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# Triacontanol Alleviated Nickel Toxicity in Maize Seedling by Controlling Its Uptake and Enhancing Antioxidant System

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## Abstract

Triacontanol (TRIA) role in improving growth, physiological activities and tolerance against abiotic stresses has been reported. Yet, the mechanism by which TRIA executes its effects remains elusive. This work therefore studied the possible role of TRIA exogenous application in counteracting the adverse effects of nickel (Ni) treated maize seedlings. Maize seedlings (15-day-old) were grown in washed sand irrigated with nutrient solution provided with 100 µM $NiCl_2$. Two concentrations of TRIA (25 and 50 µM) were applied twice as a foliar spray for Ni-stressed seedlings. Shoot and root growth attributes, Ni content, and antioxidant defence systems of maize seedlings were determined. Ni treatment reduced the shoot and root length and biomass, causing necrosis of the old leaves, greater reduction was shown in the roots. The shoot and root length was negatively correlated with their Ni content, which was consistent with their content of $H_2O_2$, but not with their malondialdehyde (MDA) content. As the roots had the greatest Ni content, maximum peroxidase (PX) and glutathione reductase (GR) activity as well as the highest ascorbic acid (ASA) and reduced glutathione (GSH) content were observed in the roots. The Ni-induced deleterious effects were alleviated by foliar application of TRIA concentrations. Also, TRIA treatment minimized root Ni content, whereas it maintained the shoots unharmed by Ni. Such mitigative effects of TRIA are explained by its key role in enhancing antioxidant capacity (expressed as $IC_{50}$), increased PX and ascorbate oxidase (AO) activity, GSH, and total phenolic contents.

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## Introduction

Heavy metal pollution is a global concern as it adversely affecting crop production. Heavy metals (HMs) are naturally occurring metals with atomic numbers greater than 20 and an elemental density greater than 5 g $cm^{-3}$ [1,2]. HMs including cadmium (Cd), lead (Pb), and mercury (Hg), are nonessential and highly toxic to plants [3,4,5]. Other metals are required for life and considered as micronutrients (i.e., Zn, Mn, Ni, Cu, etc.), but their excessive accumulation in living organisms is always toxic. Ni is one of such micronutrients with dual characteristics. For instance, several enzyme activities depend on the presence of Ni highlighting its benefit effects on plant growth and development [6]. Conversely, excess concentrations of Ni become toxic and cause disturbances in several physiological processes including photosynthesis, respiration, mineral nutrition, transport of assimilates and water relations [7]. It is documented that the adequate levels of Ni for plant species are ranged from 0.01 to 10 mg $g^{-1}$ dry weight [8].

Ni toxicity induces high levels of reactive oxygen species (ROS) which triggers lipid peroxidation, oxidation of proteins, degradation of chlorophyll pigments and DNA damage [9,10,11]. Plants evolved a complex ROS scavenging mechanism at the molecular and cellular levels to survive with HMs stress [11]. Therefore, increased stress tolerance in metal exposed plants is often associated with enhancement of antioxidant defense system comprising both enzymatic and non-enzymatic antioxidants [12,13,14]. The antioxidant enzymes comprise superoxide dismutase (SOD), catalase (CAT), peroxidase (PX), polyphenol oxidase (PPO), glutathione reductase (GR), ascorbate peroxidase (APX), and ascorbate oxidase (AO) [14]. The non-enzymatic antioxidants include phenolics, ascorbate (ASC), α-tocopherol, proline and glycinebetaine, and reduced glutathione (GSH) [15,16,17].

TRIA is one of relatively new plant growth regulators (PGRs) which has been established to play a critical role in plant growth and development when exogenously applied to various plant species [18,19]. The prominent effect of TRIA has been reported to influence the enzymes regulating growth [20], metabolic processes in plants [21], enhance photosynthetic rate and chlorophyll fluorescence [22, 23], stimulate mineral nutrients uptake [24, 25], and increase various organic compounds in plants [26]. Furthermore, TRIA has been shown to improve the plant resistance against several abiotic stresses as salinity [23, 24, 14], water stress [27], chilling [28], and HMs stress [29, 30]. To our knowledge, the role of TRIA in Ni-induced oxidative stress and antioxidant response is fragmentary studied. Maize (*Zea mays* L.) is the third most important cereals crop [31] cultivated globally and used largely as food for human and animals. Maize suffers heavily by metals which eventually reduce its growth and grain yield [32]. The present study was therefore undertaken to investigate the role of TRIA in mitigating the adverse effects of Ni stress on maize seedlings. A variety of biochemical and physiological parameters related to antioxidant defense systems was addressed.

## Materials and Methods

### *Plant Material and Growth Conditions*

Maize (*Zea mayes* L.) grains (hybrid three way cross 321) were obtained from the Agricultural Research Centre, Giza, Egypt, and kept in the dark at 4 °C before use. The grains were surface sterilized by immersion in 1% (w/v) sodium hypochlorite solution for 30 min. Maize was cultivated in sand in plastic pots (diameter 15 cm, height 30 cm, 2.5 Kg dry sand per pot). The sand was washed with 12% hydrochloric acid to remove any carbonates and contaminants, rinsed with deionized water, and then dried in an oven (70 °C, 48 h, then 200 °C, 2 h). During the first week from the sowing, seedlings were irrigated with distilled water; then nutrients were added with Ni contamination ($NiCl_2$.6 $H_2O$), in a single dose (100 µM), in a 150 ml (about 0.25 mg Ni/ 100 g soil) nutritive solution was prepared according to the composition described by Smith et al. [33]. The nutrient solutions were applied at a rate of 50 ml per pot three times a week to maintain the same quantity of nutrient solution per unit of sand. Moisture stress was avoided by watering the sand in the pots to 80 % of saturation capacity. Two concentrations of triacontanol solution (TRIA) (TriplntanoI.com), at 25 and 50 µM, were applied as foliar spray treatment at the two-leaf stage (15-day-old seedlings) twice for 7 days. At the end of experiment, 21-day-old seedlings of both the treated and the untreated (control) shoot and root

samples were collected, five plants per treatments were subjected for measuring some growth criteria and the remaining seedlings immediately frozen in liquid nitrogen and then stored at −80 °C for the analyses.

*Chlorophyll Fluorescence Measurements*

The maximum quantum efficiency of PSII ($F_v/F_m$) were determined on fully exposed leaves with a Hansatech Pocket Plant Efficiency Analyzer (Pocket PEA, Hansatech Instruments, King's Lynn, Norfolk, UK) by following Kitajima and Butler [34] method. The data were recorded using previously dark-adapted leaves for 30 min.

*Determination of Nickel Content*

Dried samples (roots and shoots from each treatment were extracted by dry ashing as described by Chapman and Pratt [35]. Ni content was determined by atomic absorption spectroscopy (Savant AA, GBC, Australia). The results were expressed as mg of metal $g^{-1}$ of sample (dry weight).

*Lipid Peroxidation and Hydrogen Peroxide Contents*

Lipid peroxidation was evaluated by measuring the production of malondialdehyde (MDA) by thiobarbituric acid reaction (TBAR)-based colorimetric method as described by Heath and Packer [36]. Hydrogen peroxide ($H_2O_2$) were determined by the methods of Velikova et al. [37].

*DPPH Radical Scavenging Assay*

The measurement of diphenylpicrylhydrazyl (DPPH) radical scavenging activity was carried out according to the method of Hatano et al. [38]. The antiradical activity was finally expressed as $IC_{50}$ (mg $g^{-1}$ F W), the extract concentration required to cause a 50% inhibition. A lower $IC_{50}$ value corresponds to a higher antioxidant activity of the plant extract. Standard curve was prepared to calculate $IC_{50}$ value using ascorbic acid.

*Enzymatic and Non-Enzymatic Antioxidants*

Antioxidant enzymes were extracted from maize shoots and roots by using a known volume of phosphate buffer (PH 7) (1:4 W/V). The crude extracts were used for enzyme assays. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed according to method for Kong et al. [39]. Catalase (CAT, EC 1.11.1.6) activity assayed following the method of Aebi et al. [40]. Peroxidase (PX, EC 1.11.1.7)) activity was determined by the method of Shannon et al. [41]. Ascorbate oxidase (AO, EC 1.10.3.3) and peroxidase (APX, EC 1.11.1.11) activities were measured by the methods of Diallinas et al. [42] and Ali et al. [43] respectively. Glutathione Reductase (GR; EC1.6.4.2) was assayed by the method of Goldberg and Spooner [44] using a commercially kit (Biodiagnostics, Giza, Egypt). Polyphenol oxidase (PPO, EC 1.10.3.1) activity was measured by the method of Gonzalez et al. [45]. Non-enzymatic antioxidants comprises total phenolics, as gallic acid equivalent (GAE), were determined according to Makkar et al. [46]. Ascorbic acid (ASA) was determined by the methods of Mukherjee and Choudhuri [47]. Reduced glutathione (GSH) was estimated according to the method given by Beutler et al. [48] using a commercially kit (Biodiagnostics, Giza, Egypt).

**Statistical Analysis**

The results were subjected to one-way analysis of variance (ANOVA) using the software package SPSS v20.0 (SPSS Inc., Chicago, USA). The comparison of the means of different treatments was carried out using Duncan's multiple range test at a significance level of 5% ($P \leq 0.05$).

**Results**

Maize seedlings exposed to Ni treatment exhibited a major decrease in shoot height, circumference and fresh weight, as well as root length (Fig. 1A and 2) and developed symptoms of Ni toxicity such as chlorosis and necrosis, especially in the older leaves (Fig. 1B). However, a slight increment root fresh weight was observed (Fig. 2C). Foliar-applied TRIA significantly improved Ni-induced reduction in growth traits, but, it did not have any noticeable effect on root fresh weight of maize seedlings (Fig. 1 and 2).

Application of TRIA (50 µM) markedly decreased root Ni content, however, both TRIA treatments (25 and 50 µM) did not show any prominent effect on shoot Ni content (Fig. 3A). Meanwhile, Ni effect was emphasized by correlating Ni content with root growth of maize seedling, as it has revealed a strong reverse correlation with root length ($R^2$= 0.9, data not shown), while a positive correlation has been obtained with root fresh weight ($R^2$= 0.9, data not shown).

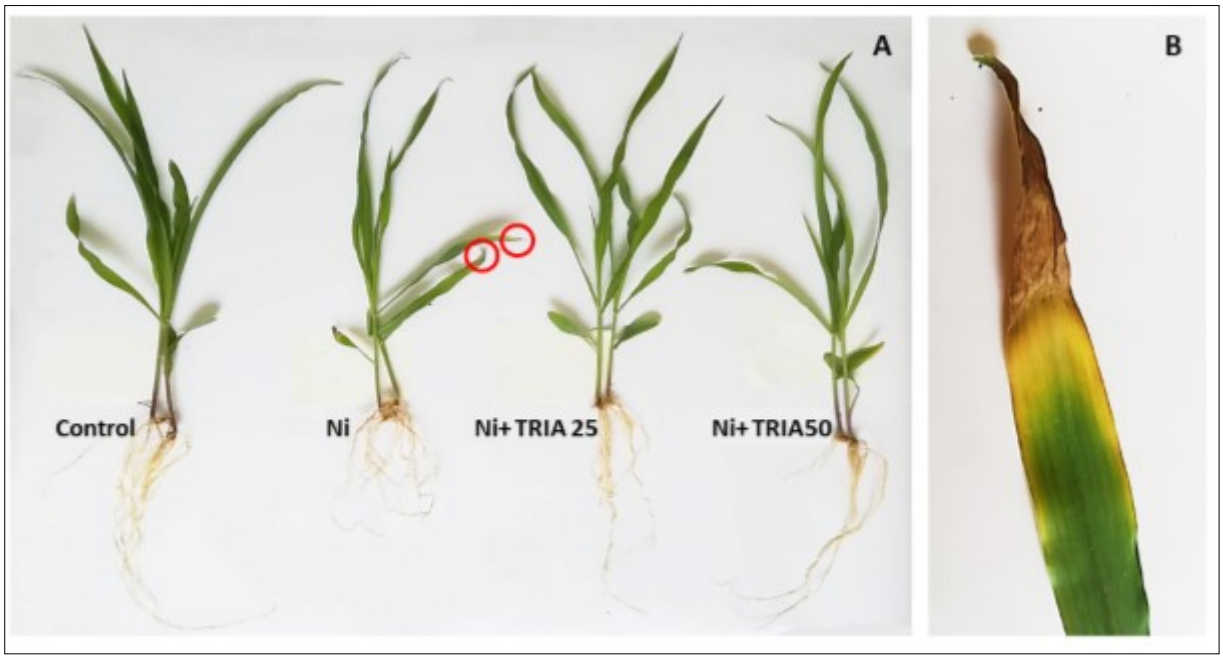


Figure 1. A) A Growth comparison of 21-day-old maize seedlings (control—untreated plants, Ni—plants exposed to Ni stress alone, Ni+ TRIA 25—plants exposed to Ni stress and treated with 25 µM triacontanol, Ni+ TRIA 50—plants exposed to Ni stress and treated with 50 µM triacontanol). B) Symptoms of injury on leaf tips, especially in mature leaves, caused by Ni exposure.

The values of the maximum quantum efficiency of PSII ($F_v/F_m$) showed nonsignificant effect for Ni treatment as well as both concentration of TRIA (Fig. 3B), which correlated with shoot Ni content ($R^2$= 0.8, data not shown). Interestingly, Ni-exposed maize shoots exhibited higher lipid peroxidation (MDA) content reached about 136% as compared with untreated control seedlings despite of low shoot Ni content (Fig. 3C). On the other hand, roots did not exhibit differences in MDA levels of Ni-exposed plants as well as TRIA-treated ones (Fig. 3C). However, TRIA treatments decreased the MDA level by about its half value as compared with untreated Ni-stressed shoot (Fig. 3C). Further, the exposure of maize seedlings to 100 µM Ni led to a significant increment of $H_2O_2$ content in the roots reached about 109% as compared with untreated control seedlings (Fig. 2D). Meanwhile, applications of 25 and 50 µM TRIA significantly reduced $H_2O_2$ accumulated in Ni-stressed root by about 28.5% and 60.5% respectively (Fig. 3D). The enhancement in root $H_2O_2$ level was positively correlated with its Ni content ($R^2$= 0.7, data not shown). While in shoots, $H_2O_2$ were shown to be reduced in Ni exposed plants, but the treatment with TRIA resulted in $H_2O_2$ content to around control levels (Fig. 3D).

The lowest $IC_{50}$ values indicating the highest antioxidant capacity was recorded in 25 µM TRIA-treated stressed shoots, whereas, the highest $IC_{50}$ value was obtained in Ni-stressed roots of maize seedlings (Fig. 4A). Which was strongly correlated with Ni content ($R^2$= 0.91, data not shown). Regarding antioxidant enzymes, roots of all treatment showed boosted activities of most enzymes (SOD, CAT, PX, APX, GR and PPO), as compared with its corresponding roots (Fig. 4). Reduction of SOD was resulted in 25 µM TRIA treated shoot, while no significant change in this enzyme activity for all root treatment (Fig. 4B). Generally, maize roots exhibited increased CAT activities compared to those of roots (ranging between 1 and1.5 folds, except for 50 µM TRIA). The treatment does not seem to affect the CAT activity in both shoots and roots. The only exception is the treatment with 50 µM TRIA, in which the treatment caused a reduction in root's CAT activity to be almost equal to that of shoot (Fig. 4C). Ni-stressed maize roots recorded the maximum PX activity (115.4% over the control level), while no significant changes of PX activity

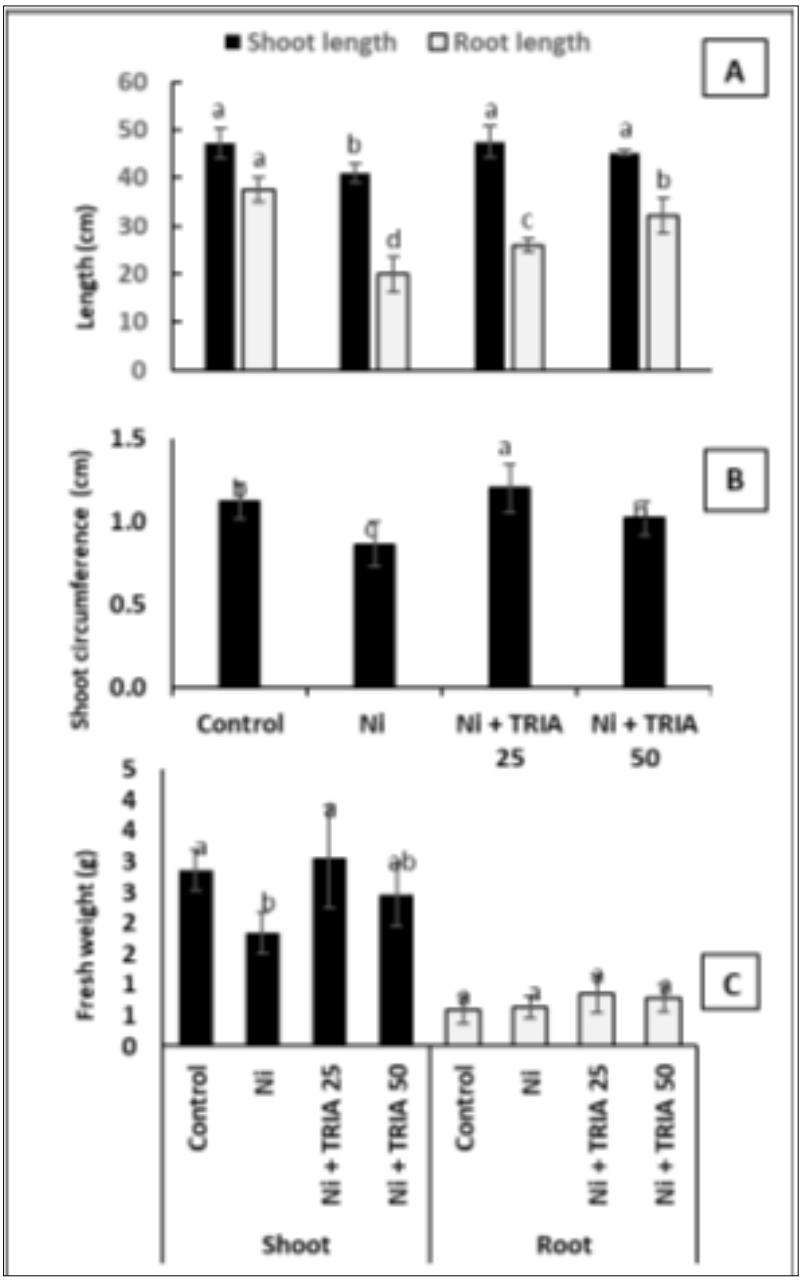


Figure 2. Effect of Nickel (Ni) alone or combined with different concentrations of triacontanol (25 and 50 µM) on A) Shoot and root length, B) Shoot circumference and C) Shoot and root fresh weights, as compared with the control untreated plants. Data are means ± SD (n=4), bars with different letters are significantly different at $P \leq 0.05$.

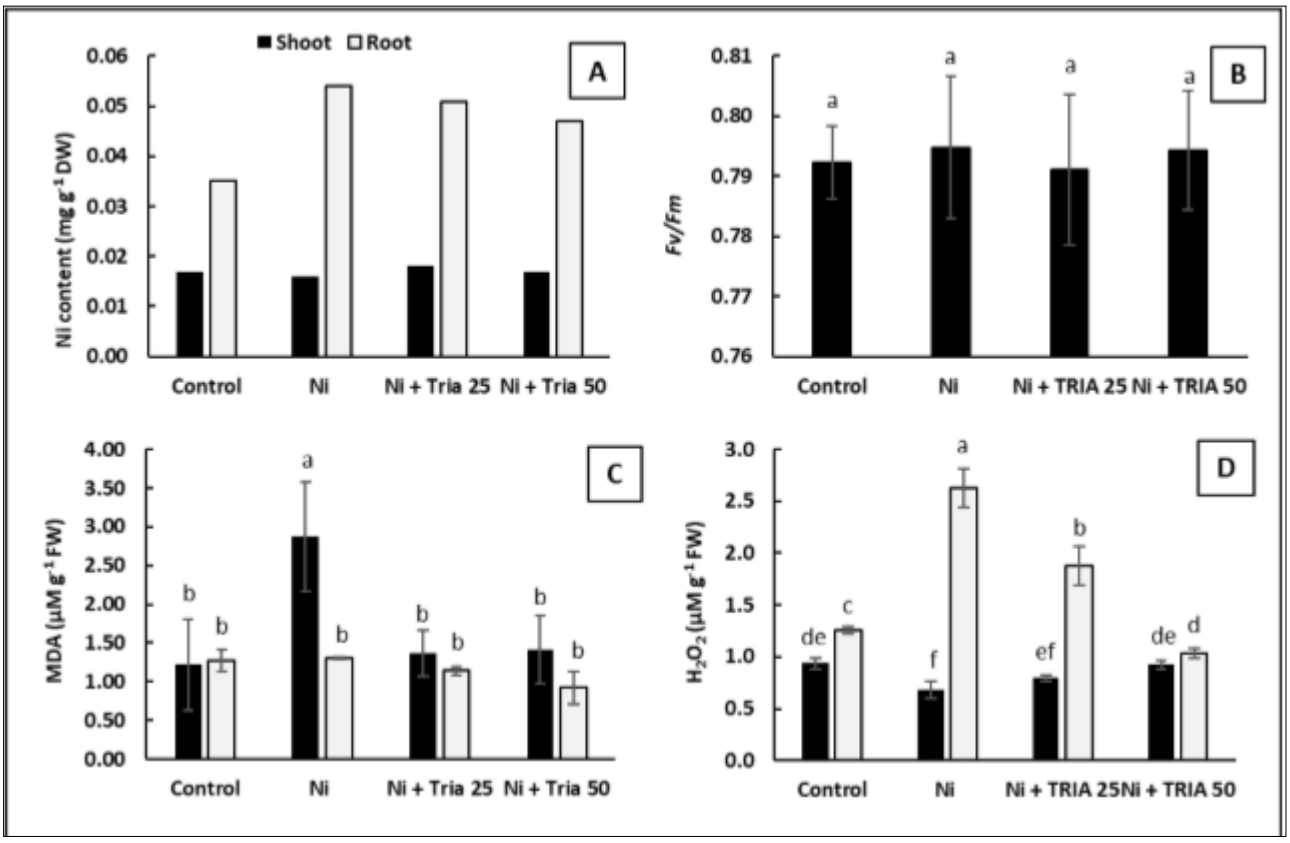


Figure 3. Effect of triacontanol (TRIA) (25 and 50 µM) on A) Nickel (Ni) content B) Quantum efficiency of the photochemical reactions in PSII *(Fv/Fm)* in leaves, C) lipid peroxidation (MDA) and D) $H_20_2$ content in shoots and roots of maize plants exposed to Ni stress, as compared with the control untreated plants. Data are means ± SD (n=3), bars with different letters are significantly different at $P \leq 0.05$.

was recorded in its corresponding shoots (Fig. 2D). Further, the TRIA application reduced the activity of PX enzyme in both Ni-stressed shoots and roots, with greatest decrease in 25 µM TRIA-treated shoots (ten folds blow the untreated control) (Fig. 4D). On the other hand, significant reductions in APX enzyme activity by 25% and 22% were noticed in 50 µM TRIA-treated shoots and roots respectively as compared with Ni-stressed untreated ones (Fig. 2E). The most pronounced induction in response of maize plants to Ni stress was observed in the case of GR activity of the maize roots, where it recorded about 9-fold higher than the control (Fig. 4F). However, no significant changes in GR activity were found in the maize Ni-stressed shoots as well as TRIA treatment (Fig. 4F). Shoots treated with 25 µM TRIA exhibited the maximum enhancement in AO activity under Ni stress (Fig. 4G). While in roots, 25 µM TRIA treatment significantly reduced the activity of this enzyme by about 61% as compared with the untreated stressed roots (Fig. 4G). Treatment with 100 µM Ni along with 25 µM TRIA reduced the PPO activity by 43.7% (Fig. 4H).

Interestingly maize roots revealed similar behavior in both ASA and GSH contents (Fig. 5A and B). As Ni caused a significant increase in ASC and GSH contents in roots of maize seedlings reached about 38.5% and 18% respectively as compared with unstressed control (Fig. 5A and B). However, both TRIA treatments (25 and 50 µM) resulted in significant reduction in ASC and GSH contents of Ni-stressed maize roots (Fig. 5A and B). On the other hand, in the shoots, treatment with Ni induced a highly significant reduction approximately 64% and 67% in ASC and GSH contents respectively as compared with untreated control (Fig. 5A and B). Meanwhile, both TRIA treatment succeeded to retrieve control levels of GSH, but for ASA in maize shoots (Fig. 5A and B). Interestingly, total phenolic

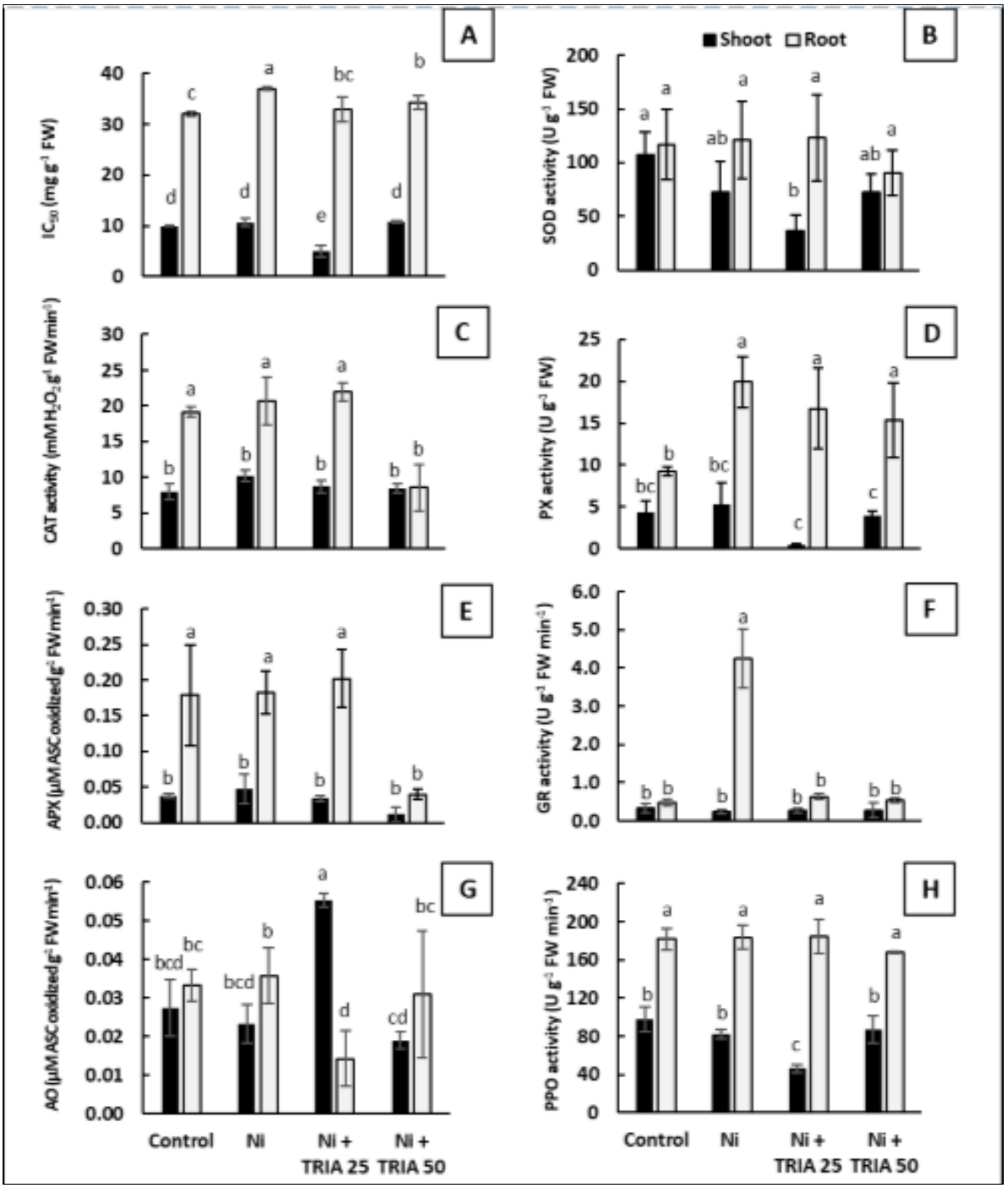


Figure 4. Effect of triacontanol (TRIA) (25 and 50 µM) on A) scavenging activity of DPPH expressed as $IC_{50}$ value B) Superoxide dismutase (SOD) activity, C) Catalase activity (CAT), D) Peroxidase activity (PO), E) Ascorbate peroxidase (APX) activity, F) Glutathione reductase (GR), G) Ascorbate oxidase (AO) activity and H) Polyphenol oxidase (PPO) activity measured in shoots and roots of maize plants exposed to Ni stress, as compared with the control untreated plants. Data are means ± SD (n=3), bars with different letters are significantly different at $P \leq 0.05$.

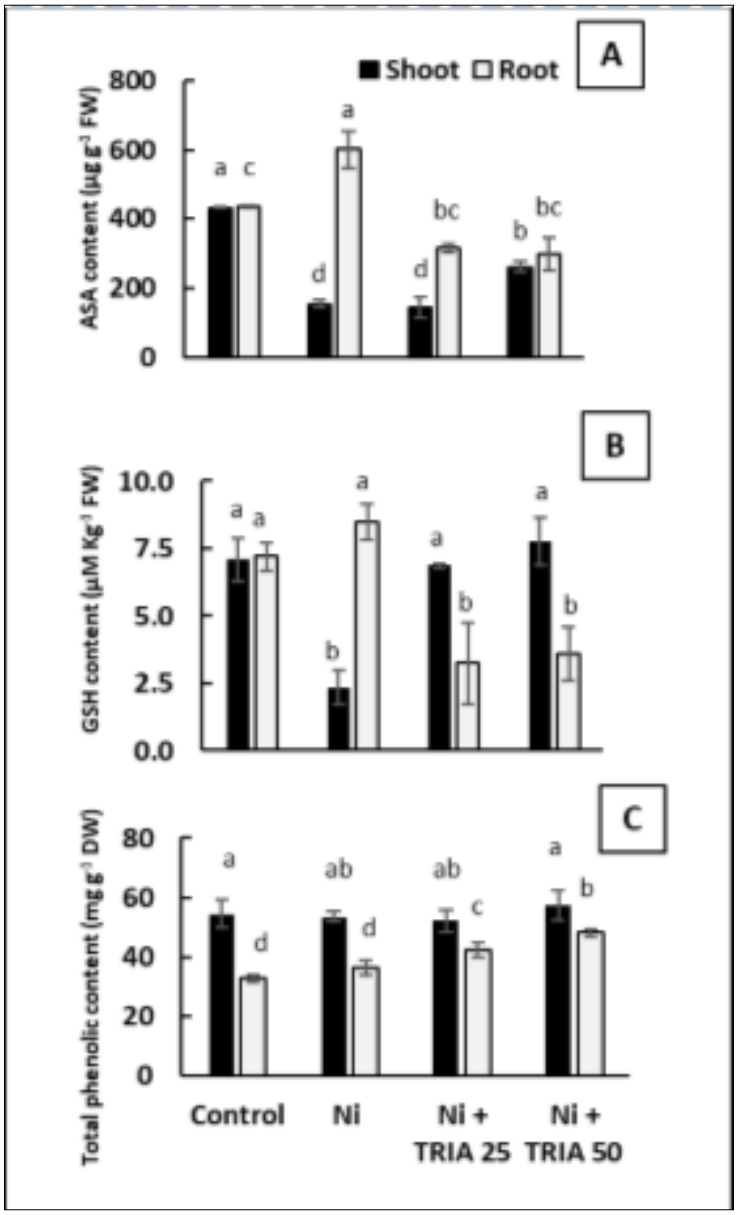


Figure 5. Effect of triacontanol (TRIA) (25 and 50 µM) on A) Ascorbic acid content (ASA) B) Reduced glutathione content and C) Total phenolic content of maize plants exposed to Ni stress, as compared with the control untreated plants. Data are means ± SD (n=3), bars with different letters are significantly different at $P \leq 0.05$.

compounds were the unique antioxidant component that accumulated in shoots more than roots, even though their contents seemed to be treatment independent (Fig. 5C). Total phenolic of roots has not been affected by Ni stress, but it is boosted by addition of TRIA in concentration dependent manner (Fig. 5C).

## Discussion

Decreased growth of maize seedlings exposed to 100 µM Ni could be attributed to inhibition of cell division [7, 49]. Several studies indicated that Ni-stressed plants showed disrupted mitotic index and had chromosome abnormalities [50, 51, 7, 52, 53]. TRIA -induced improvement in the growth of maize might be due to the synergistic interaction of TRIA with phytohormones and induction of 9-b-L (+) adenosine, cytokinin like structure, which might mainly responsible for increased growth [26]. Our results are in agreement with previous studies where TRIA induces the growth of *Brassica napus* [53], *Coriandrum sativum* [30], and *Erythrina variegata* [29] under metal stress.

Increased Ni accumulation in the roots but not in the shoots of maize seedlings could possibly be due to the contention that roots are directly contacted with soil solution including Ni, and root water absorption likely increased Ni accumulation with low translocation of Ni to the shoots [54]. Consistently, previous works on other plant species also reported a greater accumulation of Ni in the roots [55, 56, 57, 58]. TRIA treatment decreased root Ni content in concentration dependent manner, but never retrieved the control levels. Accordingly, it seems that TRIA might reduce Ni uptake from the beginning. Supporting to our proposal is the finding that TRIA could affect heavy metal ATPases or cation diffusion facilitators [1]. Although little is known about the effect of TRIA on ions absorption and membrane permeability, Ramani and Kannan [58] showed that TRIA hinders absorption of minerals from the soil. Moreover, Shripathi and Swamy [59] report changes in the composition of membrane phospholipids of cotton by TRIA treatment.

Our results showed nonsignificant effect on $F_v/F_m$ values in all treatments of maize shoots, which is consistent with low Ni content in the shoots. Absence of Ni effect on this parameter might be interpreted to short time of Ni exposure independently of the prior spraying of the seedlings with TRIA. Also, lower contents of $H_2O_2$ in the shoots of Ni stressed seedlings parallel with shoot decreased Ni concentration. In contrast, significant increase in the shoot MDA relative to the roots under Ni stress might be explained by the fact that other ROS might participate in increased MDA and that symptoms of Ni toxicity such as chlorosis and necrotic spots were visible in the older leaves of maize plants treated with Ni which may indicate Ni accumulation mostly in the oldest leaves and hence function as metal sink and protect younger leaves against its toxicity [60]. The results agree with earlier studies on Ni-stressed shoots of *Cajanus cajan*, *Brassica juncea* [61, 62], and *Zea mays* [52]. Similarly, roots of *Triticum aestivum* [54] *Solanum nigrum* [51] did not demonstrate significant changes in lipid peroxidation levels under Ni stress. The reduction in the shoot lipid peroxidation by foliar treatment of TRIA under Ni stress is in agreement with previous investigations [30, 14] that TRIA may play a key role in protecting the structure and function of cell membranes against metal toxicity *via* elevating antioxidant system. Maize shoots showed higher antioxidant capacities (expressed as lower $IC_{50}$ of DPPH), which might be attributed to non-enzymatic antioxidant compounds like total phenolic compounds and reduced glutathione that accumulated in shoots in response to TRIA treatment.

Accumulation of $H_2O_2$ and Ni in the roots of maize seedlings agrees with previous studies on roots of *Alyssum bertolonii* and *Nicotiana tabacum* [63] and *Triticum aestivum* [55, 56] illustrating Ni-stressed roots suffered from oxidative stress. Despite the higher Ni and $H_2O_2$ accumulation in roots, no significant effect on the root MDA which may be attributed to the elevated activity of antioxidant enzymes that scavenged ROS generated under stress conditions [55, 64, 65]. Minimum antioxidant capacity was observed in Ni-stressed maize roots under TRIA treatment. Similar relation between Ni concentration and antioxidant capacity has been reported by Stanisavljevic et al. [66], Kulbat and Leszczyńska [67] and Georgiadou et al. [68]. Interestingly, Ni content was found to be strongly correlated to $IC_{50}$ and PX ($R^2$ = 0.91 and 0.93, respectively) and to lesser extent to APX, total phenolic

compounds and $H_2O_2$ content ($R^2$ = 0.56, 0.55 and 0.68, respectively). This can be interpreted that accumulated Ni in stressed tissues exerted an oxidative stress leaded to $H_2O_2$ formation, which was scavenged using antioxidative enzymes and phenolic compounds. Similarly, Ibrahim et al. [69] report such correlation relationships under heavy metal stress. Moreover, our results observed a correlation between PX and both $IC_{50}$ and $H_2O_2$ content, indicating that it is the chief antioxidant enzyme in Ni-stressed roots (either treated with TRIA or no). In addition to PX, Ni-stressed root used other antioxidative mechanisms, such as glutathione/ascorbate cycle, which was evident from their enhanced levels of GR, GSH and ASA. Despite these defense mechanisms, it appears that the oxidative rate was greater than scavenging one in maize stressed roots.

TRIA enhanced enzymatic (mainly peroxidases) and non-enzymatic (phenolic compounds) antioxidants under Ni stress, which is previously reported in response to heavy metal-stressed plants [30, 14, 54]. Even though TRIA-treated plants did not use multiple and complex antioxidant systems as that observed in Ni-stressed plants alone, it seems that such antioxidant defense system was sufficient to reduce $H_2O_2$ content and counterbalanced the deleterious impacts of Ni stress. The elevated AO activity in of TRIA 25 µM treated shoots was one of the most remarkable results in this study as this was escorted by increased growth parameters and reduced ASA. Taken together, we can hypothesize that TRIA might have enhanced shoot growth *via* enhancing AO activity on the expense of ASA. AO is an ubiquitous apoplastic multi-copper oxidase enzyme that catalyze oxidation of apoplastic ASA into monodehydroascorbate (MDHA) then finally into dehydroascorbate (DHA) using oxygen as a hydrogen acceptor. This mechanism controlled by AO is the main regulator of apoplastic redox status and hence growth. The reciprocal interaction between AO expression and auxins has confirmed the role of AO in auxins signal transduction [70, 71]. Smirnoff [72] has demonstrated that increased AO activity and its product (DHA) are performing critical role in auxin signal transduction and are directly associated with cell elongation and expansion in actively growing tissues. It has been also shown that overexpression of *AO* gene is associated with a leap in AO activity and apoplastic DHA, which promote shoot elongation in tobacco and cotton plants [73,74].

In conclusion, our results showed that TRIA foliar spray did not only alleviate the toxicity and oxidative stress imposed by Ni stress, but also provoked the growth to be comparable with that of the untreated plants. We propose three mechanisms by which TRIA express its effect: extrusion of Ni from roots, enhancing enzymatic and non-enzymatic antioxidants, and stimulating growth-related enzymes.

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**Abbreviations**

AO - Ascorbate oxidase

APX - Ascorbate peroxidase

ASA - Ascorbic acid

CAT - Catalase

CDFs - Cation Diffusion Facilitators

DHA - Dehydroascorbate

DPPH - Diphenylpicrylhydrazyl

Fv/Fm - The maximum quantum efficiency of PSII

GR - Glutathione Reductase

GSH - Reduced glutathione

HMAs - Heavy Metal ATPases

HMs - Heavy metals

IC50 - Median inhibitory concentration

MDA - Malondialdehyde

MDHA - Monodehydroascorbate

Ni - Nickel

PGRs - Plant growth regulators

PPO - Polyphenol oxidase

PX - ROS

Peroxidase - Reactive oxygen species

SOD - Superoxide dismutase

TBAR - Thiobarbituric acid reaction

TRIA - Triacontanol

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