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Role of exogenous application of abscisic acid ABA in drought tolerance and evaluation of antioxidant activity in durum wheat genotypes

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Abstract: *Survival under stressful circumstance depends on the plant's aptitude to perceive the stimulus, generate and transmit the signals, and initiate various physiological and biochemical changes. This study aims to evaluate the exogenous seed treatment by abscissic acid (ABA) in durum wheat genotypes under water stress conditions. In this investigation, a hydroponic experiment was conducted to evaluate the potential role of exogenously applied abscicic acid in improving drought tolerance in wheat. Three contrasting wheat genotypes were used in this work: Hoggar, Hedba3 and Sigus. Two levels of water stress were induced: 2h and 4h, the aim of this work was to evaluate the action of seed exogenous treatment with ABA for 8 and 16h on physiological and biochemical parameters like stomatal resistance, antioxidant enzyme activity and quantification of ABA by HPLC. The results showed that water stress caused a decrease in endogenous ABA concentration in the roots of the stressed varieties with the exception of Hedba3. Furthermore, after ABA treatment for 16h, the two genotypes Hedba 3 and Hogar showed a higher accumulation of this phytohormone, compared to Sigus variety which marks a decrease in this concentration and which can be explained by the consumption of the ABA in the defense against the ROS.*

Keywords: Wheat, drought, abscicic acid, antioxydants enzyms

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Introduction

Among the main staple crops across the globe, wheat is the most important for providing daily calories and protein intake. Wheat forms the major staple food in the human diet with a contribution of 19% in world food energy and 21% in total protein eating [1]. Currently, wheat culture is severely affected by environmental stresses [2]. More than one third of the world's total cultivated area is affected by drought. Within that area 33% (99 million hectares) belongs to developing countries and 25% (60 million hectares) belongs to developed nations [3, 4, 5]. With the world population predicted to increase to 9.6 billion by 2050, the demand for wheat is only going to increase mainly in North Africa, Algeria principally [6]. Demand for wheat is estimated to increase by 60% by 2050, but production might go down by 29% as a result of climate change imposed by environmental stresses [7].

In Algeria, a country most of which is desert, climate change is a great interest. Indeed, by its geographical position, Algeria is exposed tote negative effect of climate change and greenhouse gas emissions, including floods, drought and high temperatures. Over the past 20 years, maximum temperatures have increased more than minimums. As for precipitation, for the same periods, the report shows that in autumn and winter, there is less rain in the North, and in spring in the East of the country. These predictions indicate that improving abiotic stress tolerance in wheat is vital for global food security [8].

Water accessibility is critical for wheat production, and drought is the major cause of yield losses [9]. The roots are the first organs exposed to water deficiency in the soil and are the location of drought sensing [10]. Chemical signals from the root are transported towards the shoot and initiate molecular and biochemical processes, which finally moderate the morphological responses, enabling plants to survive with the drought conditions [11].

The cellular effects of drought stress mostly include accumulation of a large amount of reactive oxygen species (ROS), imbalances of osmotic homeostasis [12]. These mechanisms rely on a complex cellular metabolism, in which almost all known phytohormones play an important role. High concentrations of ROS are highly harmful to organisms, and when the symptoms persist, irreversible damage occurs to cells, resulting in loss of physiological capacity and eventual cell death. Therefore, defense mechanisms against oxidative damage are activated during stress to regulate toxic levels of ROS [13]. Some researchers believed that drought leads to the decrease of antioxidant enzymes like: SOD, CAT and POD activity and an increase of unsaturated fatty acid peroxidation in cell membranes induced by free radicals [14].

In order to keep the plant physiological balance, to protect them against the damage of reactive oxygen, plants positively regulate their physiological function under water stress. Antioxidant enzymes can either direct eliminate reactive oxygen species in cells, which protect cells from oxidative damage, and have a

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detoxifying effect on plants at the same time [15, 16, 17].

In addition, a major change in response to water deficit increased synthesis of Abscisic acid (ABA). A recent investigations have shown that phytohormones (such as ABA, auxins, CKs, SA, JAs, BRs, etc.) have the potential to enhance the abiotic stress tolerance (including drought tolerance) of various plant species [18, 19].

Abscisic acid (ABA) promotes the closure of stomata in guard cells to maintain water in plants [20]. Also, researchers reported that seed treatment with ABA can significantly enhance the antioxidant enzymes activity in maize seedlings subjected to water stress. Furthermore, exogenous application of ABA under water stress increased the grain weight in susceptible wheat cultivars [21, 22].

Thus, our research aims to understand the role of phytohormones in drought tolerance, in durum wheat genotypes under water stress and to examine the stress response on the physiological, biochemical stress markers (malondialdehyde (MDA) and antioxidant enzymes) and endogenous ABA levels, by exogenous ABA seed treatment in drought conditions.

Material and Methods

Plant material and treatments

A laboratory experiment was conducted; the experiment was carried out in a factorial on a randomized block design with three replications. Seeds of three different wheat varieties (Hoggar, Hedba3 and Sigus) were used. Seeds of each cultivar were surface sterilized, thoroughly washed three times with distilled water and were placed in Petri plates containing sterilized filter paper. In each Petri plate, eight seeds were placed. Water was added in each Petri plate at regular intervals of time. After two days, seedlings were exposed to three different treatments i.e., control (C), 30 µM of abscisic acid (ABA) was added for 8h and 16 h. Seedlings were transferred to nutrient solution. Plants were grown in a growth chamber under controlled conditions. The day/night temperature was 26°C/18°C under a 16 h photoperiod with a light intensity of 150 Wm^{-2} (Philips Master HPI-T Plus, 400 W). The relative humidity was about 50%. After four weeks, plants were subjected to drought stress by water privation for a period of 2 and 4 h and analysis were done.

Measurements

1. Stomatal Resistance *was measured with a Portable Porometre System type Delta Devices MK3.*

2. Electrolyte leakage (EL)

Membrane stability after water stress was estimated using a conductance test, as described by Thiaw (2003). Ten discs of fresh leaf (The medium part of leaves) were cut from the fully expanded leaves (five plants per variant) and the samples were washed three times with deionized water to remove surface-adhered electrolytes. Leaf discs were placed in closed tubes containing 5 mL of deionized water and incubated at 10°C

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for 24 h. Subsequently, the initial electrical conductivity of the solution (EC1) was determined using a conductometer. The samples were then incubated in a water bath at 95°C for 20 min to release all electrolytes, cooled down to 25°C and their final electrical conductivity (EC2) was measured. The electrolyte leakage (EL) was calculated from

$$EL = (EC1/EC2) \times 100\ (\%),$$

where: EC1 and EC2 are the first and the second reading of conductance.

3. Enzyme assays

Leaf fresh materials (0.1 g) were homogenized in liquid nitrogen into microtubes by electrical homogenizer, after that, 1mL of extracting reaction including 0.1 M Sodium phosphate buffer (pH=7.8) and 1 mM ethylenediaminetetraacetic acid (EDTA) was added to homogenized leaf material. Insoluble materials were removed by refrigerated centrifuge at 15000 g for 20 min at 4°C and supernatant used as the source of enzymes assays. An aliquot of 100 µL was used for assaying the activities of catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) and stored at -80°C until assay enzyme activities.

4. Determination of catalase activity

Catalase activity was assayed by measuring the initial rate of hydrogen peroxide disappearance according to Chance and Machly (1955). For measurement of CAT activity assay solution (3 ml)was used, containing 50 mM of phosphate buffer (pH=7.0), 5.9 mM H_2O_2 and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. Decrease in absorbance of the reaction solution at 240 nm was recorded after every 20 sec. An absorbance change of 0.01 units min-1 was defined as one unit CAT activity. Enzyme activities were expressed on protein basis.

5. Determination of superoxide dismutase and peroxidase activity

The frozen leaves (0.5 g) were homogenized in 3 mL of cold solution containing 50×10^{-3} mol/L Na phosphate buffer (pH=7.8), 1×10^{-3} mol/L EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 40°C for 20min at 13000 rpm. The spectrophotometric analysis was conducted on spectrophotometer. The assay for SOD and POD were based on the method described by Beauchamp and Fridovich (1971) and Chance and Machly (1967), respectively. One unit of SOD was defined as the amount of enzyme that inhibits the nitro blue tetrazolium chloride (NBT) photo reduction by 50%. For POD, the activity was defined as the changes in absorbance per minute for 1 g FW of leaves.

6. Lipid peroxidation

For the measurement of lipid peroxidation, the thiobarbituric acid (TBA) protocol to determine the MDA produced was followed [55]. The test was performed using thirty freshly cut leaf discs (0.5 cm^2 each). The amount of MDA was calculated from the absorbance at 532 nm after subtracting the non-specific absorption at 600 nm. The extinction coefficient 155 $mmol/L^{-1}cm^{-1}$ for MDA was used.

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7. Determination of endogenous abscisic acid (µg/g)

ABA was isolated from roots of wheat cultivars, were immediately frozen after fresh weight had been recorded, fresh roots were immediately frozen and stored at -75°C until extractions for ABA analysis. The procedure of Zhou (2003) was used with some modifications in the HPLC purification and quantification. Briefly, it consists of weighing 300 mg of fresh root material of control and stressed samples, then grinding it with liquid nitrogen and placing them in 1.5 mL Eppendorf tubes. 750 µL of extraction solution was added to the grinder and proceed to the sounder for 5 min, then centrifuged for 2 min at 4°C and 10000 rpm. The extracted sample was centrifuged and the supernatant was reduced proceed to the sonicator for 30 min and centrifuged 2 min at 4°C at 10000 rpm and add the second supernatant. The exctract was filtred in a separate conical flask using Whatman filter paper N°1; the filtrate was vacuum evaporated in lyophilizer at pressure of 0.133 mBar and a temperature of - 46°C.

The dried sample was redissolved in 1mL of methanol (80%). Samples were analyzed on HPLC, using U.V. detector and C18 column. For identification of hormones, plant samples were passed through millipore filters (0.45 µ). Pure ABA (Sigma Chemicals Company Ltd., USA) was used as standard for identification and quantification of plant hormone.

ABA was identified and quantified on the basis of retention time and peak area of the standards at 254 nm. Methanol, acetic acid and water (30:1:70) were used as mobile phase. The reproducibility and linearity of the chromatography system was estimated by five consecutive injections of different concentrations of ABA. Stock solution (1000 µg/mL) of pure ABA was prepared by dissolving 25 mg of ABA in 25 mL of HPLC grade acetonitrile. The calibration standards of concentration of 1, 5, 10, 50 and 100 µg/ml were prepared by successive dilutions of the stock solution.

Statistical analysis

The data compiled were submitted to an analysis of variance (ANOVA) (Statistica Software, 2007) and means were compared by LSD test ($p \leq 0.05$).

Results and Discussion

Our results indicate that water stress has a significant effect on stomatal resistance, at all the studied genotype. A net increase in stomatal resistance (SR) is observed in all studied genotypes in the 2nd stress levels (4h). Under non-deficit conditions, the different genotypes have lower values of SR for the untreated witness with the ABA; we observe a low stomatal resistance that varies between (2.42 s/cm²). (3.5 s/cm²) and (0.47 s/cm²) (Figure 1) noted successively in varieties Hoggar, Hedba3 and Sigus. Concerning the treatment by the ABA, we note remarkable increase in this parameter, especially for 16h of ABA exogenous application, it achieves: 33 s/cm², 37.12 s/cm², 38.11 s/cm² noted at Sigus, Hogar and Hedba3 successively.

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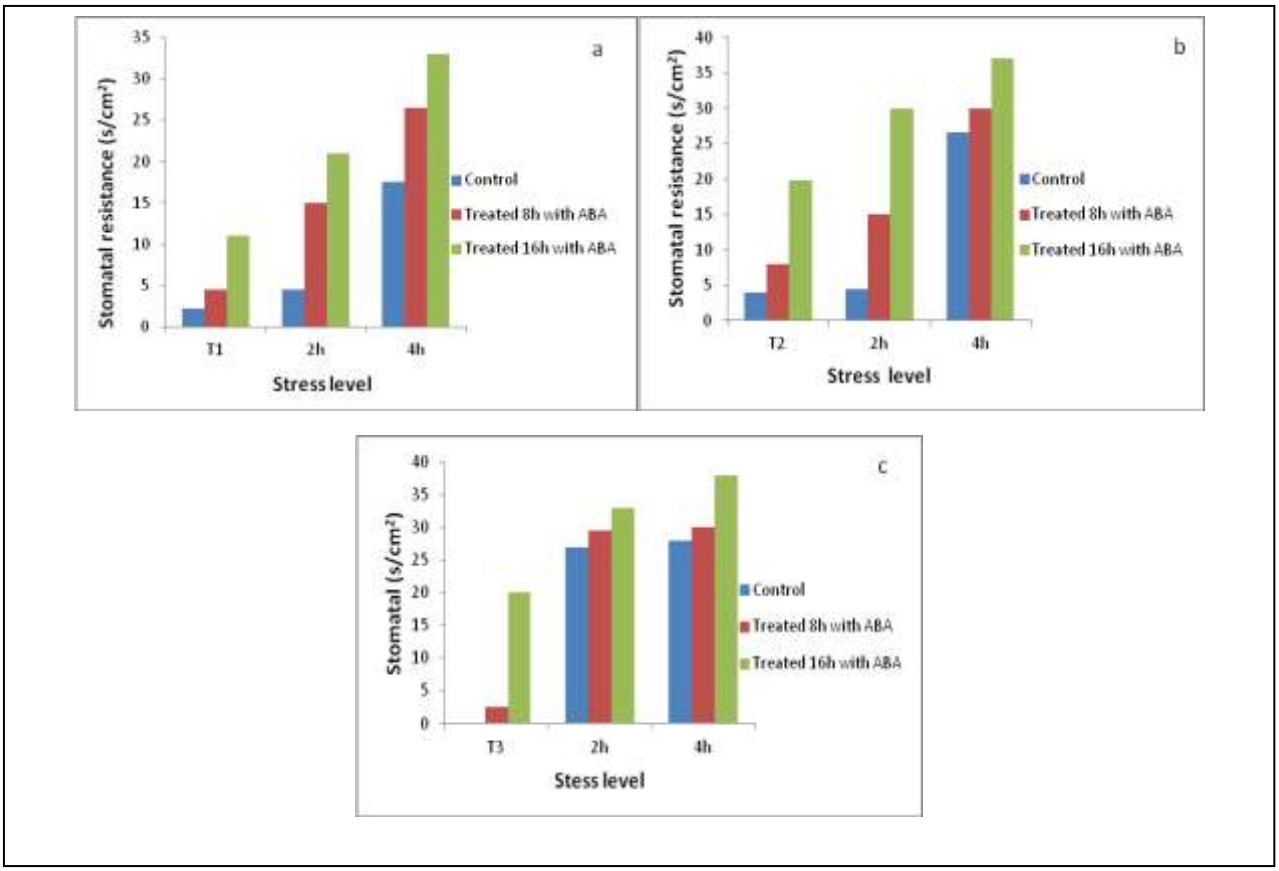

Figure 1. Variation in stomatal resistance in the three varieties studied under two levels of water stress

Drought tolerance can be improved by ABA exogenous application. This parameter increased significantly with the intensity of stress. Exogenous ABA increases stomatal resistance in all studied genotype. Thus, the duration of the treatment influences the stomatal resistance where the highest values are obtained in genotypes treated for 16 hours with ABA. It has been confirmed by Bousba et al. (2009) who reported that wheat responds to water stress by increasing stomatal resistance. Rapid stomatal closure is the best adaptation to hydric stress, it allows the plant to save available water and maintain high tissue moisture content.

Conductivity measurements indicated that, overall, the leaves of stressed seedlings lose as much electrolyte as those of controls (Figure 2). The results showed that the percentage of electrolyte leakage in stressed plants is higher than in the control plants, which is explained by an alteration in the membrane integrity of the cells during stress. Indeed, the analysis of variance reveals a highly significant difference ($p = 0.02\%$) between the percentages of electrolyte leakage recorded in stressed plants compared to controls and a non-significant effect of ABA treatment.

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It has been shown that water stress significantly increased leaf membrane damage. The percentage of electrolytes was generally higher in seedlings exposed to (4 h) of stress compared to membrane damage in plants growing at (control). Cell membranes are one of the first targets of many plant stresses and it is generally accepted that the maintenance of their integrity and stability under water stress conditions is a major component of drought tolerance in plants [24, 25, 26, 27]. The degree of cell membrane injury induced by water stress may be easily estimated through measurements of electrolyte leakage from the cells [28]. It has been also demonstrated recently that electrolyte leakage measurements may be correlated with several physiological and biochemical parameters conditioning the plant responses to environmental conditions such as antioxidative enzyme synthesis [29, 30, 31], stomatal resistance [32, 33, 34].

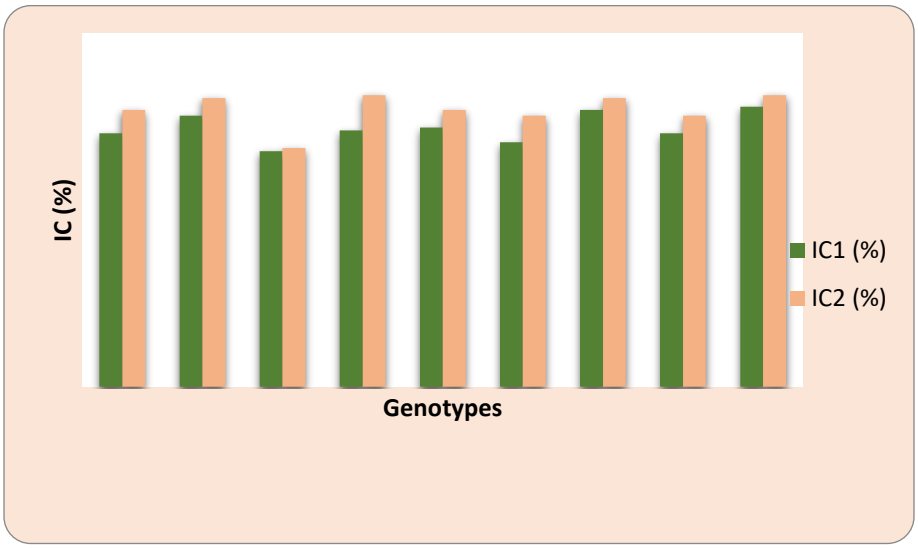

Figure 2. Percentage of membrane integrity in genotypes under stress (4 h) and controls (IC1: Controls, IC2: stressed 4 h) in all the varieties studied

Water stress generally causes a decrease in the concentration of ABA in the roots of stressed varieties (Figure 3). However, after treatment with ABA for 16 hours, the two stressed varieties Hedba 3 and Hogar showed an increase in its concentration. Contrary to the previous results, the Sigus variety shows a decrease in ABA concentration and this can be explained by its consumption for the defense against ROS.

The ABA endogenous content of the two wheat cultivars were greatly reduced by water stress and in seeds treated by ABA (16h), this was clear with the cultivars Hedba3 and Hoggar more than the Sigus genotype. These results are in agreement with those of [35, 36, 37], who showed that when exogenous treatment with ABA under water stress conditions, plants respond by accumulating damage repair proteins, enzymes and osmolytes that can help maintain membrane stability [19, 38].

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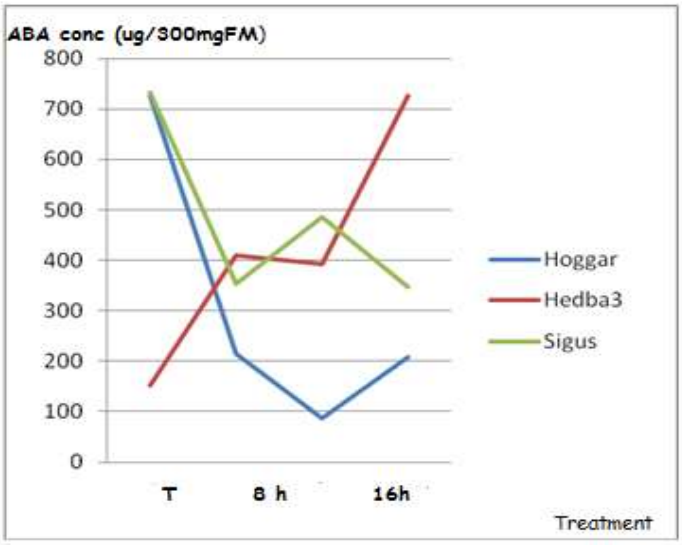

Figure 3. ABA (abscicic acid) levels in studied genotype under water stress conditions

T: control, 2h and 4h of water stress;

T: not treated by ABA, 8h and 16h duration of seed exogenous ABA application

The SOD activity differs depending on the variety and the level of water stress applied (Figure 4). SOD activity decreased in controls and stressed varieties (1st level: two hours and treated 8 hours with ABA), i.e. the Hoggar and sigus varieties; whereas the Hedba3 variety registered a slight increase before the application of water stress and a significant increase after the first stress (2 hours). This increase is due to the activation of defense mechanisms by the increase in antioxidant enzyme activity, particularly SOD caused by the accumulation of ROS. The percentage of SOD inhibition increases in the Hoggar and Sigus varieties subjected to a 16 h treatment with ABA and a 2 h water stress compared to the Hedba3, a variety which shows a decrease in the percentage of SOD inhibition.

The first water stress (2 h) causes a decrease in catalase activity in the majority of genotypes, whereas it induces a slight increase in the Hoggar variety treated for 16h with ABA (Figure 5b). After the second stress (4 h), catalase activity decreased in control genotypes (not treated with ABA) and genotypes treated 8h with ABA; this activity increased in Hedba3 and Sigus genotypes treated 16h with ABA (Figure 5c).

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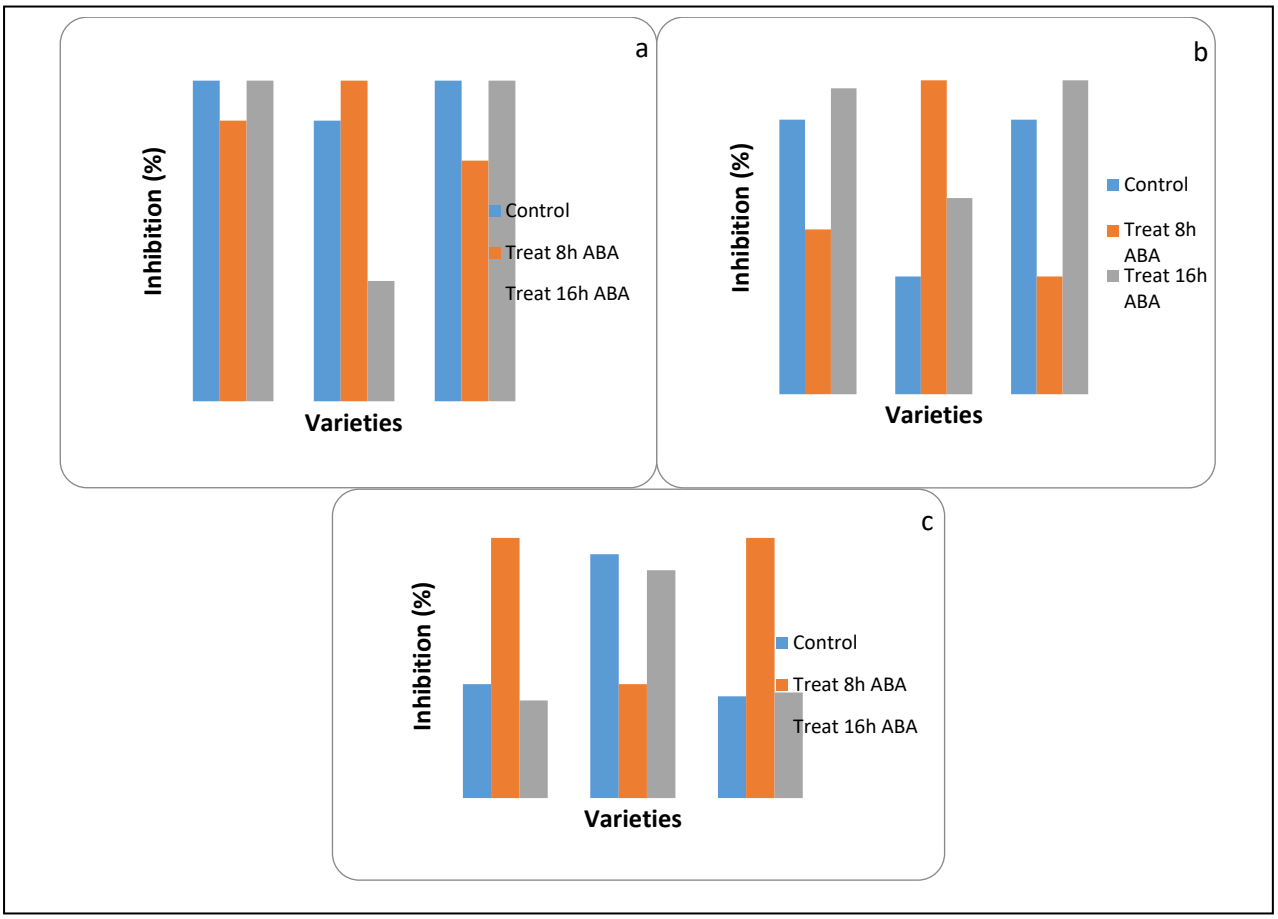

Figure 4. Percentage of SOD inhibition (a: controls, b: 2 h water stress, c: 4 h water stress)

A remarkable increase in peroxidase activity is obtained in the stressed Hoggar variety (4 h and treated 8h with ABA) (Figure 6). This activity decreases and remains low in plants subjected to two hours of stress. However, the enzymatic activity does not show major differences between the control and stressed genotypes at 2 h of water stress, after which the activity increases significantly to reach a maximum at 4 hours for the varieties treated 16 h by ABA.

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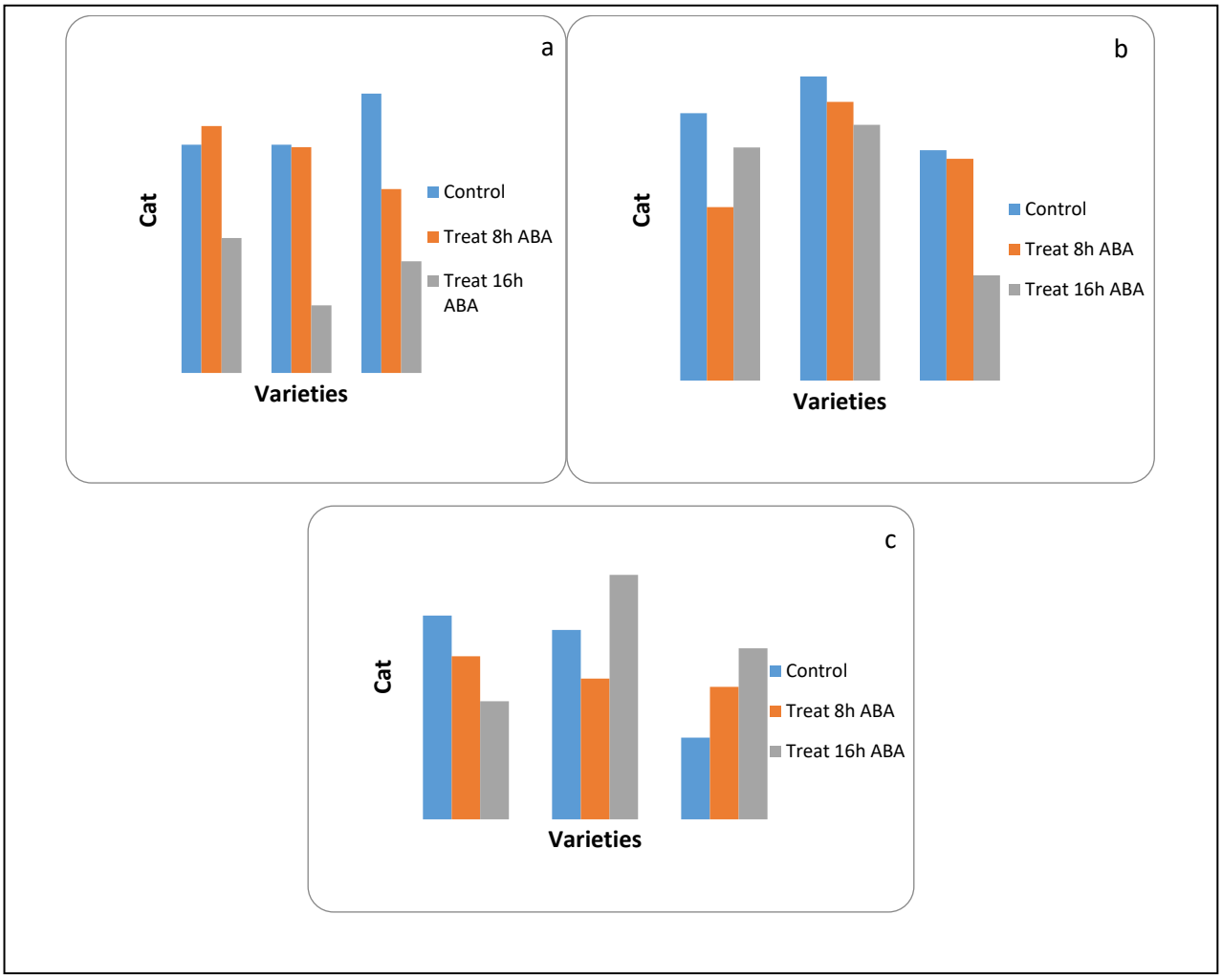

Figure 5. Variation in Catalase activity in the three genotypes under water stress

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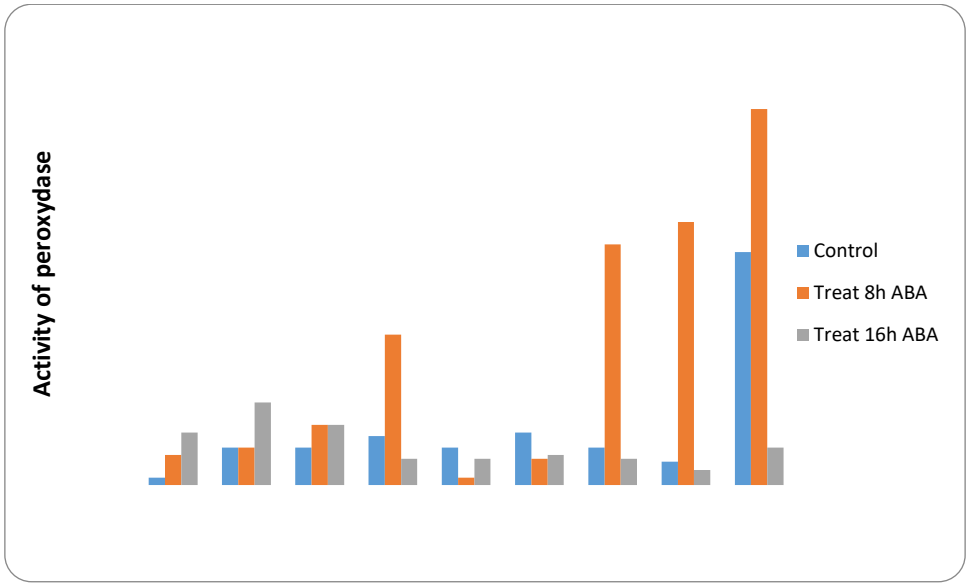

Figure 6. Variation in peroxydase activity in the studied genotypes treated with ABA and subjected to two levels of water stress

Plants are able to improve their stress tolerance often by antioxidant system. Various abiotic stresses lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress [39]. Results of the present study clearly indicate enhanced activities of the antioxydant enzymes, signifying a potential role of these enzymes in providing antioxidative defense under drought stress conditions. At higher levels of drought stress, generation of superoxide anion, increased lipid peroxidation. A higher quantity of ROS activate increase in of the activity of antioxidant enzymes, such as APX, CAT, SOD, and POD which in turn protects plants from oxidative stress [40, 41].

POX, CAT, SOD are the three major antioxidant enzymes responsible for scavenging, the reactive oxygen species generated via different mechanisms in plant cells. Activities of catalase and peroxidase showed increasing trends with increasing stress treatments in all the varieties. The increased SOD, CAT and POX activities point to a signalling role of H_2O_2 in the induction of H_2O_2 synthesis detoxifying enzymes in wheat, as reported for other works [42, 43]. The work of Rossa and al. (2002) and Chen and al. (2004), showed that catalase activity increases in plant leaves under stress conditions. Exogenously ABA induced to wheat seedlings that most of the genotypes reflected increased activity but few had decreased than control. Yang and al. (2007) found that the rules of SOD, CAT and POX activity change are similar, which indicated that these three enzymes cooperated with each other during water deficits. However, reports on the effect of stresses on

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CAT activity vary. Higher levels of antioxidant enzymes are related to drought tolerance in different plants [47]. Activities of antioxidant enzymes could be a useful tool for depicting drought tolerance of wheat, which could be useful to plant breeders for developing drought-tolerant cultivars

Table 1 shows that the variation in MDA content is a function of the variety, the treatment applied and the duration of the stress. Indeed, a decrease in MDA content in genotypes stressed for 2 hours is recorded. In the varieties subjected to the previous stresses and treated with ABA, a significant increase in MDA is noted in the controls deprived of water except for the Sigus variety (16h ABA exogenous application). The 4 h water stress induces a decrease in MDA content for all varieties after 8h of ABA treatment and an increase after 16 h ABA exogenous application. Comparison of the MDA content shows that the varieties Hedba3 and Sigus accumulated higher levels of MDA than the Hoggar variety during severe stress.

Table 1. Variation in MDA activity in the three studied genotypes

(a: Controls, b: subjected to a 2-hour water stress, c: subjected to a 4-hour water stress)

Treatmemt	Not stressed			Stressed 2h			Stressed 4h		
Varieties	Hoggar	Hedba3	Sigus	Hoggar	Hedba3	Sigus	Hoggar	Hedba3	Sigus
Control	0.0031	0.002	0.005	0.00308	0.00205	0.00107	0.00205	0.00309	0.00302
Treat 8h ABA	0.001	0.00205	0.001	0.00202	0.00305	0.00201	0.00309	0.002	0.00205
Treat 16h ABA	0.00103	0.00207	0.00508	0.00308	0.00205	0.00109	0.00105	0.00205	0.00208

Formation of MDA is an oxidative effect of ROSs such as H_2O_2 on membrane lipids; therefore, MDA level increased in drought- stressed plants during stress period, but decreased in treated ones with ABA. MDA content can indicate the extent of oxidative stress in plants. It increased under drought conditions. MDA level also increased in ABA exogenous application for 16 h, but had significant differences with drought-stressed group and ABA treatment duration. According to Mickky and Aldesuquy (2017), during oxidative stress, the level of MDA is proportional to the degree of lipid peroxidation; hence it is used as a biomarker for measuring oxidative stress. MDA is generally correlated with the reduction of total antioxidative enzymes.

The level of invariant MDA appears to be a characteristic of drought-tolerant plants, and the degree of cellular oxidative damage in plants exposed to abiotic stress is a function of the ability of plants to protect themselves against oxidative agents [49]. MDA has long been accepted as an indicator of abiotic stress and can therefore be used as a biomarker of oxidative stress [50].

Conclusions

Our results indicate that water deficiency causes an increase in stomatal resistance and membrane integrity in all three genotypes depending on the intensity of stress and the treatment applied. Analysis of

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endogenous ABA in the roots of seedlings subjected to different levels of water stress shows that during oxidative stress, the concentration of this phytohormone increases in the roots to help decrease ROS. Also, the exogenous supply of abscisic acid is also important in the response of plants to water stress. The activity of catalase decreased under applied stresses and this may indicates that the exogenous effect of ABA helped the plant to catalyze the dismutation of hydrogen peroxide.

On the other hand, the activity of peroxidase increased significantly with the intensity of the stress and the treatment applied to reduce the oxidized substrates during this stress. The increase in SOD activity shows a significant production of ROS observed in all three varieties, MDA is generally correlated with the reduction of total antioxidative enzymes. In this study the accumulation of MDA is greater under severe stress and with low ABA treatment, which could be explained by a higher peroxidation of membrane lipids under water stress conditions.

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