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Changes in the antioxidant and glyoxalase enzyme activities in leaves of two Moroccan sorghum ecotypes with differential tolerance to nitrogen stress

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Abstract

Nitrogen stress as well as other stresses can negatively impact the plant development and metabolism. Generally, stress factors increase the reactive oxygen species (ROS) and methylglyoxal (MG) production, which may, in the absence of effective protective mechanisms, induce irreparable metabolic dysfunction and death. The effect of different amounts (from deficiency to excess) of nitrate, ammonium or nitrate combined to ammonium, on enzyme activities of antioxidant and methylglyoxal detoxification systems of two sorghum ecotypes (3P4 and 4P11) was studied. The N supply was performed per pot during the sowing step using potassium nitrate and/or ammonium sulfate. Six N treatments were applied using 120, 240 and 480 Kg ha^{-1} of ammonium or nitrate and three other treatments were applied using 120 kg ha^{-1} nitrate combined to 120, 240 and 480 kg $ha^{-1}$of ammonium. The specific activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR), glyoxalase I (Gly I) and glyoxalase II (Gly II) were investigated. Results showed that, ammonium excess and N-deficient conditions increased the contents of malondialdehyde (MDA), and induced the enzyme activities of ROS and MG detoxification systems, supporting the sorghum's ability to counteract the negative effect of N stress (deficit and excess). We have also shown that the SOD, CAT, GR and Gly I enzyme activities were higher in the 4P11 ecotype compared to the 3P4 ecotype. These results indicate that sorghum ecotypes exhibit differential tolerance to N stress and suggest that the 4P11 ecotype has higher capacity to cope with N stress.

Keywords: *Sorghum bicolor*; C_4 plants; nitrogen deficiency; ammonium toxicity; antioxidant enzymes; glyoxalase system.
Abbreviations: CAT_catalase; EDTA_Ethylenediaminetetraacetic acid; Gly I_glyoxalase I; Gly II_glyoxalase II; GR_glutathione reductase; GSH_glutathione; MDA_malondialdehyde; MG_methylglyoxal; N_nitrogen; NBT_nitroblue tetrazolium; NH_4^+-N_ammonium; NO_3^-N_Nitrate; P_phosphorus; PMSF_phenylmethylsulfonyl fluoride; POD_peroxidase; PVP_polyvinylpyrrolidone; ROS_Reactive oxygen species; $O_2^{-\circ}$_superoxide radical; SOD_superoxide dismutase; TBA_thiobarbituric acid; TCA_trichloroacetic acid.

Introduction

Appropriate fertilization is a prerequisite to meet high yields and optimum quality of crops. Unlike most of mineral elements essential to plant development that are needed in trace amounts, some key elements (macroelements) like nitrogen (N) and phosphorus (P) are required in large amounts. These elements are regularly insufficient in the soil to allow optimum growth of crops. Of all mineral elements, N is the one that organisms require in greatest quantity, because it is a constituent of macromolecules such as proteins (Yang et al., 2012). Adequate N fertilization is therefore of importance for better growth of crops.

Nitrate (NO_3^--N) and ammonium (NH_4^+-N) are the most important forms of N that plants use for growth and development. However, nitrate is very mobile and easily moves with water, causing a diminution in the levels of N available to plants and leads to many problems related to environment and human health (Domínguez-Valdivia et al., 2008; Khajuria and Kanae, 2013). For these reasons, ammonium is of great importance because it can replace nitrate and thus, increase the amounts of N available for plants and decrease the negative impact of N on groundwater. However, it is now well known that ammonium is toxic to many plant species, leading to a reduction in growth and alteration in content of amino acids, mineral cations and organic anions (Li et al., 2011).

Only few studies about antioxidant responses in crop-plants grown under differential N supply have been conducted (Nimptsch and Pflugmacher, 2007; Yañez-Mansilla et al., 2014; Yañez-Mansilla et al., 2015). Asada and Takahashi (1987) reported that N excess is generally related to the accumulation of ammonium in plant tissues, whereas N deficiency may increase the production of ROS. Polesskaya et al. (2004b) reported that NH_4^+ increased antioxidant enzyme activities. They also reported that N deficiency leads to an oxidative stress due to an increase in the Mehler reaction and an energetic imbalance related to a decrease in

N assimilation. Accordingly, excess production of ROS disturbs the normal redox environment of cells and lead to lipid peroxidation of cell membranes (Asada and Takahashi, 1987). To maintain ROS under control and reduce the oxidative damage caused by N stress, plants have evolved different protective mechanisms, including the antioxidative enzymes such as SOD, POD, CAT, and GR.

Methylglyoxal (MG) is a natural metabolite and a highly reactive cytotoxic compound. The overproduction of MG under abiotic stress has been reported in several studies (Yadav et al., 2008). Besides, it was turned out that the overproduction of MG is related to lipids, proteins and DNA damage (Yadav et al., 2005). The MG is detoxified mainly by the glyoxalase system composed of two enzymes, Gly I and Gly II (Yadav et al., 2005). While Gly I catalyzes the transformation of MG to S-D-lactoglutathione by using one molecule of reduced glutathione (GSH), Gly II catalyzes the formation of D-lactic acid from the S-D-lactoylglutathione. This reaction allows the regeneration of one molecule of GSH (Yadav et al., 2008). Thereby, the MG detoxifying system could play an important role in plant tolerance to abiotic stress by recycling GSH and thus maintaining GSH homeostasis (Yadav et al., 2008). Many studies have reported the involvement of Gly I and Gly II in the regulation of environmental stresses such as salinity (Hasanuzzaman and Fujita, 2013). However, the effect of N stress on the enzymes of MG detoxifying system has been not studied yet.

Sorghum bicolor (L.) Monech is considred to be the 5th most important cereal in the world; it is known for its resistance to drought and its low demand for inputs. Sorghum is also considered as a good model for physiological and molecular studies in C_4 metabolism (El Omari et al., 2010). Numerous studies recorded the improving effect of nitrogenous fertilizers on yield of forage crops (Hussein and Sabbour, 2014). Beyaert and Roy (2005) obtained the highest yields of forage sorghum at 125 kg N ha^{-1}, and Ketterings et al. (2007) found that optimum N levels for brown midrib forage sorghum was between 125 and 145 kg N ha^{-1} in the northeastern U.S. A recent study showed that *Sorghum-sudangrass* plants are tolerant to high levels of inorganic N supply and this tolerance is accompanied by an increase in the capacity of N assimilation in roots (El Omari et al., 2010). In this way, sorghum could be a good model to study the implication of antioxidant and glyoxalase system enzymes in the tolerance to N stress.

The response of plants to the N availability depends on the genotype and the N sources and levels (Chardon et al., 2012). Previous works have shown that *sorghum-sudangrass* acclimation to conditions of ammonium supply, as well as N deficiency in growth medium, produced different phenotypes. These changes involved plant growth, plant and cell morphology, the content of amino acids and proteins in leaf and root tissues, and some key enzymes of N and carbon metabolism (El Omari et al., 2010; El Omari and Nhiri, 2015). The goal of this work is to study whether such significant changes in plant metabolism, as induced by the changes in N supply, activate the antioxidant and glyoxalase system enzymes in sorghum plants. The second objective is to study the effect of the N source (ammonium, nitrate or both) and doses, on two sorghum ecotypes, to examine if there is a differential response depending on the N source and the genotype.

Results

Lipid peroxidation as affected by different nitrogen sources and levels in the two sorghum ecotypes

The effect of N treatments on lipid peroxidation levels in leaf tissues, measured as the content of MDA, is represented in Figure 1. Lipid peroxidation in plants grown without N supply was approximately 50% higher ($p \leq 0.05$) compared to plants grown under moderate N doses. However, unlike nitrate, which had no remarkable effect on the lipid peroxidation; increased ammonium concentration in the medium resulted in a profound increase in lipid peroxidation levels. The effect of the combination of ammonium and nitrate was more pronounced and increased the MDA content to levels similar ($p > 0.05$) to those of the N-deficient plants. The MDA content was similar in both ecotypes (Figure 1).

Antioxidant enzyme activities as affected by different nitrogen sources and levels in the two sorghum ecotypes

The SOD activity showed differential results in response to N levels and source. However a similar trend in SOD activity was observed in both ecotypes (Figure 2A). The highest specific SOD activity was observed at 0 kg N ha^{-1}. Nitrate leads to a slight increase in SOD activity, but only at 480 kg ha^{-1}. Nevertheless, SOD activity strongly increased with increasing ammonium supply. Under N stress conditions (deficiency or ammonium excess), the activity of SOD was significantly higher in the 4P11 ecotype compared to the 3P4 ecotype.

The effect of N on CAT activity was shown in Figure 2B. The activity of CAT was significantly induced in plants treated with ammonium as single N source. It reaches its maximum at 480 kg NH_4^+-N ha^{-1} for both ecotypes. However, CAT activity was reduced when nitrate was combined with the same doses of ammonium. Plant without N supply showed high CAT activity in comparison with plants treated with moderate N doses (120 kg N ha^{-1}). Figure 2B also shows that the 4P11 exhibited higher CAT activity mainly under N deficient and high ammonium level conditions.

Plants treated with nitrate as sole N source showed the lowest POD activity for both ecotypes (Figure 2C). However, in the leaves of N-deficient plants, and those grown under high levels of ammonium, POD activity almost doubled compared to plants treated with nitrate. Ammonium or ammonium plus nitrate induced significantly the POD activity which reaches its maximum at A3 for both ecotypes. As for SOD and CAT, the combination of nitrate and ammonium reduce the POD activity in both ecotypes. It should be also noted the POD activity was significantly higher ($P \leq 0.001$) in the 3P4 ecotype compared to the 4P11 ecotype in almost all treatments.

In Figure 2D, the GR activity significantly varied in response to the ecotypes as well as by the effect of N source and concentration. Ammonium-fed sorghum plants had higher GR activity than nitrate-fed plants. In fact, GR activity (Figure 2D) increased significantly in plants exposed to ammonium or ammonium plus nitrate compared to N untreated plants. For both ecotypes, GR activity reaches its maximum at A2 after which the activity in the 3P4 ecotype decreased but for the 4P11 ecotype, this activity remains constant. Higher GR

Table 1. The treatments applied to sorghum plants. Six N treatments were applied using 120, 240 and 480 Kg ha^{-1} of ammonium or nitrate and three other treatments were applied using 120 kg ha^{-1} nitrate combined to 120, 240 and 480 kg ha^{-1} of ammonium. Pots without N supply were considered as control and abbreviated as (-N).

Nitrate		Ammonium		Nitrate + Ammonium		
Treatment	Nitrate supply (kg/ha)	Treatment	Ammonium supply (kg/ha)	Treatment	Nitrate supply (kg/ha)	Ammonium supply (kg/ha)
N1	120	A1	120	NF1	120	120
N2	240	A2	240	NF2	120	240
N3	480	A3	480	NF3	120	480

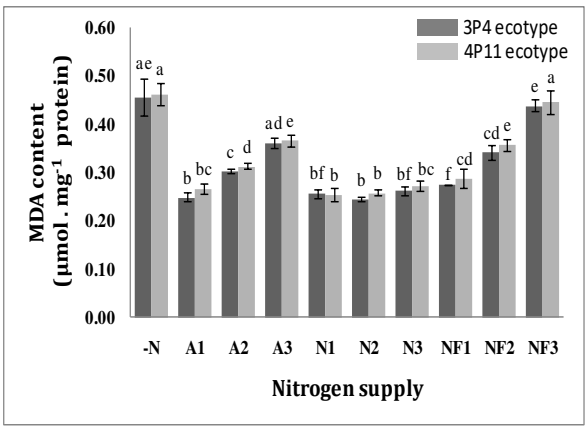

Fig 1. Concentration of malondialdehyde (MDA) in the leaves of two sorghum ecotypes (3P4 and 4P11) growing at different levels and sources of inorganic N. Six N treatments were applied using: 120 (A1), 240 (A2), 480 (A3) Kg ha^{-1} of ammonium and 120 (N1), 240 (N2), 480 (N3) Kg ha^{-1} of nitrate and three other treatments were applied using 120 kg ha^{-1} nitrate combined to 120 (NF1), 240 (NF2) and 480 (NF3) kg ha^{-1} of ammonium. Pots without N supply were considered as control and abbreviated as (-N). Each value represents the mean of six replicates. Bars represent the standard error. Different letters indicate significant differences among N treatments, for the same ecotype, at the 5% level. The signs *, † and ‡ indicate significant difference between the two varieties for same N treatment at P≤0.05, P≤0.01 and at P≤0.001, respectively.

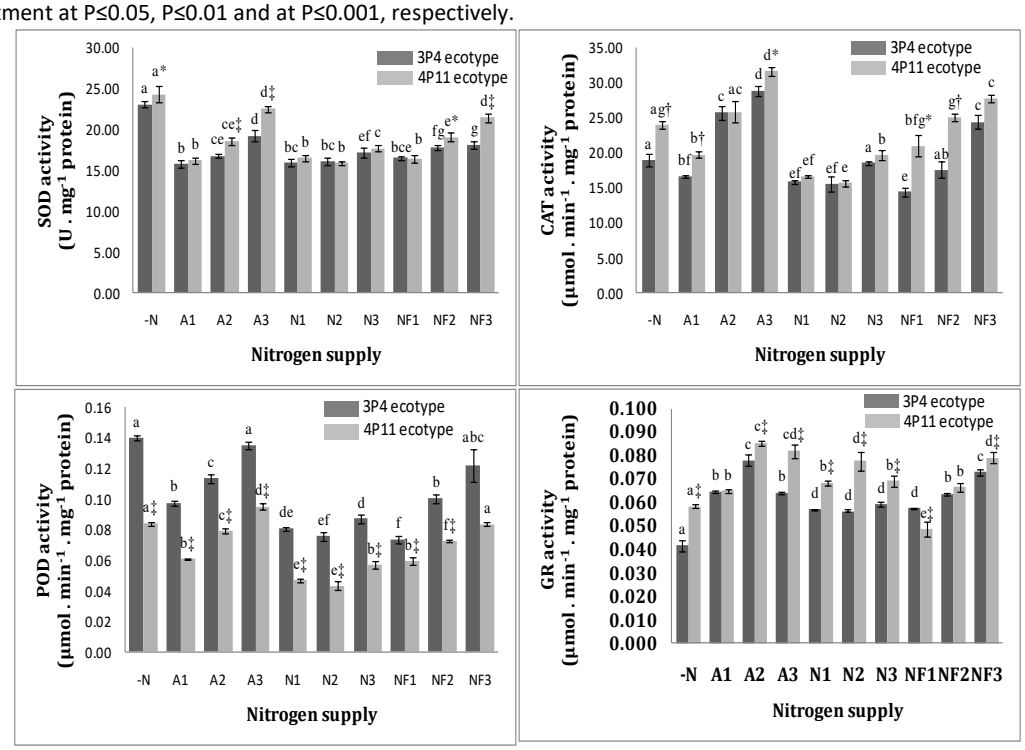

Fig 2. Activities of antioxidative enzymes; superoxide dismutase (SOD, A), catalase (CAT, B), peroxidase (POD, C) and glutathione reductase (GR, D) in the leaves of two sorghum ecotypes (3P4 and 4P11) growing at different levels and sources of inorganic N. three N treatments were applied using: 120 (A1), 240 (A2), 480 (A3) Kg ha^{-1} of ammonium and 120 (N1), 240 (N2), 480 (N3) Kg ha^{-1} of nitrate and three other treatments were applied using 120 kg ha^{-1} nitrate combined to 120 (NF1), 240 (NF2), 480 (NF3) kg ha^{-1} of ammonium. Pots without N supply were considered as control and abbreviated as (-N). Each value represents the mean of 5-6 replicates. Bars represent the standard error. Different letters indicate significant differences among N treatments, for the same ecotype, at the 5% level. The signs *, † and ‡ indicate significant difference between the two varieties for same N treatment at P≤0.05, P≤0.01 and at P≤0.001, respectively.

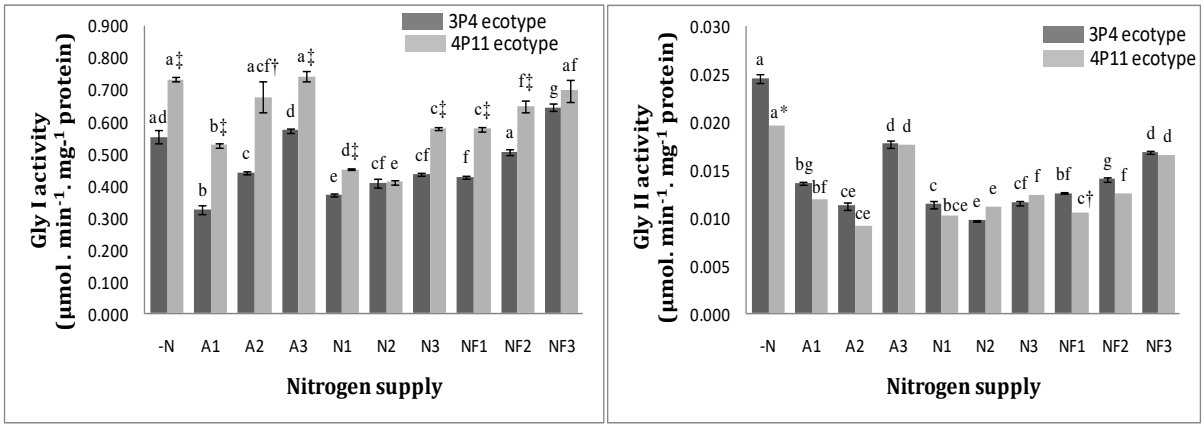

Fig 3. Activities of glyoxalase system enzymes; glyoxalase I (Gly I, A) and glyoxalase II (Gly II, B) in the leaves of two sorghum ecotypes (3P4 and 4P11) growing at different levels and sources of inorganic N. Six N treatments were applied using: 120 (A1), 240 (A2), 480 (A3) Kg ha^{-1} of ammonium and 120 (N1), 240 (N2), 480 (N3) Kg ha^{-1} of nitrate and three other treatments were applied using 120 kg ha^{-1} nitrate combined to 120 (NF1), 240 (NF2) and 480 (NF3) kg ha^{-1} of ammonium. Pots without N supply were considered as control and abbreviated as (-N). Each value represents the mean of 4-6 replicates. Bars represent the standard error. Different letters indicate significant differences among N treatments, for the same ecotype, at the 5% level. The signs *, † and ‡ indicate significant difference between the two varieties for same N treatment at P≤0.05, P≤0.01 and at P≤0.001, respectively.

activity was observed for the 4P11 ecotype compared to the 3P4 ecotype.

Glyoxalase system enzyme activities as affected by different nitrogen sources and levels in the two sorghum ecotypes

Results showed that, independent of the N source and ecotype, there was a significant decrease in the Gly I activity at 120 kg N ha^{-1} dose compared to N untreated plants (Figure 3A). The Gly I activity subsequently increased in plant treated with 240 and 480 kg ha^{-1} of ammonium or ammonium plus nitrate treatments, reaching a level similar or even higher to those detected in N untreated plants. Despite the increase in enzyme activity of nitrate-treated plants, the maximum activity reached remains significantly lower compared to N-deficient condition. The Activity of Gly I was significantly higher in the 4P11 ecotype compared to the 3P4 ecotype.

The effect of N source and rate on the on the Gly II activity was shown in figure 3B. For the 3P4 and 4P11 ecotypes, the Gly II activity significantly decreased by 119 and 116 % when ammonium rate increased from 0 to 240 kg ha^{-1}. However, high level of ammonium (480 kg ha^{-1}) caused an increase in the Gly II activity, this activity remains lower compared to the level observed in N untreated plants. Furthermore, the effect of nitrate on the Gly II was less marked compared to that of ammonium. For the 3P4 ecotype, Gly II activity was 116, 153 and 112% lower at N1, N2 and N3 treatments in comparison to plants with no N supply. However, for the 4P11 ecotype, this decrease was about 93, 77 and 59% lower compared to the N untreated plants. The Gly II activity increased with increasing ammonium doses in plants treated with ammonium combined to nitrate.

Discussion

Nitrogen deficiency or excess increase the production of ROS in plants, which results in lipid peroxidation of cell membranes (Asada and Takahashi, 1987; Pompelli et al., 2010). In oxidative stress, MDA content can be used as an indicator of lipid peroxidation. Several studies have reported that increased level of MDA was correlated with oxidative damages caused by different abiotic stresses (Garg and Manchanda, 2009). In our results, N deficiency (-N) and ammonium excess (A3) lead to the highest lipid peroxidation in the two sorghum ecotypes studied here (Figure 1). Moreover, nitrate showed no significant increase in leaves lipid peroxidation among nitrate supply rates. Many studies have reported that the leaf lipid peroxidation was increased under both N deficiency and N excess stress conditions. In fact, it was demonstrated that N deficiency increased MDA concentration in *Vaccinium corymbosum* (Yañez-Mansilla et al., 2014; Yañez-Mansilla et al., 2015). Similar increase of MDA was observed in *Myriophyllum spicuatum* and *Potamogeton crispus* leaves under ammonium stress (Jiao et al., 2009; Yin et al, 2016). The N starvation led to the highest lipid peroxidation in leaves, without difference between the ecotypes. Indeed, N addition, significantly reduced lipid peroxidation in leaves of both ecotypes, without difference among nitrate supply rates. The increasing content of MDA and thus, of lipid peroxidation products in plants under higher ammonium levels signified that the damage extended as the ammonium level increased. The increases in MDA content in the same way for both ecotypes may suggest two things: (i) these ecotypes respond in the same way to N stress, and/or (ii) the lipid peroxidation is not a solid indicator of N stress.

Superoxide dismutase is recognized as the primary plant defense barrier against ROS (Alscher et al., 2002), which catalyzes the dismutation of superoxide anion radicals (O_2^-) to hydrogen peroxide (H_2O_2). Our results showed that for both ecotypes, the highest specific SOD activity was observed at 0 kg N ha^{-1} (Figure 2A). Previous studies showed a significant increase in SOD activity in N-deficient mulberry plants and rice seedlings (Tewari et al., 2007; Lin et al., 2011). Moreover, in the present work we observed slight increases in the SOD activity in response to nitrate supply,

but only at the highest dose. In contrast, ammonium supply at rates from 240 kg ha^{-1} to 480 kg ha^{-1} markedly increased the SOD activity in both cultivars. Similar outcomes have been reported previously for spinach, sunflower and wheat plants grown under ammonium excess (Rios-Gonzalez et al., 2002; Polesskaya et al., 2004b; Domínguez-Valdivia et al., 2008). From these results, it is possible to suggest that both N deficiency or ammonium excess conditions triggered SOD activity in order to combat ROS production as it was proposed by Alscher et al. (2002). The increase of SOD activity under N deficiency may also be related to the low rate of CO_2 fixation under these conditions (Polesskaya et al., 2004a). In fact, it was shown that the rate of CO_2 is lower in plants cultivated under N deficiency as compared to control plants without differences in PSII functioning (Polesskaya et al., 2004a). Furthermore, it was reported that this imbalance is accompanied by the over-reduction of carriers in the electron-transport chain which increases the probability of O_2^{-}° generation through the Mehler reaction (Fryer et al., 1998). According these antecedents, the high SOD activity detected here could be related to the enhanced O_2^{-}° generation during photosynthesis in the leaves of the N-deficient sorghum plants. The negative impacts of a high ammonium accumulation in plant tissues might be a consequence of an increase in ROS production in cell membranes due to failure in photosynthesis (Pompelli et al., 2010). It is now well known that N stress (excess or deficiency) can trigger ROS production, affecting cell membranes as well as the integrity of chloroplasts and photosynthetic machinery (Buchanan et al., 2000). Thus, enzymatic antioxidant defenses could help to protect the photosynthetic apparatus against N-induced oxidative stress. Our results also showed that, under N stress conditions (deficiency or ammonium excess), the activity of SOD was significantly higher in the 4P11 ecotype compared to the 3P4 ecotype, suggesting that this antioxidant enzyme may be strongly related to a higher tolerance of 4P11 ecotype to N stress.

Hydrogen peroxide, generated by the SOD enzyme, to prevent cellular damage, is a toxic compound and must be eliminated by conversion to H_2O. In this way, CAT and POD regulate H_2O_2 level in plants (Hossain et al., 2015). In leaves of ammonium treated and N deficient sorghum plants, the activities of POD and CAT were significantly higher than those detected in nitrate-grown plants (Figure 2B and 2C). The increased on POD and CAT activities in N deficient plants observed here are in accordance with results obtained by Tewari et al. (2007) in mulberry plants. These authors related POD and CAT increments with increased H_2O_2 concentration. In addition, we found that POD and CAT activities were significantly induced when plants were supplied with ammonium, especially when this N source was used as sole nitrogen source. Our findings are in accordance with the earlier observations of 5 for *Myriophyllum*. Moreover, Medici et al. (2004) tested the effect of N supply on the activities of the antioxidant system enzymes in the roots of some crop species, including *Zea mays* and *Hordeum vulgare*, and obtained similar results. In this work, the activity of CAT was generally higher in the 4P11 ecotype especially under N deficient and high ammonium level conditions, suggesting that this ecotype has more catalase to protect against oxidative damages caused by N stress particularly, N deficient stress. Associating the results obtained for the SOD activity and the CAT activity we can consider that the 4P11 ecotype is more tolerant to N stress compared to the 3P4 ecotype. In contrast to the result obtained for CAT, activity of POD was much higher in the 3P4 compared to the 4P11 ecotype. The drastic increase in the activity of this enzyme in the 3P4 ecotype might not be related to an effective protective mechanism by this enzyme, but it could be due to an accumulation of a damage lasting for a long time.

In N-deficient plants, GR activity was lower compared to the others treatments conditions (Figure 2D). A similar result was found by *Polesskaya* et al. (2004b), who showed a low GR activity in N-deficient wheat compared to plants supplied with nitrate or ammonium. The results presented here, showed also that both N sources induced GR activity in sorghum plants. Increased GR activity has been observed in sunflower plants grown under ammonia stress (Rios-Gonzalez et al., 2002). The increases on GR activity may be due to the high demand for reduced glutathione (GSH) for plant growth (Mullineaux and Creissen, 1997). In fact, GR is essential to maintain high levels of GSH in plant cells. This molecule is used in numerous redox reactions in the cell, including those involved in protein and DNA synthesis and amino acid transport (Noctor et al., 2002; Frendo et al., 2013). The high GSH/GSSG (GSSG: oxidized glutathione) ratio is also essential to accelerate the H_2O_2 scavenging pathway, principally under stress environment (Pang and Wang, 2010). In the present work, the increased GR activity was greater in NH_4^{+}-N treated plants compared to NO_3^{-}-N treated plants which confirm the importance of the GR in maintaining high levels of GSH to be used in reactions involved on growth rate and antioxidative processes in higher plants (Noctor et al., 2002).

Glutathione produced by the GR enzyme may be involved in the detoxification of methylglyoxal (MG) *via* a reaction catalyzed by Gly I and Gly II. In this way, different studies have shown that the activities of these enzymes are affected by various abiotic stress conditions including salt and heavy metal (Hossain and Fujita, 2009; Kaur et al., 2014). In the present study, we observed an increase of the Gly I and Gly II specific activities in plants grown under both N deficient conditions and N excess, especially when ammonium was used as N source (Figure 3A and 3B). This is the first report indicating that N stress can induce increases of glyoxalase system enzymes in higher plants. It has been reported that high activities of Gly I and Gly II can protect plants against the negative impacts of methylglyoxal which is formed during abiotic stress (Hossain and Fujita, 2009; Saxena et al., 2011, Alvarez et al., 2013). In fact, overexpression studies of the glyoxalase system enzymes in plants have shown that glyoxalases may avoid excessive accumulation of MG under stress conditions, by maintaining intracellular antioxidant pools (Hasanuzzaman and Fujita, 2011; Hasanuzzaman et al., 2011; Ghosh et al., 2014). In addition, it was reported that glyoxalase enzymes increased the tolerance of plants to drought-induced oxidative damage by maintaining the GSG/GSSG ration (Hasanuzzaman and Fujita, 2011). Therefore, the increases in Gly I and Gly II activities observed here, allow suggesting that this system seems to be essential for the detoxification of MG under N stress in sorghum plants. Our findings also indicated that Gly I was much more induced by ammonium compared to the Gly II. In addition, a slight decrease was observed in the Gly II between 120 kg N

ha^{-1} and 240 kg ha^{-1}. A decrease in Gly II activity was also reported in preceding studies conducted on onion callus and mung bean under Cd stress (Hossain and Fujita, 2009; Hossain et al., 2010). These authors suggested that the decrease observed could be related to the proteolytic degradation or the inactivation of enzymes. However, in this study, we observed an increase of the Gly II at 480 kg N ha^{-1} suggesting that the decrease observed at A1 and A2 might not be due to the proteolytic degradation of enzymes. The induction of Gly I activity was higher in the 4P11 ecotype compared to the 3P4 ecotype. One possible explanation of these results is related to an increase of GSH, which acts as a co-factor of Gly I (Hasanuzzaman et al., 2014) due to the consequent mitigation of oxidative stress in plants grown under N stress, which is in line with the results obtained for the GR activity.

Materials and methods

Plant material and growth conditions

Two sorghum ecotypes (3P4 and 4P11) were chosen for this study. These ecotypes were collected from the North Western of Morocco and used after three cycles of selection. They were chosen for the present study because they have been shown to display different growth rates under various N treatments in a preliminary experimental trial.

Sorghum seeds (*Sorghum bicolor*) were sterilized with 5% of NaOCl for 15 minutes and washed thoroughly with sterile water. Plants were then cultivated in 18-cm plastic pots (3000 cm^3) containing 2 kg of a soil that had never received N fertilizer. Twenty seeds per pot of each ecotype were planted. After one week, the plants were thinned to 15 plants per pot. The plants were grown in a controlled environment chamber at 28°C day/21-22°C night, with a photoperiod 16/8 h (light/dark). Before sowing, the soil of each pot was carefully homogenized. Both ecotypes were cultivated in the same conditions and received the same treatments. The N supply was performed per pot during the sowing step using 500 mL of pH-adjusted solutions (pH = 6 ± 0.2) of potassium nitrate (KNO_3) and/or ammonium sulfate ($(NH_4)_2SO_4$,). During the experiment, all pots were watered to near saturation twice a week and were randomized every day. Six N treatments were applied using 120, 240 and 480 Kg ha^{-1} of ammonium or nitrate and three other treatments were applied using 120 kg ha-^{1} nitrate combined to 120, 240 and 480 kg $ha^{-1}$of ammonium. Pots without N supply were considered as control and abbreviated as (-N). All the pots received a basal dose of 60 kg P ha^{-1} and 60 kg K ha^{-1}.

Table 1 indicates the treatments applied to sorghum plants. The experiment was arranged in a randomized complete block design (RCBD) with 3 replications. After five weeks, samples from fully expanded leaves were harvested randomly, liquid N_2 frozen and stored at -80 °C for enzyme activity analysis.

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating the malondialdehyde (MDA) content according to Heat and Packer (1968) using 0.4 grams of frozen leaves.

Enzyme extraction

Using a pre-cooled mortar and pestle, frozen leaves (0.4 grams) were homogenized in 50 mM ice-cold phosphate buffer (pH 7.6) containing 14 mM β-mercaptoethanol, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) , 1% (w/v) polyvinylpyrrolidone (PVP) and 10% (w/v) glycerol. The homogenates were centrifuged at 15,000 g for 20 min and the supernatants were used for determination of enzyme activities. All procedures were performed at 0–4ºC.

Enzymatic measurements

The activity of SOD (EC 1.15.1.1) was assayed according to the method described by Beauchamp and Fridovich (1971). The CAT (EC: 1.11.1.6) activity was assayed according to the method described by Aebi (1974), whereas POD (EC: 1.11.1.17) activity was measured as described by Pütter (1974). The activity of GR (EC: 1.6.4.2) was measured by the method of Yannareli et al. (2007). Glyoxalase I (EC: 4.4.1.5) assay was carried out according to Racker (1951) and Glyoxalase II (EC: 3.1.2.6) activity was determined according to the method of Principato et al. (1987). The total soluble protein content of the enzyme extracts was determined following the method of Bradford (1976), using bovine serum albumin (BSA) as a protein standard.

Statistical analysis

The used data are mean values ± S.D. Results were subjected to a one-way analysis of variance (ANOVA) followed by the *Tukey* test using *PASW statistics (version 18).*

Conclusion

In conclusion, N stress (ammonium excess and N deficient conditions) increased the contents of MDA and in turn induces the activities of SOD, POD, GR and CAT and the glyoxalase system in both sorghum ecotypes. The POD and CAT activities of sorghum leaves increased even under excessive rates of ammonium (480 kg ha^{-1}), supporting the sorghum's ability to alleviate the effect of ammonium stress. It was clear that the 4P11 ecotype has higher levels of SOD, CAT and GR enzymes, and also higher Gly I activity in comparison to the 3P4 ecotype under both N deficient and ammonium excess conditions. These results suggest that the 4P11 ecotype has higher capacity to cope with N stress. The work presented here could help to identify the way in which antioxidant enzymes and glyoxalase systems are affected by N stress. These results will also be used in the identification of biochemical markers useful for the selection of genotypes characterized by higher levels of tolerance to N stress.

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