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Efficacy of natural aluminosilicates in moderating drought effects on the morphological and physiological parameters of maize plants (*Zea mays* L.)

D. Kojić[a*], S. Pajević[a], A. Jovanović-Galović[a], J. Purać[a], E. Pamer[a], S. Škondrić[b], S. Milovac[a], Ž. Popović[a], G. Grubor-Lajšić[a]

[a]Department of Biology and Ecology, Faculty of Science, University of Novi Sad, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia. [b]Faculty of Science, University of Banja Luka, Mladena Stojanovića 2, 78 000 Banja Luka, Republic of Srpska - Bosnia and Herzegovina. [*]Corresponding author: danijela.kojic@dbe.uns.ac.rs

Abstract

Natural aluminosilicates, zeolites and phyllosilicate clays are widely used in agriculture as additives to animal nutrition and as fertilizers for soil conditioning and remediation. Antitoxic nutrient (ATN) is a unique combination of naturally-occurring phyllosilicates, zeolite, bentonite and activated charcoal (60:20:1), processed using a specific technology. In the present study, the effects of ATN treatment on the morphological and physiological parameters of maize plants under drought stress were investigated. Seeds were separated into two experimental groups: a control group (no ATN treatment) and an ATN-treated group (~0.01g ATN/seed). After 16 days of growth, these two groups of young plants were further subdivided into two subgroups: a watered group and a water-deficient group. Over the next 20 days, the following morphological and physiological parameters were measured: plant fresh weight, weights of individual vegetative plant parts, leaf area (LA), leaf water potential, proline content, nitrate reductase activity (NR; NADH:nitrate oxidoreductase; EC 1.6.6.1), glutathione S-transferase (GST; EC 2.5.1.18) activity and total nitrogen content. Results from water potential and proline content measurements suggest that ATN exhibits a protective effect on the roots and leaves of water-stressed maize plants, which is more pronounced in the latter. Interestingly, GST activity was detected in plant roots only, and was stimulated under drought conditions.

Keywords: antitoxic nutrient, zeolite, bentonite, water stress.

Abbreviations: ATN- antitoxic nutrient; ATN O- optimal irrigated group with ATN; ATN D- drought exposed group with ATN; CO- optimal irrigated control group; CD- drought exposed control group; FW- fresh weight; GST- glutathione S-transferase; LA- leaf area; LSD-the least significant differences; NR- nitrate reductase; ROS- radical oxygen species.

1. Introduction

Drought is a worldwide problem, and the study of drought has become one of the main research directions of global plant biology. In particular, great effort has been expended to develop drought-resistant plants by producing transgenic plants using different types of gene technologies, or genotypes possessing full physiological potential under limited soil water conditions (Casadebaig *et al*., 2008; Hong-Bo *et al*., 2006; Stella and Christoph, 2006; Sang-Eun *et al*., 2005). As a result of this research, the introduction of drought-resistant transgenic plants for commercial use has also become possible (Hilbeck *et al*., 2005).

Thus, a deeper understanding of plant physiological mechanisms operating under drought conditions is needed. Drought resistance in plants involves many biochemical, molecular and structural mechanisms, including the biosynthesis of different biomolecules (Bajguz and Hayat, 2009; Hong-Bo *et al*., 2006; Fiehn, 2002). For example, one well-known response of plants to water deficiency is osmotic adjustment via accumulation of the amino acid proline, which acts as a compatible solute, an osmoprotectant, and a protective agent for cytosolic enzymes and cellular organelles (Hong-Bo *et al*., 2006).

In addition, the activities of many enzymes are affected by drought conditions. For example, nitrate reductase activity has been shown to be highly sensitive to water stress, and a significant decrease in nitrate reductase activity was observed in many plant species under drought conditions (Casadebaig *et al*., 2008; Foyer *et al*., 1998). Nitrate reductase activity is induced by nitrogen content in plant tissue, and is regulated at the transcriptional level by the availability of its' substrate, NO_3^-, and by glutamine, the end product of the nitrogen assimilation pathway (Downs *et al*., 1993). Water deficits induce an abrupt reduction in the uptake and nitrate flux rates from roots to leaves. In fact, during prolonged periods of drought, a decrease in water availability for transport-associated processes leads to changes in the concentrations of many metabolites, followed by disturbances in amino acid and carbohydrate metabolism, which accompany a decrease in nitrate reductase activity (Huber *et al*., 1994).

Drought stress also increases the levels of radical oxygen species (ROS) in plant cells, resulting in lipid peroxidation and protein damage (Taylor *et al*., 2004). Glutathione S-transferase (GST) is an essential enzyme which utilizes glutathione to catalyze glutathione-dependent detoxification reactions, reducing organic hydroperoxides and protecting protein sulfuric groups (Edwards *et al*., 2005).

In recent years, there has been increasing interest in the application of natural aluminosilicates in agricultural technology. Several studies have demonstrated that both bentonite and zeolite have desirable properties and a large water holding capacity; while in treatment soil they improved the level of available nutrient content (Coppola *et al*., 2003; Pisarovic *et al*., 2003; Xiubin and Zhanbih, 2001; Mumpton, 1999). The unique physical and chemical properties of natural aluminosilicates make them particularly suitable for agricultural use. Zeolites and bentonite are naturally occurring structured and phyllosilicate minerals, respectively, with high cation exchange and ion adsorption capacities.

Zeolites are hydrated aluminosilicates of alkaline and alkaline-earth minerals with an infinite, open three-dimensional structure (Mumpton, 1999) that is able to lose or gain water reversibly and exchange extra framework cations, both without crystal structure changes. Zeolite structures are made up of a framework of $[SiO_4]^{4-}$ and $[AlO_4]^{5-}$ tetrahedrons linked to each other via shared oxygen atoms. The substitution

of Si^{4+} by Al^{3+} in tetrahedral sites results in a more negative charge and higher cation exchange capacity (Iskander *et al*., 2011). Technologies based on zeolites use slow releasing fertilizers (zeopones) as soil conditioners and remediators (Coppola *et al*., 2003; Pisarovic *et al*., 2003; Mumpton, 1999). In fact, zeolites have been reported to improve soil characteristics by increasing moisture content (Xiubin and Zhanbih, 2001). Thus, zeolites could potentially act as water moderators by absorbing water and slowly releasing it: preventing root rot and moderating drought cycles.

Bentonite is a 2:1 mineral, with a high surface area and cation exchange capacity. Each layer of bentonite is composed of 2 silica sheets and 1 octahedral sheet, with individual layers held together by van der Waals forces. Because of the relatively weak nature of these interlayer forces water can easily penetrate between layers, while cations balance charge deficiencies present in the structure (Iskander, 2011). The primary effect of bentonite addition is to improve the water holding capacity and moisture content of soil, contributing to the stimulation of biological activity. However, mixing bentonite into soil also increases both mineral nutrient and colloid content; and the resulting higher colloid content decreases nutrient leaching (Magdolna *et al*., 2011). In experiments conducted by the University of München, where bentonite was mixed into the soil of salad seedlings, a 3% dose had a favorable effect on the fresh weight of seedlings, while a higher dose was found to be detrimental to plants (Magdolna *et al*., 2011).

Interestingly, activated carbon has also been shown to affect nitrogen and other nutrient concentrations in soil media in the absence of plants. Moreover, addition of activated carbon to soil was found to increase plant biomass, possibly because of greater nitrogen availability (Lau *et al*., 2008).

The present study was conducted to test the hypothesis that natural aluminosilicates can moderate drought effects in plants. Specifically, we examined the effects of an antitoxic nutrient (ATN), composed of a mixture of zeolite (> 90% clinoptilolite), bentonite (> 83% montmorillonite) and small amounts of charcoal (60:20:1) on the growth of maize plants (*Zea mays* L.) under unfavorable water conditions. To the best of our knowledge, this is the first study to investigate the effects of ATN treatment on plant growth under drought conditions. However, in an unrelated study, ATN dietary supplementation has been shown to have beneficial effects on both the growth performance and meat quality of broiler chickens (Prvulović, 2008). In order to investigate the effects of ATN treatment on drought resistance, maize plants were grown in sand culture in the presence or absence of ATN, under well-irrigated or drought stress conditions. Results were quantified by measuring several morphological and physiological parameters, including proline levels and total nitrogen content, leaf water potential as well as nitrate reductase and glutatione S-transferase activity.

2. Materials and methods

2.1. Plant material

Experimental plant material consisted of young maize plants (*Zea mays* L.) grown in sand culture. Sand particle size was 0.01-0.075 mm. Before seeding, seeds were sterilized with 3% H_2O_2 for 15 minutes and rinsed with distilled water. After imbibition in distilled water for 24 hours, seeds were transferred to individual pots containing sterilized sand (700 mL). Using a chamber growth method, maize plants were exposed to a 16h light and 8h dark regimen, under constant ambient temperature (25^{o}C). The experiment consisted of 120 pots containing 5 seeds each. The

two main experimental groups were organised as follows: the first group (the control) consisted of 60 pots with seeds covered in sand; the second group (ATN-treated group) consisted of 60 pots with seeds covered with 0.01 g ATN per seed. ATN is mixture of zeolite, bentonite (origin from southern Serbia) and activated charcoal (60:20:1). After seeding, pots were irrigated with 10 mL distilled water to maintain optimal soil humidity conditions during the seedling period. From the 12th to the 15th day, plants were watered alternately with 20mL of distilled water and Reid-York nutrient solution (Reid and York, 1958). On the 15th day of growth, control and ATN-treated plants were further subdivided into two subgroups, each consisting of 30 randomly selected pots. Four different experimental treatments were as follows:

CO – control, optimal irrigation
CD – control, drought
ATN O – ATN, optimal irrigation
ATN D – ATN, drought

Plants under optimal irrigation conditions (CO and ATN O) were continuously watered to maintain at least 70% soil (sand) humidity (Kamara and Jackson, 1997). For water stress conditions (CD and ATN D), plants were subjected to drought for 5 days; after 5 mL of distilled water was given once, before watering with 10 mL of distilled water per pot for 7 days. Plants were then watered with 5 mL of distilled water per pot until the end of the experiment. This treatment resulted in a soil (sand) humidity of 20%, indicating drought conditions.

After 35 days of growth, plant material was harvested and prepared for physiological and biochemical analyses.

2.2. Morphological and physiological analyses

Plants were separated into roots, stems and leaves. The fresh weight (g) of whole plants and individual vegetative parts (roots, stems and leaves) were measured. Leaf area (LA) per plant was measured by using a portable leaf area meter (LI-COR 3000, Lambda Instruments Corporation, Lincoln, Nebraska, USA). Leaf water potential was determined using the pressure chamber described by Scholander *et al*. (1965).

Free proline content in roots and leaves was measured according to Bates *et al*. (1973). Briefly, plant material was homogenized in 3% aqueous sulfosalicylic acid and the resulting homogenate was centrifuged for 10 minutes at 10000 rpm. The supernatant was used to estimate proline content as follows. A reaction mixture consisting of 2 mL of supernatant, 2 mL of acid ninhydrin (1.25 g ninhydrin in 30 mL glacial acetic acid and 60 mL 6 M phosphoric acid) and 2 mL of glacial acetic acid was boiled at 100 °C for 1 h. Reactions were then terminated in an ice bath. The resulting reaction mixture was extracted with 4 mL of toluene and the absorbance of the proline-ninhydrin chromophore was measured at 520 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as μg proline g^{-1} of fresh weight material.

Nitrate reductase (NR) activity was determined by the method described by Downs *et al*. (1993). For the assay, 0.5 g of root or leaf material was collected and incubated for 30 minutes at 36°C in 5 mL of incubation solution containing 0.1 M potassium phosphate buffer, pH 7.5 and 0.02 M KNO_3. Nitrite was determined in 0.8 mL aliquot of the incubation solution by adding 0.6 mL 0.02% N-1-naphthyl-ethylendiamine

dihydrochloride and 0.6 mL 1% sulfanilamide in 1.5 M HCl. After shaking, the reaction mixture was left to stand for 30 minutes, and then was diluted to 5mL with distilled water and absorbance was read at 540 nm. Nitrite concentrations were determined from a standard curve and calculated on a fresh weight basis as µmole of NO_2^- g^{-1} h^{-1}.

The total nitrogen concentration in the dry matter of roots and leaves was determined using the standard microkjeldahl method (Nelson and Sommers, 1973).

Glutathione S-transferase (GST) activity was measured as described by Habig *et al*. (1974) using 1-chloro-2,4-dinitrobenzene as a substrate. GST activity was assayed in 1.25 mL reaction medium containing 120 mM phosphate buffer, pH 6.5, 120 µM reduced glutathione and 1 mM CDNB in the final volume. The reaction was started after addition of 10 µL of sample and absorbance at 340 nm was followed for 3 minutes. One unit of GST activity was defined as the amount of GST enzyme that utilizes 1 nmol GSH min^{-1} at 25°C, pH 6.5.

The total protein concentration in the supernatant was determined using the method of Bradford (1976) with bovine serum albumin as a standard.

2.3. Statistical analyses

Data were analyzed by two-way analysis of variance (Two Way ANOVA) using Duncan's test as a post hoc test for separation of means. Means in table and figures with the same letters are not significantly different at $p<0.05$.

3. Results and discussion

3.1. Morphological parameters

The data obtained in this study clearly suggests that ATN has a positive effect on the drought resistance of maize plants under the experimental conditions tested. Fresh weights (FW) of whole plants and their vegetative parts for control and ATN groups are shown in Table 1. As can be seen, ATN clearly has a positive effect on biomass production. The significant increase in FW observed in maize plants from the ATN treated group could be due to a nutritional effect from ATN or an effect related to the structure of the substrate. In support of the former, Leaf Areas (LA) were found to be significantly higher in the ATN-group versus the control-group in optimally irrigated plants (Table 1).

Table 1: Fresh weight (FW) of whole plants and excised vegetative parts and leaf areas of control-group and ATN -group (optimal irrigated and under drought stress). Note: Means with the same letters in a column do not differ significantly at $p<0.05$.

treatment		Fresh weight (g)			Leaf area (cm^2)
		whole plant	root	leaf	
Control-group	optimal irrigated	4.90 B	1.87 B	0.31 B	133.30 B
	drought	2.73 D	1.17 C	0.15 D	85.12 D
ATN-group	optimal irrigated	7.68 A	2.59 A	0.44 A	200.00 A
	drought	4.26 C	1.69 B	0.22 C	96.40 C
$LSD^*_{(AxB)}$ 5%		0.55	0.28	0.06	6.49

* least significant difference

FWs for whole plants and their vegetative parts decreased significantly (~1.5-fold) under drought conditions for both the ATN and control group plants. A drought-induced reduction in leaf area was also observed for both groups, and leaf expansion was decreased in the presence of drought stress, due to the inherently slow rate of osmotic adjustment and the low membrane extensibility of leaf cells. It has been well-documented that differences in turgor pressure can result in smaller leaf areas in plants grown under drought stress versus well-watered plant (Iersel and Nemali, 2004). Our results confirm the remarkable effects of drought stress on leaf area, and highlight the protective effects of ATN in the ATN treated group on leaf water potential. Although leaf areas were observed to decrease more in the ATN-group during drought (ATN D was 48% of ATN O in comparison with C D, which was 63% of C O), leaf areas for ATN D were still significantly larger than those of the control-group under water stress conditions. The effect of ATN on the absorption and retention of ammonia-nitrogen and potassium ions from clinoptiolite, which supplies necessary nourishment (Englert and Rubio, 2005), could explain the higher mass and larger leaf area values observed for the ATN group versus the control group.

3.2. Physiological parameters

Water potential values for the ATN and control groups (Figure 1) were similar under optimal irrigation conditions (no statistically significant differences were observed). In contrast, although water stress treatment resulted in decreased water potential in both plant groups; this decrease was significantly less (i.e. less negative) in the ATN D group compared to the CD group (-875.0 kPa vs. -641.7 kPa). The higher water potential observed for the ATN-group during drought suggests that these plants experienced less intense stress conditions due to the presence of ATN in the substratum.

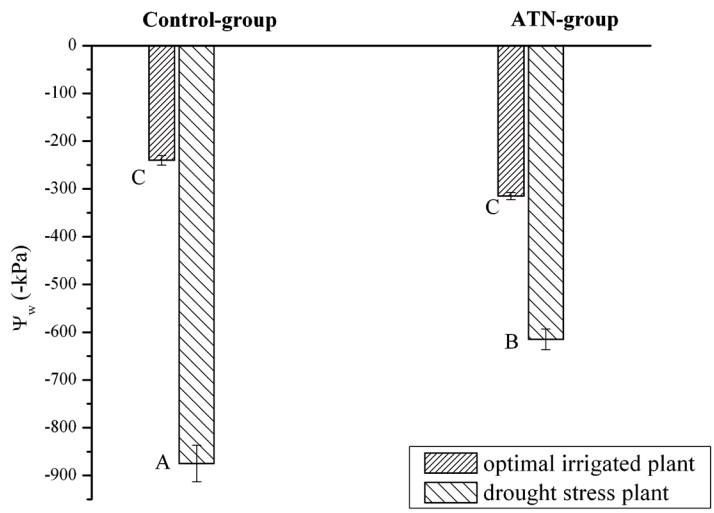

Figure 1. Leaf water potentials for irrigated maize plants (control and ATN group) and plants subject to drought-stress (control and ATN group). Means with the same letters are not significantly different at $p<0.05$.

The free proline content of well-irrigated and drought-exposed plants from both the control and ATN-group (leaves and roots) is shown in Figure 2. The free proline content in the leaves of well-irrigated control-group plants was 16.5 μg g^{-1} of their fresh weight; 1.7-fold higher than in the ATN-group. In contrast, the free proline content in the roots of well-irrigated control and ATN-group plants did not differ significantly (approximately 7 μg g^{-1} of their fresh weight). In both groups, exposure to drought stress conditions resulted in an increase in free proline content: 2 times higher in the leaves of both groups; and 3 times higher in the roots of control plants vs. 7 times higher in roots of ATN plants.

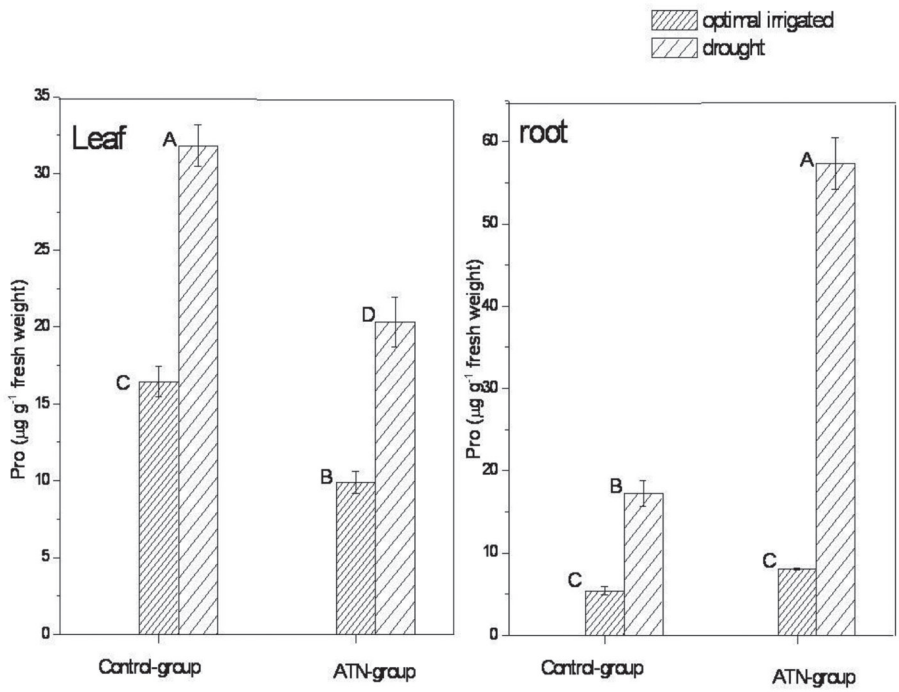

Figure 2. The effect of drought-stress on proline accumulation in the leaves and roots of ATN group vs. control group plants. Means with the same letters are not significantly different at $p<0.05$.

Our results are in agreement with earlier reported results that proline content was increased under drought stress conditions in all of the examined groups (Figure 2). The presence of ATN was associated with a somewhat lower proline content in leaves under drought conditions compared to the control group, suggesting a positive effect from ATN during drought conditions. This reduced free proline content in leaves suggests a reduced level of physiological suffering in plants under drought conditions, when ATN is present. This effect might be attributable to the increase in humidity connected with the presence of natural aluminosilicates (Xiubin and Zhanbin, 2001). Although drought conditions induced a significant increase in free proline con-

tent in the roots of both the control and ATN groups, this increase was more dramatic in the ATN-group. The extremely free proline content found in the roots of ATN-group plants increases the protective effect of proline, which acts as an osmotic substance in plant tissue: making the plant more efficient at absorbing water from dry soil (Kevrešan *et al*, 1998; Handa *et al*., 1986).

Nitrate reductase (NR) activity levels and the nitrogen content in the leaves and roots of both groups (ATN and control) are shown in Figure 3. These results demonstrate that drought conditions induced a significant decrease in nitrate reductase activity in the roots of both groups (control and ATN group), along with lower N uptake in the roots of the ATN-group.

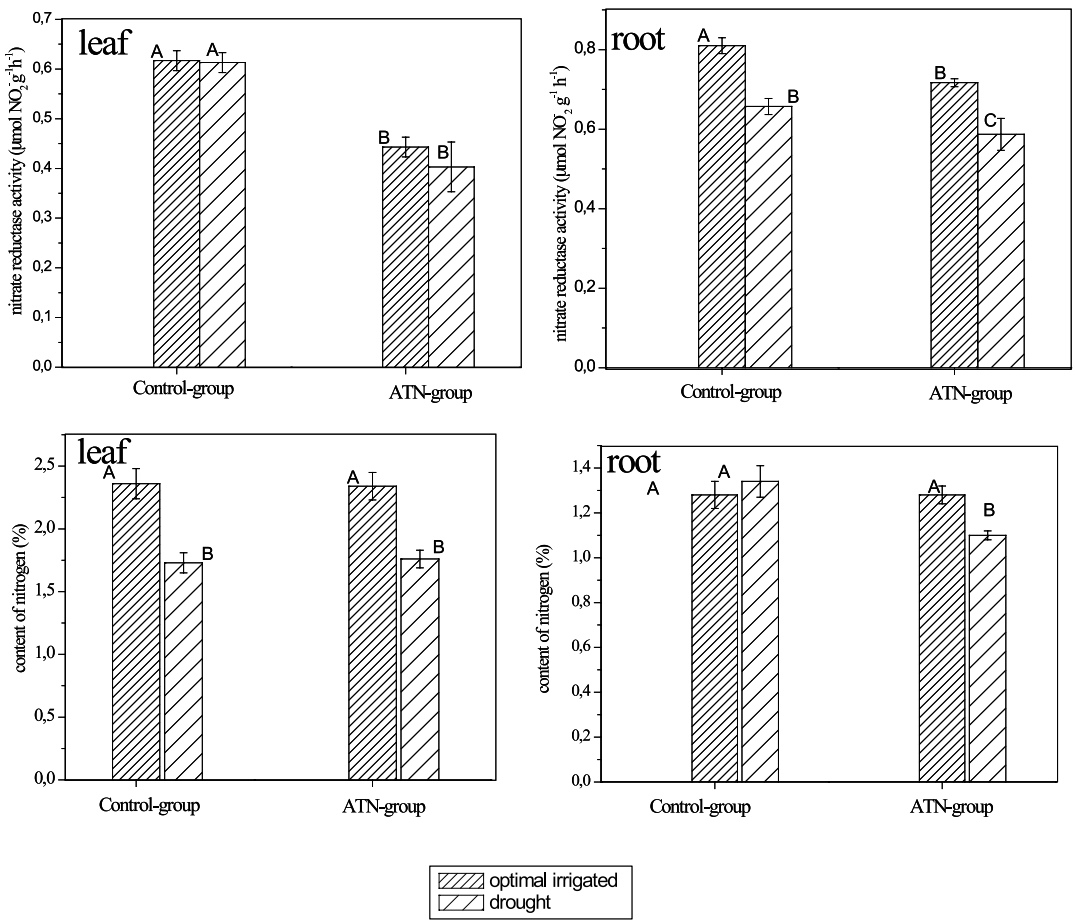

Figure 3. Nitrate reductase activity in leaf and root parts, and nitrogen content in leaf and root parts of control and ATN groups (optimally irrigated vs. under drought stress). Means with the same letters are not significantly different at $p<0.05$.

In our study, GST activity was detected in only plant roots (Figure 4). GST activity in plants from both groups (control and ATN) was found to be increased during drought conditions, and was not affected by the presence of ATN. Specifically, GST activity in the control group was observed to increase from 255.5 to 711.6 U/mg protein, and in the ATN group from 179.4 to 645.4 U/mg protein.

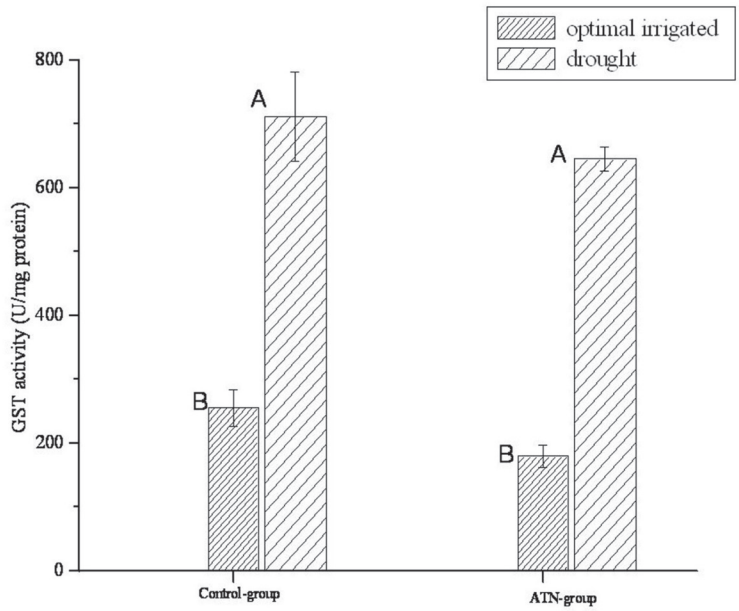

Figure 4. Glutathione S-transferase (GST) activity in the roots of control and ATN groups (optimal irrigated vs. under drought stress). Means with the same letters are not significantly different at $p<0.05$.

The significant increase in GST activity measured under drought conditions in both groups (control and ATN) is in agreement with the induction of oxidation process in cells provoked by drought, while ATN treatment appears to have had no effect on GST activity levels.

4. Conclusions

In the present study we demonstrate that ATN treatment has a significant positive effect on biomass and certain physiological stress parameters in maize plants, under both well-irrigated and drought conditions. Plants treated with ATN have a higher whole plant and vegetative parts fresh weight. Furthermore, increased water potential, reduced proline accumulation and reduced nitrate reductase activity were recorded in the leaves of ATN treated plants; whereas higher proline accumulation and less nitrate reductase activity were observed in the roots. Together our data suggest that plants treated with ATN may potentially have increased drought resistance potential, although further investigation is required.

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