe stress. Higher PEG osmolarity changed catalase activity in cells (Fig. 5a). This noted enzyme was increased 1- and 2.5-fold at moderate and severe drought stress, respectively, as compared to controls. Both APX and POD of stressed leaves increased its activities being in maximum at water potential of -2.1 MPa of nutrient solution (severe stress), as compared to the controls (Fig. 5b-c).

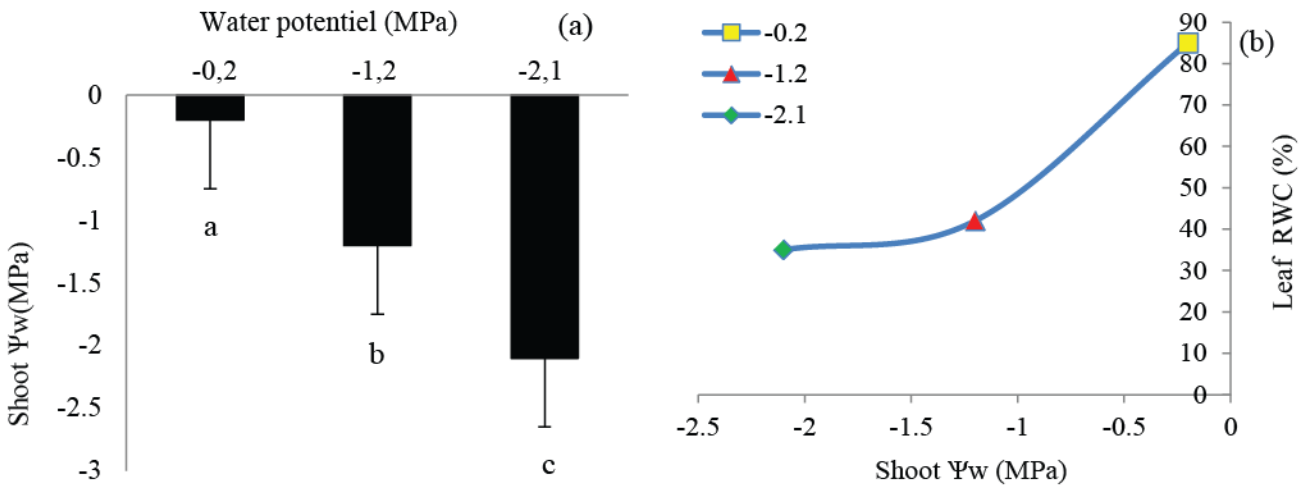

Fig. 2. Changes in water potential of shoot (Ψw, MPa) (a), and (b) correlation between shoot water potential and relative water content in leaves (RWC, %) of *Anthyllis sericea*. Values are from three treatments with three replicates. Data represent mean ± SE (n = 3). Different letters indicate the values are significantly different among treatments ($p<0.05$, Tukey's test).

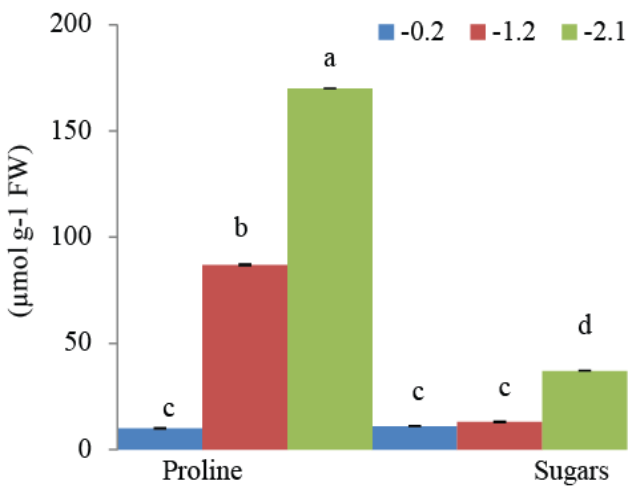

Fig. 3.Changes in proline and sugars content (µmol g^{-1} FW) of *Anthyllis sericea* leaves. Data represent mean ± SE (n = 3). Different letters indicate the values are significantly different among treatments ($p<0.05$) according to Tukey's test.

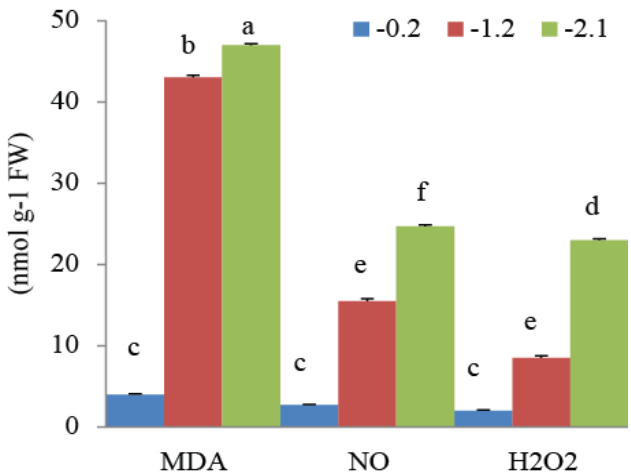

Fig. 4. Effect of water stress on lipid peroxidation content (MDA), NO and H_2O_2 (nmol g^{-1} FW) in leaves of *Anthyllis sericea*. Data represent mean ± SE (n = 3). Different letters indicate the values are significantly different among treatments ($p<0.05$, Tukey's test)

Discussion

Exposure of plants to the temperature, heavy metals, flooding, salt stress, water deficiency, raises the higher production of reactive oxygen species, H_2O_2, O_2 and OH. Plants defend themselves against the toxic alterations; cells promote a complex defense system of enzymatic antioxidant acting to cope with the intercellular oxidative damages (Halliwell & Gutteringe, 2007).

Desert plant species are considered an important model to understand the tolerance mechanisms to deficit water. In this work, drought developed a growth restriction in leaf formation and significant biomass reduction in shoots and roots in *A. sericea* plants when subjected for the severe stress phase, although no significant change were observed in RWC measured in earlier phase of stress application, except in severe stress conditions. Similar results have been observed in some species as chickpea plants (Khanna *et al.*, 2014) soybean (Liu *et al.*, 2004). Drought considerably affected RWC of several plant species such as *Oudneya* (Talbi *et al.*, 2015). Based on leaf RWC variations, shoot Ψw concentrations and growth reduction observed in *A. sericea*, enable this plant to tolerate stress under higher PEG potentials and continue normal growth and development. RWC is a common indicator for drought tolerance in *A. sericea*.

Drought induced oxidative damage in *A. sericea* is manifested through high production of MDA and H_2O_2, especially under severe treatment. The increase level of osmoprotectants observed in leaves of plants subjected for higher potentials of deficit water is an important indicator of oxidative stress. MDA is formed by ROS induced by drought stress in plants. Increased lipid peroxidation level in stressed leaves exhibited an increased oxidative damage observed in degradation of poly-unsatured lipids. MDA accumulation has been well documented in several species exposed to drought stress (Sánchez-Rodríguez *et al.*, 2010). A similar increase was also observed in H_2O_2 concentration during moderate and severe stress period. This association between oxidative damage and H_2O_2 concentration in

stressed plants has been demonstrated in different species (Wei *et al.*, 2013). H_2O_2 in stress conditions regulates stomatol closure, auxin accumulation(Bright *et al.*, 2006) and antioxidant enzymes expression (Fujita, 2006). Hydrogen peroxide acts as a signaling molecule involving regulation of cell response under biotic and abiotic stress. The increase of H_2O_2 induced a similar increase in NO content in the stressed leaves. Recent results support the systemic activity of ROS and NO. Interestingly, a rapid NO production in leaves precedes H_2O_2 induction; NO has been shown to be implicated in the signaling way of H_2O_2 synthesis, thus justifying the observed timing of induction of the two signaling molecules. Similar results were reported in *Medicago* plants(Filippou *et al.*, 2011), transgenic *Arabidopsis* plants (Shi *et al.*, 2014) and *Oudneya* species (Talbi *et al.*, 2015).

In this work a crucial link is provided between the increases of ROS in the stressed leaves and the excess amounts of NO, this highlighting the primary role of NO in drought stress responses as a essential and protective signaling molecule.

Proline accumulation in *A. sericea* leaves exposed for stress has been observed in similar trend with the previous results. Interestingly in this work, proline level was raised up clearly under severe stress conditions. Similar findings were showed in *Ziziphus* species (Clifford *et al.*, 1998) and coconut palm leaves (Gomes *et al.*, 2010)and other crop species including *solanum lycopersicum* (Ali & Rab, 2017). Plants were accumulated higher proline exhibited higher tolerance to drought by maintaining cellular stability of membranes and protein structure. Proline accumulation reduced ROS damages during water stress period.

Oxidative stress and H_2O_2 accumulation allowed plants to synthesize antioxidant enzymes such as CAT, APX and POD in cytosol, mitochondria and peroxisome of cells. Indeed, the maintenance of CAT activity in stressed leaves of *A. sericea* when subjected for water deficiency appeared to be increased to scavenge the ROS damages. Although no data is available on the effect of drought stress at the level of antioxidant enzymes system in *A. sericea*. CAT enzyme dismutates H_2O_2 into H_2O and O_2 in peroxisomes during stress conditions. The significant induction was observed with CAT activity in tissue under moderate and severe stress period in comparison with well watered conditions, indicative the early response to applied stress. The higher activity of catalase activity might be correlated with the increased levels of H_2O_2 and NO in cells of leaf tissues. The induction of CAT activity in response to water stress has been shown in different species (Sharii *et al.*, 2012). Ajithkumar & Panneerselvam (2014) has proved that the expression of CAT activity is significantly varied according to the degree and the intensity of the drought stress application in plants. The authors reported in *Panicumsumatrense*, that drought increased the compatible solutes in cells. Therefore, stress application caused an increase in CAT and SOD activities that allowing a remarkable tolerance characters to water stress in plants. The data presented here indicate that CAT enzyme has a primary role in H_2O_2 detoxification in peroxisomes roots and leaves in earlier phase of stress application.

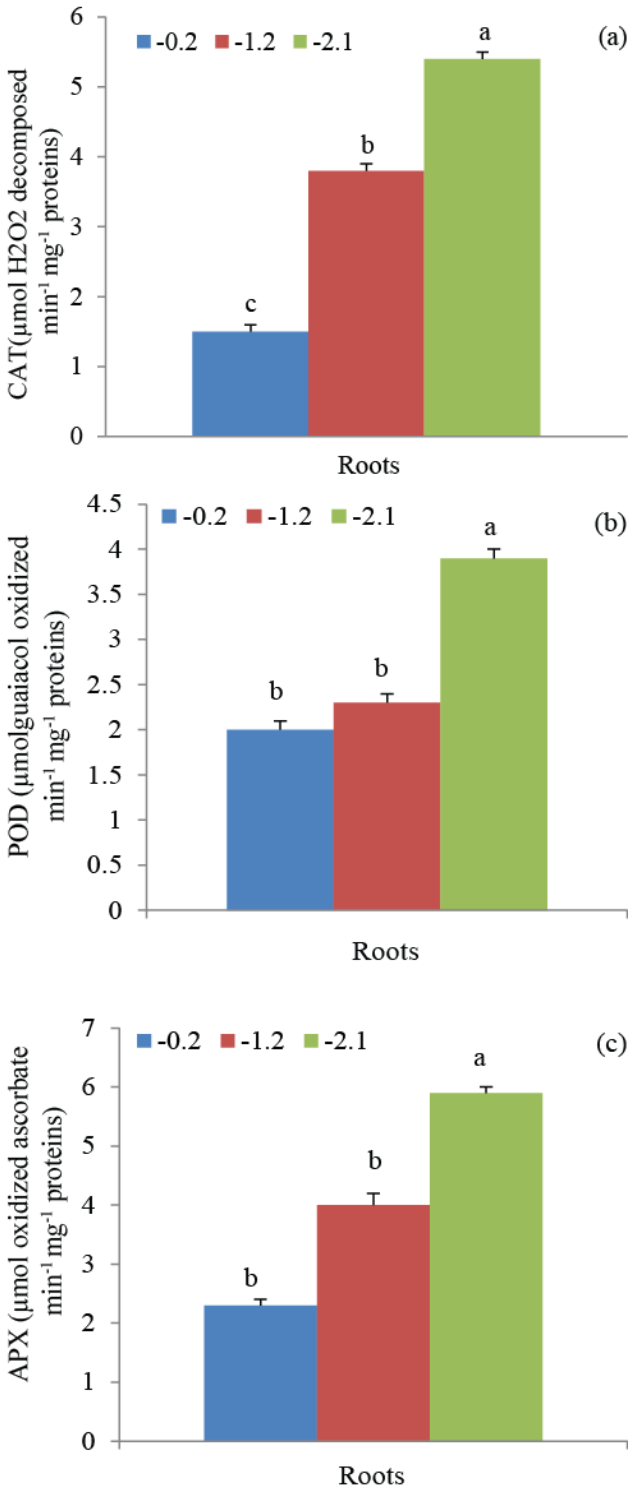

Fig. 5. Activities of catalase (CAT) (a), guaiacol peroxidase (POD) (b) and ascorbate peroxidase (APX) (c) in *A. sericea* leaves. Data represent mean ± SE (n = 3). Different letters indicate the values are significantly different among treatments ($p<0.05$, Tukey's test).

Another antioxidant enzyme seems to be analyzed in *A. sericea* when subjected for drought, is the cytosolic APX enzyme. The activity of this one has been well demonstrated with different plants in drought stress responses. Stressed *A. sericea* plant showed a clear increase of APX activity under stress. For instance, Zarei *et al*. (2012) recorded a higher APX amount under

drought stress when applied with PEG solution in transgenic tobacco plants. In another report, Sofo*et al.* (2005) measured the induction of APX activity in wild amound (*Prunus* sp.,) when subjected for deficit water. These results demonstrated that APX enzyme was one of the key members of ROS scavenging system that avoid the increased production of H_2O_2 damages in cells and induce tolerance drought in plants.

In addition to CAT and APX, the POD enzyme is a very efficient defensive enzyme to protect cells from toxic compounds such as H_2O_2 (Chaparzadeh *et al.,* 2004). Interestingly in *A. sericea*, POD activity recorded an increase in leaves during moderate and severe stress conditions. The resistance of plant species to stress conditions has been involved with the significant induction of POD and CAT activities. Similar results have been observed with some drought tolerant species, such as *Triticum aestivum* (Omar, 2012; Hasheminasab *et al.*, 2012) and *Phaseolus mungo* (Pratap & Sharma, 2010) had a higher activity of POD activity than the drought sensitive species. According to Bian& Jiang (2009), the significant increase of POD activity in drought-stressed plants was also recorded, others pronounced that the highest POD activity during water stress involve reduction in plant cells, which can reduce the shoot growth (Dichio *et al.,* 2002).

The present findings carried out, in this study, generated the scavenging system of *A. sericea* in drought stress conditions. *A. sericea* exhibited higher induction of enzymatic antioxidant system which allows it's survival and stability during continuous water deficiency conditions in desert.

Conclusions

Due to drought stress, plants have organized different mechanisms to protect and defend themselves during their development against oxidative damages with is taken out by the decrease in the growth. Increased antioxidant enzymes activities and solutes accumulation and other metabolic adjustment, not identified yet, indicate that this attribute enhance adaptative responses to tolerate the imposed severe conditions in the Saharan region to maintain plant stability.

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