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Exogenous potassium nitrate alleviates salt-induced oxidative stress in maize

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ABSTRACT: The effects of the exogenous potassium nitrate application on major antioxidant enzymes, photosynthetic pigment content, malondialdehyde, hydrogen peroxide and free proline were investigated in salt-stressed (75 mM NaCl) maize genotype (ADA 9510). Plants were grown in growth chamber for ten days. After five days of applications (control, 0 mM NaCl), S75 (75 mM NaCl), potassium nitrate (3 mM KNO_3) and S75 + potassium nitrate (75 mM NaCl + 3 mM KNO_3), plants were harvested. The results showed that salt stress significantly decreased chlorophyll a, chlorophyll b and total chlorophyll contents and increased the activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase. Malondialdehyde, hydrogen peroxide and free proline contents were increased by salt stress. These results showed that salinity led to the oxidative stress and destruction of photosynthetic pigments in maize leaves. The exogenous potassium nitrate application, on the other hand, caused to the increased chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid, elevated level of ascorbate peroxidase and glutathione reductase, and decreased malondialdehyde, hydrogen peroxide and free proline content. This kind of changes may indicate that the exogenous potassium nitrate application activates the antioxidant defence system and counteract the oxidative stress. Thus, it may be concluded that the exogenous potassium nitrate application improves salt tolerance and encourage the growth of maize plants under salt stress at early seedling stage.

Keywords: Antioxidant enzymes; Maize; Potassium nitrate; Salinity; Salt tolerance.

1. INTRODUCTION

It has been well known that excess accumulation of salt ions in soils is increasingly becoming a problem for agricultural activities, which is due to nutritional disorder and oxidative stress involved in ion toxicity and osmotic stress. Oxidative stress leads to inactivation of the enzymes, inhibition of the protein synthesis, peroxidation of the lipids, degradation of the photosynthetic pigments and damage in the membrane systems [1]. Plants have evolved an antioxidant system to counteract oxidative stress. This system includes both enzymatic and non-enzymatic elements such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, ascorbic acid, glutathione, α-tocopherol and anthocyanins [2]. The balance between production and detoxification of free radicals determines the survival of plants under salinity conditions [3].

In environments containing high level of soluble salts, the ability of plants to grow and complete their life cycle is known as salt tolerance [4]. Soluble salts in soil could be removed by washing technique.

This method, however, is impractical because of being expensive. The second method has been indicated to select and cultivate salt tolerant plant species and genotypes [5]. In recent years, considerable improvements have been made in crop plants through conventional selection and breeding methods [6]. Many scientists have suggested that selection is more convenient if the plant species possesses distinctive indicators of salt tolerance at the whole plant, tissue or cellular level [6, 7]. However, there are no well-defined plant indicators for salinity tolerance that could practically be used by plant breeders to improve salt tolerance in crop plants. In addition, the strategies of plant breeding and genetic engineering are long-term and complex endeavors to develop salt tolerance that still has limited success [8]. Therefore, salinity tolerance in plants is very complex and has yet been understood well. In addition, variation in the degree of salt tolerance occurs not only amongst species but also among cultivars of the single species.

At the present time, some alternative chemical approaches have been applied to improve salt tolerance in plants. Potassium, for example, is an essential macro-element that affects growth and development in plants. It has also been reported that potassium is enable plants to withstand various biotic and abiotic stress factors including salinity [9]. Application of potassium mitigates the adverse effect of salt stress through regulating stomatal movement and osmotic adjustment and maintaining the membrane-ion charge balance and protein synthesis [10]. Also, potassium has been known to contribute to the restriction of sodium uptake by plants under salt stress. Potassium content in plant tissues, on the other hand, progressively decreases with an increase in salinity and maintenance of the adequate levels of potassium has a vital role for plant survival in saline conditions [11]. Chakraborty et al. [12] and Kaya et al. [13], for example, have found that external potassium applications led to the elevated level of potassium in the leaves and improved salt tolerance in different peanut cultivars and *Cucumis melo* L. plants, respectively. It has been reported that ABTS and DPPH test demonstrated the increased antioxidant capacity in soybean plants under medium salinity as a result of foliar potassium application [14]. Seçkin et al. [15] have declared that plant tolerance to salinity is closely related to the elevated activities and capacities of antioxidant enzymes and antioxidant molecules, respectively. In this case, it is very important to give special attention to investigate the effects of the interaction between salt stress and exogenous potassium application on antioxidant system in plants. However, limited number of researches are available in this area.

Therefore, the aim of the present study is to investigate the influence of salinity in the presence and absence of potassium nitrate on photosynthetic pigments and proline, cellular damage effects (MDA and H_2O_2) and antioxidant enzyme activities (superoxide dismutase, ascorbate peroxidase and glutathione reductase) in a maize cultivar (ADA9510).

2. MATERIALS AND METHODS

2.1. Plant materials, growth conditions and experimental design

Maize (*Zea mays* L.) cultivars, ADA9510, were grown in growth chamber in plastic pots containing Hoagland nutrient solution. The average temperature for day/night was 25/18°C respectively, relative humidity was 40-50%, the photoperiod for the day/night cycle was 16/8 h respectively, and the maximum photosynthetically active radiation was about 200 µmol photon m^{-2} s^{-1}. After 10 days of growth, the applications were done as follows: Control (0 mM NaCl), S75 (75 mM NaCl), potassium nitrate (3 mM KNO_3) and S75+ potassium nitrate (75 mM NaCl + 3 mM KNO_3). Control plants were watered with Hoagland nutrient solution only. The seedlings were harvested after 5 days of applications and leaves are kept at -80°C until analysis.

2.2. Photosynthetic pigment analysis

Photosynthetic pigments were extracted from leaf segments in 3 mL 100% acetone. The absorbance of the extracts was measured at 470, 644.8 and 661.6 nm using a Shimadzu mini 1240 UV visible spectrophotometer. The concentrations of chlorophyll a, chlorophyll b, total chlorophyll (a+b) and total carotenoids (x+c) were calculated according to the procedure of Lichtenthaler [16].

2.3. Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) analysis

MDA and H_2O_2 content were determined by the method of Heath and Packer [17] and Ohkawa et al. [18], respectively. Fresh leaf material (0.1 g) was homogenized in 6 ml of 5 % TCA (4°C) and centrifuged at 10 000g for 15 min and the supernatant was used in the subsequent determination. To 0.5 ml of the supernatant were added 0.5 ml of 0.1 M Tris-HCl (pH 7.6) and 1 ml of TCA–TBA reagent. The mixture was heated at 95°C for 60 min and then quickly cooled in an ice bath. After centrifugation at 10 000 g for 5 min to remove suspended turbidity, the absorbance of supernatant at 532 nm was recorded. Non-specific absorbance at 600 nm was measured and subtracted from the readings recorded at 532 nm. Concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}. For determination of hydrogen peroxide, 0.5 ml of 0.1 M Tris-HCl (pH 7.6) and 1 ml of 1 M KI were added to 0.5 ml of supernatant. After 90 min, the absorbance was read at 390 nm. A standard curve for hydrogen peroxide was prepared to determine hydrogen peroxide concentration in each sample.

2.4. Free proline analysis

Approximately 10 mg powdered dry leaf material was extracted in 4 mL distilled water on a hot plate at 100ºC for 10 min according to Bates et al. [19]. Extracts were filtered and the same procedure was repeated two times. The liquid phase of the homogenate was collected and centrifuged at 3500 rpm for 10 min. Two mL of the supernatant was reacted with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid at 100°C for 1 h. The reaction mixture was mixed with 4 mL toluene and vortexed for 20 s. The chromophore containing toluene was separated and the absorbance of the pink upper phase was recorded at 520 nm against toluene blank. A standard curve for proline in the range of 0.2-1 µmol mL^{-1} was prepared to determine free proline concentration in each sample.

2.5. Antioxidant enzyme activities

For determination of enzyme activities, 0.3 g fresh leaves material from non-acclimated and cold-acclimated leaves were powdered with liquid nitrogen and suspended in specific buffer with proper pH values for each enzyme. The homogenates were centrifuged at 14,000 rpm for 20 min at 4°C and resulting supernatants were used for enzyme assay. The protein concentrations of leaf crude extracts were determined according to Bradford [20], using BSA as a standard.

Superoxide dismutase (SOD; EC 1. 15. 1. 1) activity was determined by the method of Beyer and Fridovich [21], based on the photo reduction of NBT (nitro blue tetrazolium). Extraction was performed in 1.5 mL homogenization buffer containing 10 mM K_2HPO_4 buffer (pH 7.0), 2% PVP and 1 mM Na_2EDTA. The reaction mixture consisted of 100 mM K_2HPO_4 buffer (pH 7.8), containing 9.9 x 10^{-3} M methionine, 5.7 x 10^{-5} M NBT, 1% triton X-100 and enzyme extract. Reaction was started by the addition of 0.9 µM riboflavin and mixture was exposed to light with an intensity of 375 µmol m^{-2} s^{-1}. After 15 min, reaction was stopped by switching off the light and absorbance was read at 560 nm. SOD activity was calculated by a standard graphic and expressed as unit mg^{-1} protein.

Ascorbate peroxidase (APX; EC 1. 11. 1. 11) activity was determined according to Wang et al. [22] by estimating the decreasing rate of ascorbate oxidation at 290 nm. APX extraction was performed in 50 mM Tris–HCl (pH 7.2), 2% PVP, 1 mM Na_2EDTA, and 2 mM ascorbate. The reaction mixture consisted of 50mM KH_2PO_4 buffer (pH 6.6), 2.5 mM ascorbate, 10 mM H_2O_2 and enzyme, containing 100 µg proteins in a final volume of 1 mL. The enzyme activity was calculated from initial rate of the reaction using the extinction coefficient of ascorbate (E = 2.8 mM cm^{-1} at 290 nm).

Glutathione reductase (GR; EC 1. 6. 4. 2) activity was measured with the method of Sgherri et al. [23]. Extraction was performed in 1.5 ml of suspension solution, containing 100 mM KH_2PO_4 buffer (pH 7.0), 1 mM Na_2EDTA, and 2% PVP. The reaction mixture (total volume of 1 mL) contained 100 mM KH_2PO_4 buffer (pH 7.8), 2 mM Na_2EDTA, 0.5 mM oxidised glutathione (GSSG), 0.2 mM NADPH and enzyme extract containing 100 µg protein. Decrease in absorbance at 340 nm was recorded. Correction was made for the non-enzymatic oxidation of NADPH by recording the decrease at 340 nm without adding GSSG to assay mixture. The enzyme activity was calculated from the initial rate of the reaction after subtracting the non-enzymatic oxidation using the extinction coefficient of NADPH (E = 6.2 mM cm^{-1} at 340 nm).

2.6. Statistical analysis

Experiments were a randomised complete block design with three independent replicates. Analysis of variance (ANOVA) was performed using SPSS 20.0 statistical software for Windows. To separate significant differences between means, Duncan test was used at *P = 0.05.

3. RESULTS AND DISCUSSION

Accumulation of the soluble salts in soils is a serious environmental constraint for normal plant growth. Optimizing mineral supplementation to the saline soils may be one of the most important approaches for exploiting the genetic potential of crop plants. Potassium has an essential role in regulating the cellular functioning of plants under optimum and stressful conditions [24]. Potassium is believed to improve signalling mechanism for regulating plant responses to biotic and abiotic stress factors [25]. Therefore, it may be used as a potential tool to improve growth and productivity of crop plants under salt stress. Present investigation was carried out to gain an insight in the ameliorative effect of potassium nitrate against salt-induced oxidative stress. The results in the present study clearly showed that maize plants exhibited a remarkable decrease in chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid content under salinity (Fig. 1a, b, c and d). In accordance with our results, it has been reported that salinity reduces photosynthetic pigment content in several crop plants such as pea, wheat, rice and tomato [6, 26-28]. Bybordi [29] has indicated that reduction in the photosynthetic pigment content in plants under salt stress may be due to inhibition of the biosynthetic pathway of the pigments by the accumulated sodium ions in the leaf tissue. In addition, Taibi et al. [30] has reported that the decreased photosynthetic pigment level in salt-stressed plants has been considered as a typical symptom of oxidative stress and was attributed to the activation of its degradation by the proteolytic enzyme chlorophyllase. Similarly, it has been demonstrated both salt-induced down-regulation of chlorophyll synthesizing enzymes and up-regulation of chlorophyllase activity in plants [31, 32]. As a result, reduction in photosynthetic pigment content may result from either slow synthesis or the accelerated breakdown in salt-stressed maize plants in this study. Another possibility is that salt stress may interfere with the ultrastructure of chloroplast including plastid envelope and thylakoids and hence salinity may lead to the release of photosynthetic pigments from the thylakoid membranes as reported by

Dolatabadian and Jouneghani [33]. It has been indicated by Bybordi [29] that chloroplastic membranes seldom remains intact under salt stress conditions. In the present investigation, exogenous potassium nitrate application has been found to mitigate the adverse effects of salinity on photosynthetic pigment content in maize leaves. In maize plants under 75 mM salt stress, for example, exogenous potassium nitrate treatment caused to the increased level of chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid as compared to 75 mM salt only (Fig. 1a, b, c and d). Similar results were obtained in jojoba and barley plants [34, 35]. Our results showed that balance between pigment biosynthesis and degradation was switched by the exogenous potassium nitrate application in favour of biosynthetic pathway and that chlorophyll biosynthetic pathway is responsive to the exogenous potassium nitrate. In addition, it may be concluded that exogenous potassium nitrate application is capable of protecting the photosynthetic apparatus and inducing chlorophyll synthesis in salt-stressed maize genotype used in this study. It has been noticed that stimulation of the synthesis of IAA (indole-3-acetic acid) and GA_3 (gibberellic acid) in plant tissues results in the accelerated rate of photosynthetic pigment biosynthesis [36]. It may be an alternative explanation for the elevated photosynthetic pigment levels that exogenous potassium nitrate application affected hormonal status in maize plants under salt stress. However, this hypothesis was not tested in the present study. Carotenoids, on the other hand, has been known to be protective pigments against oxidative stress and thus avoiding chlorophyll loss. In our study, the exogenous potassium nitrate application stimulates total carotenoids, indicating the presence of a such protective mechanism in maize plants under salt stress condition [1].

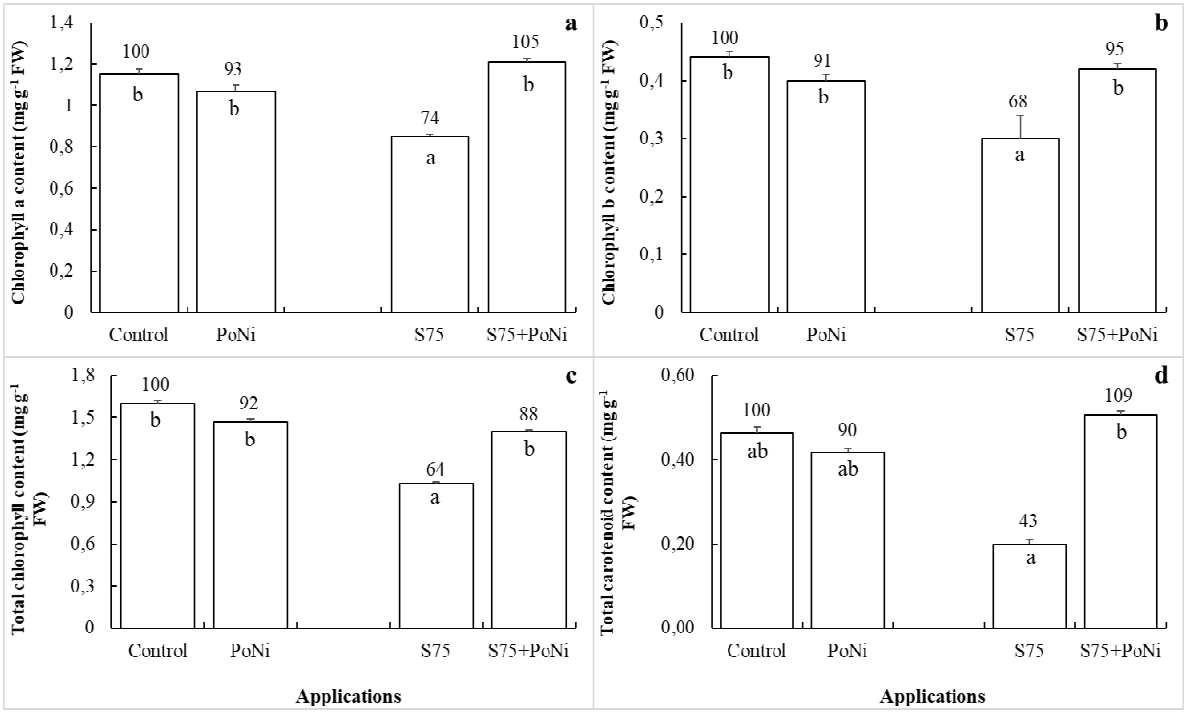

Figure 1. Effect of the exogenous potassium nitrate on (a) chlorophyll a, (b) chlorophyll b, (c) total chlorophyll and (d) total carotenoid content of maize plants under salt stress. (PoNi: Potassium nitrate; S: Salt; columns with different letters mean significant differences between the treatments according to Duncan's multiple range test ($P<0.05$) and numbers on the columns indicate % change relative to control, control=100).

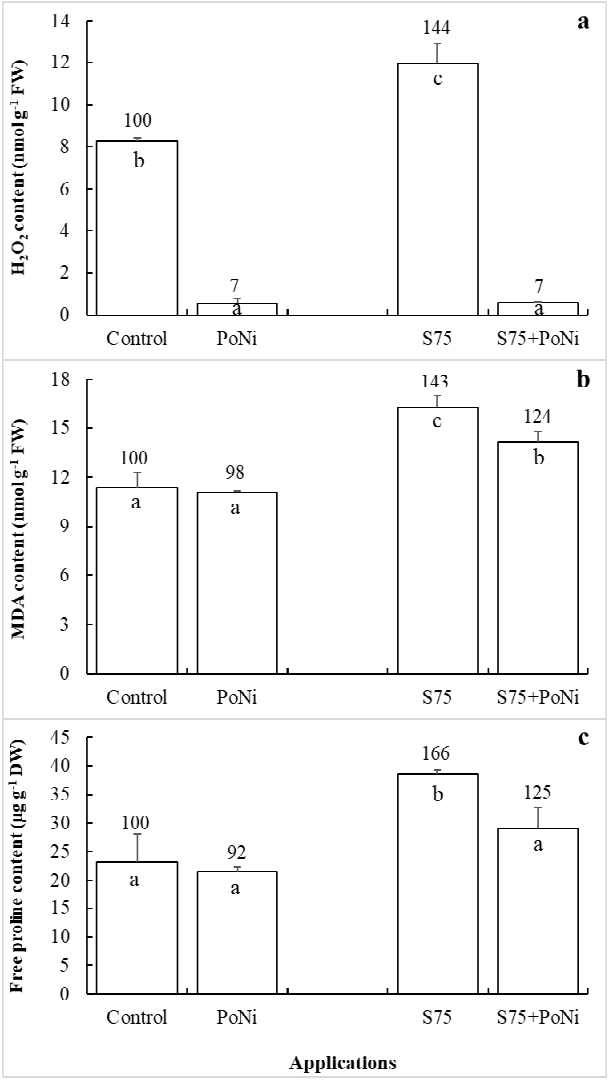

Figure 2. Effect of the exogenous potassium nitrate on (a) H_2O_2, (b) MDA and (c) free proline and content of maize plants under salt stress. (PoNi: Potassium nitrate; S: Salt; columns with different letters mean significant differences between the treatments according to Duncan's multiple range test ($P<0.05$) and numbers on the columns indicate % change relative to control, control=100).

SOD is in the first line of the antioxidant defense system and are responsible for the dismutation of the superoxide radical [2]. APX, on the other hand, reduces H_2O_2 into H_2O and O_2 using ascorbate as the electron donor. GR catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to the reduced form GSH. APX and GR are associated with H_2O_2 scavenging via ascorbate-glutathione cycle [1]. In the present study, salt stress-induced SOD activity (Fig. 3a) may indicate both accelerated production of superoxide radical and its efficient detoxification in the leaves of maize [37]. In harmony with this result, APOD and GR activity in the salt-stressed maize leaves was significantly higher than control (Fig. 3b and c), suggesting an efficient scavenging of H_2O_2 produced by SOD. H_2O_2 content in the leaves of salt-stressed maize plants, on the other hand, was found to be remarkably higher than control plants probably due to the exceeded antioxidant capacity of maize plants by 75 mM salinity (Fig. 2a). In parallel, MDA content in the salt-stressed maize plants was significantly higher than control (Fig. 2b) indicating that membrane damage occurred because of oxidative stress caused by salinity. It has been indicated that an increase in the

antioxidant enzymes helps plants maintain their growth under stress and may be regarded as indicators of salt tolerance [38]. In our study, the exogenous potassium nitrate application has been found to activate the ability of dismutation of superoxide and detoxification of H_2O_2 in maize plants under salinity, as indicated by the increased activities of SOD, APOD and GR, respectively. In those plants, less oxidative stress was observed and membrane integrity was better preserved as confirmed by lower level of H_2O_2 and MDA. These results clearly explain the elevated level of photosynthetic pigments in the same plants. These findings are also in agreement with the argument that antioxidant activity in plants under salinity are improved by potassium application [39-42].

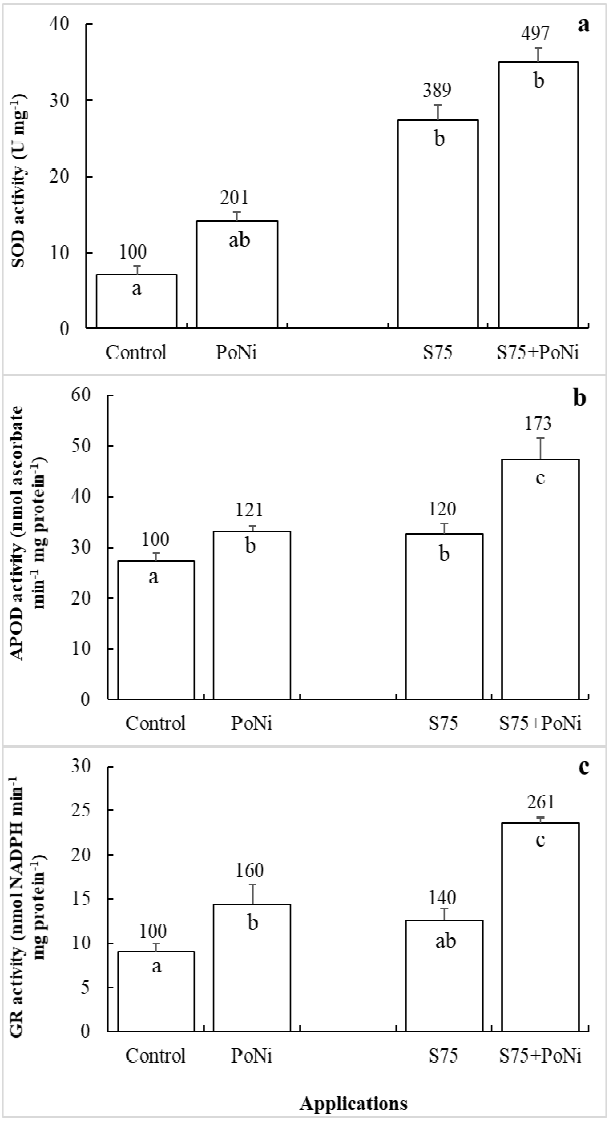

Figure 3. Effect of the exogenous potassium nitrate on (a) SOD, (b) APOD and (c) GR activity of maize plants under salt stress. (PoNi: Potassium nitrate; S: Salt; columns with different letters mean significant differences between the treatments according to Duncan's multiple range test ($P<0.05$) and numbers on the columns indicate % change relative to control, control=100).

Proline is a water-soluble amino acid. It has been shown that proline accumulation in plant tissues under salt stress regulates osmotic potential [43]. It has also been declared that proline is involved in free

radical scavenging [44]. Munns [7] has indicated that salinity up-regulated the enzymes involved in proline biosynthesis. In the present study, salt stress led to the increased proline content in maize leaves in comparison with control (Fig. 2c), probably due to induction of proline biosynthetic enzymes. The exogenous potassium nitrate application in maize plants under salt stress, however, caused lower level of proline. Our results are in line with the previous findings demonstrating the effect of potassium on proline content [45, 46]. It is probable that exogenous potassium nitrate application stimulated the scavenging of free radicals and prevented biosynthesis of extra proline [33].

In conclusion, the exogenous potassium nitrate application increased significantly salt tolerance in maize plants. This is demonstrated by the fact that the exogenous potassium nitrate application increased photosynthetic pigment content. In addition, the exogenous potassium nitrate application increased SOD, APOD and GR activities and decreased H_2O_2 and MDA accumulation in the leaves of maize plants under salt stress, indicating the lowered level of oxidative stress. Thus, the exogenous potassium nitrate application may be involved in the activation of the antioxidant defense system and had positive effect on maize growth at early seedling stage.

Authors' Contributions: AD organized experimental design, and wrote the manuscript; AD and ED carried out analysis and measurements, evaluated the data. All authors read and approved the final manuscript.

Conflict of Interest: The author has no conflict of interest to declare.

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